THE EFFECT OF DRYING AND STORAGE ON THE QUALITY OF COSMECEUTICAL SPECIES *LEUCOSIDEA SERICEA* AND *GREYIA FLANAGANII*

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I declare that the above dissertation/thesis is my own work and that all the sources that I have used or quoted have been indicated and acknowledged by means of complete references.

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Abstract

The use of plants for cosmeceutical applications is becoming more important since "safer" and more "natural" skin products are gaining popularity. The effect of different drying methods and storage conditions on metabolite changes and biological activity of two species with cosmeceutical application namely *Greyia flanaganii* and *Leucosidea sericea* were investigated using ^{1}H-NMR metabolomics. The multivariate analysis (PCA and OPLS-DA), and ^{1}H-NMR sample spectra were used to analyse the significant differences ($P<0.05$) resulting from the different treatments. The effect of these treatments on anti-tyrosinase and anti-bacterial (against *Staphylococcus aureus)* activity of *G.flanaganii* and *L.sericea* ethanol leaf extracts respectively, was further investigated to assess the quality. Four different drying methods adopted were freeze drying, oven drying at 50°C, air drying at room temperature and sun drying in a greenhouse. The dried leaf extracts were stored in three different conditions of fridge, freezer and shade conditions and samples from each storage condition taken for analysis at three and six months of storage. The chemical constituents of the leaf extracts of both species were not affected by the drying method and the storage condition, but the concentrations of the metabolites changed. The treatments did not show a significant difference ($P<0.05$) on the biological activity of the extracts. However, *G.flanaganii* plant material harvested from the University of Pretoria exhibited a higher anti-tyrosinase activity than material harvested from Mothong heritage site. In *G. flanaganii* freshly freeze and oven dried extracts exhibited a higher anti-tyrosinase activity with fifty percent inhibitory (IC_{50}) activity of 16.8±0.69 µg/ml and 15.73±0.85 µg/ml respectively than the activity of sun and air dried with IC_{50} values of 33.08±0.78 µg/ml and 36.86±2.01 µg/ml respectively. The metabolite concentrations and anti-tyrosinase activity dropped significantly after storage. *Leucosidea sericea* oven and freeze dried extracts, exhibited good anti-bacterial activity with a minimum inhibitory concentration (MIC) value of 0.25 µg/ml and 0.125 µg/ml respectively as compared to sun and air dried extracts with the same MIC value of 0.5 µg/ml. Freeze dried samples showed the best anti-bacterial activity (MIC 0.125 µg/ml) compared to other drying methods. Fridge and freezer storage conditions enhanced the activity of stored sample

Chapter 1

Introduction

1.1 Potential of the new plant species

An ethanolic extract of *Greyia flanaganii* leaves showed significant ($P<0.05$) anti-tyrosinase activity exhibiting an IC_{50} of 32.62 µg/ml in previous studies (Mapunya *et al*., 2011). The total extract was further investigated for its toxicity and effect on melanin production by melanocytes cells and showed significant inhibition of 20 % ($P<0.005$) of melanin production at 6.25 µg/ml and low level of toxicity ($IC_{50}<400$ µg/ml). Bioassay guided fractionation led to the isolation of seven compounds. The data indicates that *G.flanaganii* extract and its isolated phenolic constituents could be possible skin anti-hyperpigmentation agents (Mapunya *et al*., 2011). There is a need to further study the pre-treatments, drying and storage, of the species to assist in the commercial use of the species as an anti-hyperpigmentation agent in cosmeceuticals.

The plant *L. sericea* (Rosaceae) is a single species of the genus *Leucosidea* found in the Eastern Cape, Free State and KwaZulu-Natal provinces of South Africa (Van Wyk *et al*., 1997). It is used against various ailments including severe inflammation of the eyes and in the treatment of ophthalmia (Aremu *et al*., 2010). Previous studies have reported *in vitro* anti-microbial, anti-oxidant, acetyl-cholinesterase inhibiting (Aremu *et al*., 2010) and moderate anti-cancer activity (Fouche *et al*., 2008) of *L. sericea*. Some known compounds namely phytol-acetate, triacontanol, phytol, alpha kosin and (E)-3, 7, 11, 15 tetramethylheptadec-2-ene-1, 17-diol have been isolated after promising anti-acne activity of the crude extracts was discovered. The ethanol extract of leaves and one of the isolated compounds, alpha kosin exhibited significant minimum inhibitory concentration with MIC values 156.7 µg/ml and 19 µg/ml respectively against anti-acne by inhibiting the bacterium, *Propionibacterium acnes* (Richa *et al*., 2013). Due to the difficulty of growing and maintaining, and ease of contamination of *P. acnes*, bioassays were performed on *S. aureus*, which is a more dependable and repeatable test organism which also causes skin infections, related to *P. acnes*. This study therefore explored the effects of different drying and storage methods of *L. sericea* leaves on bacterial activity (*S. aureus*), to make informed recommendations for commercial producers.

1.2 Processing factors

Metabolites in plant leaf extracts are affected by growing conditions, harvesting and post harvesting conditions. The care of plant material during shipping, storage conditions prior to manufacture and preparation of the herb and final product as well as mixing with other herbs are other factors that may substantially alter solubility, biological availability, pharmacokinetics, pharmacologic activity and toxicity (Tiwari *et al*., 2013). The magnitude and duration of heating, also has a strong influence on the stability of phytochemicals.

Processing operations are the most controllable factors to optimize in order to reduce the loss of phytochemicals in herbs. Optimising pre-harvest factors may be difficult due to varying environmental conditions and differences in farm management practices (Tiwari *et al.,* 2013). Both drying and storage techniques influence the retention of phytochemicals.

Despite popularity of traditional medicine in Africa, the continent remains behind in terms of regulation of safety and quality control of its medicinal plant industry (Fennell *et al*., 2004). There is heightened interest in essential aspects that affect the industry, with storage and drying practices and consequent effects on plant efficacy being of high priority. The importance of raw plant material pre-treatments, such as drying and grinding processes affect the yield and quality of the extract (Lenucci *et al*., 2010). Environmental conditions and harvesting season as well as processing conditions for example drying, have a significant effect on the content of lipophilic compounds in the plant material (Guan *et al*., 2005; Ratti., 2001).

1.2.1 Drying

Drying is a very common preservation method used in foodstuffs and the quality of the final product is strongly dependent on the technique and the process variables used (Guan *et al*., 2005). The reduction of water activity by moisture removal also implies significant reduction of weight and volume, minimizing packaging, transportation and storage costs (Lenucci *et al*., 2010). The food industry has evolved and often carries out freeze and air drying processes, under controlled conditions, to achieve the objective of long term storage.

Once collected, the leaves (or plant material) need to be stabilised, usually by drying before use. However the drying methods which include thermal drying (microwave, oven, sun drying) and non-thermal drying (air drying and freeze drying) of cosmeceutical species affect the:

- Anti-oxidant properties
- Total phenolic content
- Ascorbic acid equivalent anti-oxidant capacity
- Ferric reducing power
- Lipid peroxidation inhibition activity
- Ferrous ion chelating ability (Tiwari *et al.*,2013)

It is reported that all methods of thermal drying resulted in drastic decline in total phenolic content, ascorbic acid equivalent antioxidant capacity and ferrous ion chelating ability (Chan *et al.*, 2008). For non-thermal drying methods significant losses were observed in air dried leaves. The high retention of phenolics by air drying was verified by Hossain *et al*. (2010). Until now, different drying methods have been applied to different raw materials and each possesses its own characteristics. In a similar study on anti-oxidant capacity of persimmon leaves, the results in terms of total phenol content, flavonoids and anti-oxidant capacity indicated that air drying at 100^0C would be the optimal process for the stabilization of persimmon leaves (Martinez., 2014).

Some studies report that freeze drying increases the extraction of bioactive compounds of different products in comparison to air drying (Hossain *et al*., 2010). This is because freeze drying is based on dehydration by sublimation of a frozen product. Nevertheless, freeze drying has always been recognized as the most expensive process for manufacturing of a dehydrated product and its application depends on the use of the final product.

1.2.2 Storage

Storage conditions including storage time, temperature and light influence the retention of phytochemicals (Tiwari *et al*., 2013). Most of the phytochemicals are unstable due to various storage parameters including presence of occlude oxygen or exposure to oxygen, high temperatures, light, enzyme activation during storage and co-pigmentation with other phytochemicals (Tiwari., 2009). A number of studies have reported the significance of long term storage effects on the retention of bioactive compounds in the finished product obtained from plants. Garcia-Viguera *et al*. (1999) demonstrated a significant loss (36% - 43%) of total anthocyanin in fruits and vegetables with increased storage time at temperatures of 37^0C and 20^0C. The anti-microbial activity of methanol extracts of the selected species (fresh and short term stored) were compared by Laher *et al.* (2013), and it was observed that fresh samples of the

majority of the plant extracts (*Ocimum basilicum, Senna petersiana,* and *Hypoxis hemerocallidea*) indicated better anti-bacterial (*Staphylococcus aureus* and *Escherichia coli*) and antifungal (*Candida albicans*) activities in comparison to the stored samples. Phytochemical analysis indicated that fresh samples showed higher chemical concentrations in many plant parts. It is reported in another study that after one week storage, antioxidant properties of dried *Etlingera elatior* leaves remained significantly higher than those of fresh control leaves (Hossain *et al*., 2010). In general, the degree of changes in pharmacological and phytochemical activity due to storage is plant part and species specific (Laher *et al*., 2013).

1.3 Phytochemicals

Phytochemicals are important natural bioactive compounds of herbs and are widely recognized for their health benefits. This study analysed different factors influencing the phytochemical composition in the selected species at different processing stages in the production chain. Available literature correlates the level of and phytochemicals profiles with many factors including cultivar type, environmental and agronomic conditions, harvesting, processing operations and storage factors (Durante *et al*., 2013). The optimization of processing such as drying and storage factors is an essential step to reduce the degradation of phytochemicals for the potential health benefits.

Literature sources indicate that the level of phytochemicals is dependent on several pre and post-harvest stages and production chain. To retain pharmacological properties of phytochemicals in extracts, the manufacturer must optimize relevant processing steps to restrict the loss of phytochemicals.

It is known that both the method of drying and the temperature at which the process takes place substantially affect the properties of the final product. A lot of research has been done on the effect of drying and storage in the food industry (Chan., 2009; Berk., 2013; Durante *et al.*, 2013). However there are few studies on these effects on medicinal plants, and no studies in literature that examine how drying variables can affect the quality of *G. flanaganii* and *L. sericea* leaves, especially with reference to preservation of their cosmeceutical properties. Previous work on tyrosinase activity of *G.flanaganii*, the samples were air dried and shielded from the sun at ambient temperatures. This leaves a gap to assess other drying methods and condition variables hence the purpose of this study.

1.4 Aim of study

To assess the effects of four drying methods (freeze drying, oven drying, air drying and sun drying) and three storage conditions (shade storage, fridge storage and storage in a freezer) for two storage periods (3 months and 6 months) on the metabolite profile and biological activity of *G. flanaganii* and *L .sericea* cosmeceutical species.

The ultimate aim is to determine the best drying and storage conditions in respect to conserving the main bioactive properties.

1.5 Objectives

- To evaluate the effects of different storage period (at 3 months and 6 months) on metabolic profile and bioactivity (anti-tyrosinase activity and *Staphylococcus aureus* inhibiting activity) of *G. flanaganii* and *L. sericea* respectively.
- To evaluate the effects of four drying techniques (freeze drying, air drying, oven drying and sun drying) on metabolic profile and bioactivity (anti tyrosinase and *Staphylococcus aureus* inhibiting activity) of *G. flanaganii* and *L. sericea* respectively

1.6 Layout of the study

The dissertation is laid out in seven chapters where the first chapter introduces the study and outlines the aims and objectives of the study. The second chapter is a literature build up of different drying and storage techniques reviewed from previous studies. In the same chapter information on skin problems caused by hyperpigmentation, *S. aureus* infection and the promising use of natural plants in ameliorating such problems is presented. The discussion of the metabolomic composition of *G.flanaganii* and *L. sericea* species dried and stored differently is contained in chapter three and four respectively. The quality assessment of the two species in terms of the *anti-bacterial* activity of *L.sericea* and *anti-tyrosinase* activity of *G. flanaganii* leaf extracts after the different drying and storage treatments is discussed in chapter five and six respectively. The last chapter seven summarises the study and recommendations are made based from this study.

Chapter 2

Literature study

2.1: Drying techniques

Drying is a fundamental requirement to achieve a high quality product. Because of high investment and energy costs, drying is also a large expense in medicinal plant production (Chan *et al*., 2008). Drying process increases the shelf life by slowing microorganisms' growth and preventing certain biochemical reactions that might alter the organoleptic characteristics (Sellami *et al*., 2010). A preliminary step for the development of herbal drugs includes dehydration of the fresh material to the target moisture content for further processing. Besides quality, the macro and micro alterations occurring during drying may also affect the ability of the plant material to absorb or desorb water vapour.

Natural drying (drying in the shade) and hot air drying are still the most widely used methods because of their lower cost. According to Chan *et al.* (2008), processing methods of medicinal plants may also improve the remediation properties or induce formation of new compounds having remediation properties. During drying several changes occur, depending on the method and conditions of drying and the plant species. In recent years, various methods such as microwave drying and freeze drying has gained popularity as an alternative drying method for a variety of food products (Wang *et al*., 2006). Apart from the protection of the bioactive compounds present, special attention should be given to the structural modifications that occur during drying and the changes that can be induced to the physicochemical properties of the plant. Therefore, determining a suitable drying method to achieve quality and preservation of bioactives in cosmeceutical species remains key in the industrial production of cosmetics.

Drying method and processing conditions greatly affect not only the degradation of bioactive compounds but also their extractability. In the case of total phenols, a higher concentration was found in dry leaf extracts compared to fresh leaf extracts of persimmon leaves (Martinez *et al*., 2014). This result could be related to an increase in the extractability of such compounds as a consequence of the matrix changes during the drying process. The structure of the dry leaves is more open and interconnected than in fresh leaves, meaning that the solvent can penetrate more easily, providing a greater surface for mass transfer, and resulting in a more efficient extraction of

these compounds. The method, temperature and drying time are process variables that significantly affect the structure of the resultant matrix, regardless of whether the final humidity or water activity is the same. Water removal during drying of plant material is accompanied by significant deformation, which degrades the vegetal matrix and the functionality of its cell walls and membranes. The varying disintegration of this matrix results in a greater or lesser exposure of bioactive compounds to oxidation reactions, hence the importance of establishing the most suitable drying conditions in each case (Chan *et al*., 2014).

Control of the drying conditions has several objectives including:

- shortening the drying time
- maximizing product quality, particularly retention of volatile aromas
- preventing melting and collapse

Common drying methods can be divided into thermal and non-thermal methods. Thermal drying include techniques such as sun drying, microwave drying and oven drying while non-thermal include freeze drying and air drying. Thermal drying is the application of heat to evaporate water from biosolids either directly or indirectly. They differ in operational principles and have varying effects on the quality of the resulting product. Sang *et al*. (2014), observed that plant species, which are exposed to full sunlight, possess strong anti-oxidant and high tyrosinase inhibition ability.

In a study on the effect of thermal and non-thermal drying methods on anti-oxidant properties of ginger species, it was observed that thermal drying of leaves of *Alpinia zerumbet, Etlingera elatior, Curcuma longa* and *Kaempferia galangal,* led to drastic decline in anti-oxidant properties and ferric reducing power. Freeze drying led to significant increase in total phenolic compounds for *E. elatior* and *A. zerumbet* (Chan *et al*., 2009). All methods of thermal drying (microwave, oven, sun drying) resulted in drastic declines in total phenolic content, ascorbic acid equivalent, anti-oxidant capacity and ferric reducing power in all the species. Of non-thermal drying methods, significant losses were observed in air dried leaves. Similarly in a study on legume species, freeze drying treatment was useful in retention of anti-oxidant activity and phenolic content of forage legume leaves (Sang *et al*., 2014). The team recommended novel drying technologies like freeze drying, spray drying and vacuum drying to maintain vitamin C, carotenoids, phenolic compounds and anti-oxidant vitamins which are sensitive to heat and light.

The degradation of phytochemicals upon thermal treatments has been reported by many authors. Chan *et al.* (2009) found that thermal processing can affect the phytochemicals by thermal breakdown, which affects the integrity of cell structure, thereby resulting in the migration of components, leading to breakdown by various chemical reactions involving enzymes, light and oxygen.

2.1.1 Freeze drying (Lyophilisation)

Freeze drying is the removal of water by sublimation from frozen state (ice). In this process, material is first frozen and then subjected to a high vacuum, whereby the water ice evaporates without melting. Freeze drying is carried out at low temperature, thus preserving colour, appearance and minimizing thermal damage to heat sensitive compounds. Since the entire process occurs in solid state, shrinkage and other kinds of structural changes are largely avoided. Typically freeze drying is carried to a final moisture content of 1 -3 % (Ratti., 2001).

Freeze drying in comparison to other drying methods shows an advantage due to the best preservation of compounds, however, this method has two significant disadvantages as it is expensive and time consuming (Ratti., 2001). This was supported by the findings of Nyuk *et al*. (2013) where freeze dried powder of *Mangifera indica* had higher anti-oxidant properties than those from hot air, vacuum and infrared drying techniques. NMR analysis on microalgae species showed the highest intensity of bioactive metabolites obtained for homogenized extracts pre-treated with freeze drying (Nyuk *et al*., 2013).

Freeze drying is a better method of moisture removal with the final product of the highest quality compared to air drying. It is economically feasible only in the case of high added value products and whenever the superior quality of the product justifies the higher production cost (Ratti., 2001). It usually maintains the biochemical characteristic and quality of the starting material; however the high cost of this process is a major weakness (Ratti., 2001). Use of freeze drying treatment has been reported to retain features that are closer to the characteristic appearance of fresh plant. Lyophilisation is often considered to be the most adequate drying technique for processing temperature sensitive compounds. Freeze dried kale leaves retained higher polyphenols, vitamins and anti-oxidant activity than air dried material after 12 months of storage (Korus., 2011).

There is no thermal degradation in freeze drying and neither does the process allow degenerative enzymes to function. Freeze drying is known to have high extraction efficiency because ice crystals formed within plant matrix can rapture cell structure, which allows exit of cellular components, access of solvent and consequently better extraction (Asami *et al.*, 2003).

2.1.2 Oven drying

Oven drying is a cheaper method, but often leads to the degradation of thermolabile compounds and or oxidisable substrates such as carotenoids, tocochromarols and lipids. In order to overcome these limitations, oven drying is generally carried out under vacuum, at moderate temperature (40°-60°C). The study by Pitso *et al*. (2015) showed that drying *L. sericea* leaves at higher temperatures (oven and microwave) led to a significant reduction in essential oil yield. Again in another study of drying effects on skins from grapes Torres *et al.* (2010), lyophilized skins maintained their volatile and phenolic composition in comparison with the original skins, better than those which were oven dried at 60°C.

2.1.3 Air drying

Generally air drying is favoured due to processing cost and speed (Katsube *et al*., 2009). However due to long drying period and high temperature, it can cause a decline in density and water absorbance capacity and shifting of solutes from the internal part of the drying material to the surface. Heat transfer is slow due to low heat capacity of air drying.

Natural drying (drying in shade) and hot air drying are still most widely used methods because of their lower cost. Natural drying has many disadvantages due to the inability to handle the large quantities and to achieve consistent quality standards. In addition, hot air drying presents some drawbacks such as low energy efficiency and lengthy drying time during the last stage of drying (Gornas *et al*., 2014).

The high retention of phenolics by air drying was verified by Hossain *et al*. (2010). From the study on the effect of drying method on the antioxidant capacity of Lamiaceae herbs by Hossan *et al*. (2010), it was observed that air dried samples had significantly higher total phenols, rosmarinic acid content and antioxidant capacity than freeze dried and vacuum oven dried samples throughout the storage period of 60 days.

From literature, the information reported on anti-microbial activity of *L. sericea* (Fouche *et al*., 2008; Aremu *et al*, 2010; Mapunya *et al.*, 2011; Sharma *et al.*, 2013) were obtained from extraction and isolation of compounds from air dried and powdered leaves.

2.1.4 Sun drying

Sun drying has always been an economically better alternative than mechanical and other traditional drying systems (Laher *et al*., 2013). Solar energy has been used for the preservation of agricultural produce for generations all over the world (Okechukwu., 1999) quoted in Nyuk *et al*. (2013). In natural drying or open sun drying, the materials are put on compact earthen floor, mate, concrete, floor and road in the full sun (Nyuk *et al*., 2013). Literature on sun drying reveals the shortcomings of open sun drying. There is quality loss due to insect infestation, enzymatic reactions, microorganism growth, and mycotoxin development. It is highly labour intensive, prone to theft, damage and infection by birds. It also suffers with lack of process control and treatment uniformity. Thus to overcome the above shortcomings of open sun drying; several types of solar dryers have been developed over the years (Laher *et al*., 2013).

The direct exposure to sun rays can greatly reduce quality of herbs. Naturally dried products are the cheapest whereas the quality is far below the international standards (Hossain *et al*., 2010). Therefore, open sun drying does not provide the opportunity to produce a higher quality of dried products. Due to the rapid increase in the price of conventional fuel and the depletion of it, solar energy is a useful source of energy which can be employed in the drying process. Gornas *et al.* (2014) have reviewed various existing solar dryers. Natural convection solar dryers have become more suitable for the rural sector and remote areas as they work on single energy options and do not require any other external energy source. Gornas *et al*. (2014) recommended that parts of the plant are dried into trays under direct sunlight at a temperature between 25 – 35°C for 3 days to achieve moisture content < 12 %. This was after their study on the effect of harvest time, sex, drying and extraction method on sea buckthorn leaves as valuable sources of lipophilic anti-oxidants. Sellami *et al*. (2010) in their study on the effect of different drying methods and on qualitative and quantitative changes in the essential oil of *Laurus nobilis* leaves recommended sun drying in the greenhouse for 3 days with about 27 hours of daylight, mid-day temperature in the greenhouse reaching 35°C.

2.1.5 Microwave drying

Microwave drying could be considered as a great alternative reaching a similar preservation rate of lipophilic compounds as freeze drying but at a lower costs and much shorter drying time even comparing with conventional drying (Gornas *et al*., 2014). In a study on effects of drying techniques on sea buckthorn leaves, the best preservation of bio-compounds was provided by freeze and microwave drying (Gornas *et al*., 2014). In a study by Chan *et al.* (2014) it was found that microwave treatment resulted in significant increase in tyrosinase inhibition of *Anacardium occidentale.*

2.2 Storage

Storage method, storage period and storage temperature affect quality of different species differently (Singh *et al*., 2014). Storage conditions (temperature, air conditions, moisture content) and time of storage affect the biological activity of active ingredients. Different storage conditions can lead to divergent stability of herbal extracts both on physical and chemical properties. An alteration of physical stabilities such as flavour, colour and viscosity can affect appearances while chemical instability can bring about inappropriate quality and efficacy of the extract (Stafford *et al*., 2004).

Processing and subsequent storage conditions may have a positive or negative influence on the stability of phytochemicals. There have been some reports of some healers that store air dried plant materials for prolonged periods under dark conditions (Stafford *et al*., 2004). According to Eloff. (1999), some chemical compounds that account for anti-microbial activity are fairly stable in the dry state (stored) and therefore can be used after storage for prolonged time periods. Amoo *et al*. (2012) discovered greater anti-microbial activity in stored material, and this was attributed to increase in phytochemicals such as phenolic compounds. According to Stafford *et al*. (2004) it is speculated that bark, roots and underground storage organs have a longer storage life in comparison to leaf material.

Temperature is the most important factor influencing the shelf life and quality of plant material (Chan *et al*., 2008). Effect of storage on Mycosporine like Amino Acids (MAA) such as porphyra -334 incorporated into a commercial *Aloe vera* was evaluated. Mycosporine like Amino Acids were reported to be quite stable and did not degrade during the storage of dried macroalgae packed in airtight bags under cool, dry and dark conditions (Bhatia *et al*., 2014). However MAA content

in the egg masses was reduced by 20% after 3 months at 37°C. Post-harvest storage of roses at low temperature cause a loss of quality after short and long term storage (Serrano *et al*., 1992) as a result from low temperature induced alterations in metabolic processes. Inadequate post-harvest storage and processing techniques often lead to high levels of microbial contamination and significant stock losses especially in informal markets.

2.2.1. Shade storage

Plant samples may be stored dry at ambient temperatures in the presence of a desiccant e.g. silica gel. However, ambient temperatures cannot guarantee against metabolite degradation in dry samples especially if rehydration occurs (Wei *et al*., 2010).

In a study of stored pigeon pea leaves at room temperature (25°C), the contents of 7 phenolic compounds gradually increased along with the storage period. Comparatively low temperature favoured significant accumulation of phenolic compounds (Wei *et al*., 2010). The contents of 7 phenolic compounds showed a notable increase in the first 45 days and showed a sharp decline towards the end of storage period at 120 days.

A study on bioactive content of *Moringa oleifera* leaf extract under different storage conditions showed that after six months of storage, the bioactive content and free radical scavenging activity of the extract kept at 25+2°C with 60+-5% relative humidity slightly decreased (Vongsak *et al*., 2013). High temperature and humidity negatively impacted on the contents of bioactive constituents, thus extracts and products from *M.oleifera* should be kept in a cool place to prevent significant changes in chemical, physical and biological properties.

2.2.2. Freeze storage

Storage at 4°C reduces physical changes within the samples (for example adsorption, aggregation) to a minimum but at these temperature conditions, chemical reactions may occur (Tiwari *et al*., 2013). In contrast, at -80° C, chemical reactions can be avoided but physical changes can take place. Most plant, fungal and animal tissues will remain indefinitely stable for extraction of nucleic acids and proteins if maintained at -70 to -80°C (Bhatia *et al*., 2015). However, storage at 4°C was discovered to be a better storage temperature than -80°C for metabolic fingerprinting following final extraction.

Plant tissue samples have been stored at -20°C, after quick freezing at -70°C, with apparently no DNA degradation after 6-8 months (Vongsak *et al*., 2013). However, these authors observed a marked DNA degradation when leaf tissue was stored directly at -20°C.

2.3 Bioassays

Bioassays can be defined as the use of biological systems to detect bioactivity of crude extract, chromatographic fraction, mixture or pure compounds (Chan *et al*., 2008). They are crucial in assessing activity and pharmacological actions of plant extracts and their ethno-medicinal uses (Guan *et al.*, 2005). A limitation of a bioassay is that they provide no data on individual compounds. Modern strategies involve bioassay-guided isolation and identification of active compounds from natural sources, where the focus is more on bioactivity. There is more focus on isolating target compounds (assay-guided isolation) rather than trying to isolate all compounds present in any extract.

2.3.1 Anti- bacterial activity

From previous studies, five known bioactive compounds from *L.sericea* leaves were obtained namely **1** phytol acetate , **2** triacontanol, **3** phytol, **4** alpha kosin and **5** (E)-3,7,11,15-tetramethylheptadec-2-ene-1,17-diol (Sharma *et al*., 2013).

The Minimum inhibitory concentration (MIC) values measured for the activity of the crude extract and compound 5 was considered significant against *P.acnes*. The other compounds did not show any growth inhibitory activities at highest concentration tested (500 µg/ml) (Sharma *et al*., 2013). However, petroleum ether and dichloromethane leaves extract of the same species was found to be active against *Bacillus subtilis* and *Staphylococcus aureus,* respectively with MIC values of 0.025 µg/ml (Aremu *et al*., 2010). Threshold MIC values of 100 µg/ml and 10 µg/ml have been recommended for plant extracts and pure compounds respectively to rate them as having significant anti-microbial activity (Sharma *et al.*, 2013). In another study *L.sericea* was found to have an MIC value 0.08 mg/ml against *S.aureus* (Adamu *et al*., 2014)

The above scientific studies justify the use of the species in cosmeceutical formulations. However, the findings were obtained from the extraction and isolation of bioactive compounds from air dried and powdered leaves.

2.3.2 Anti-tyrosinase activity

Tyrosinase exists widely in plants and animals and is involved in the formation of melanin pigments (Sharma *et al*., 2013). Tyrosinase is the enzyme responsible for the conversion of the substrate tyrosine to melanin in melanocytes providing pigmentation to skin. Tyrosinase is a copper containing enzyme that catalyses two distinct reactions of melanin synthesis i.e. hydroxylation of tyrosine to L-3, 4-dihydroxyphenylalanine (L-DOPA) and oxidation of L-DOPA to o-dopaquinone.

Various compounds that bind to the active sites of tyrosinase to inhibit its activity, have been developed as agents to lighten the skin and ameliorate unwanted pigmentation. Melanin is a pigment responsible for the colour of the eyes, hair and skin in humans. The pigment is secreted and produced through a physiological process called melanogenesis, by the melanocytes cells in the basal layer of the dermis. It is formed through a series of oxidative reactions involving the amino acid tyrosine in the presence of the enzyme tyrosinase, the key enzyme in melanin biosynthesis (Halder *et al*., 2004). Over-activity of tyrosinase leads to overproduction of melanin. There are several noteworthy tyrosinase inhibitors obtained from natural sources reported in literature which are used for the disorder hyper pigmentation of the skin.

Tyrosinase inhibitors are used in treatment of melasma, post inflammatory, hyperpigmentation and Addison disease. However, these inhibitors suffer from toxicity and lack of efficacy. Even the most popular drug hydroquinone used as a tyrosinase inhibitor (Briganti *et al*., 2003), causes DNA damage and a carcinogenic effect. Adverse effects have been reported in long term usage of hydroquinone and therefore, the use of hydroquinone in cosmetic products was banned in the European Union and is under scrutiny by the United States Food and Drug Administration (FDA).

Tyrosinase inhibition has been indicated as an important target for inhibition of melanin production (Akhtar *et al*., 2014). Most of the enzyme inhibitors such as kojic acid, tropolone and arbutin are structurally similar to L-DOPA and tyrosine, which act competitively for tyrosinase (Akhtar *et al*., 2014) Naturally derived compounds have a major contribution for the development of effective inhibitors for treatment with low side effects. Chalcones from a group of polyphenols, which are widely distributed in plants, possess diverse pharmacological activities including anti-inflammatory, anti-pyretic, analgesic, bactericidal, and anti-cancer and anti-oxidant (Chan *et al*.,

2014). Naturally occurring compounds such as flavonoids, stilbenes, resorcinols and chalcones have been known as potential tyrosinase inhibitors.

Effective tyrosinase inhibitors are becoming increasingly important in the cosmetic industry for skin hyperpigmentation (Guan *et al.*, 2005). Currently, arbutin and aloesin are used in the cosmetic industry as anti-hyperpigmentation agents because they show strong tyrosinase inhibition properties and do not exhibit side effects.

It has been reported that the ethanol leaf extract of *G. flanaganii* exhibited significant anti-tyrosinase activity (IC_{50}) of 32.62 µg/ml when tyrosinase was used as a substrate. The total extract also showed significant inhibition of melanin production at 6.25 µg/ml and low level of cytotoxicity with IC_{50} < 400 µg/ml (Mapunya *et al*., 2011). Out of the seven isolated compounds from the plant, the compound 2', 4', and 6'-trihydroxytrihydro chalcone exhibited significant anti-tyrosinase activity exhibiting IC_{50} of 69.15 µM (Mapunya *et al*., 2011).

2.4 Metabolomics

Metabolite profiling provides information about a plethora of metabolites and thus is an efficient tool to screen plants for novel bioactive compounds from phyto-resources (Chan., 2009). Metabolomics gives a comprehensive quantitative and qualitative analysis of all metabolites within cells, tissues or organs. High resolution ^{1}H- NMR is one method constituting a promising tool with the potential to detect and identify a large number of compounds. It is thus, a leading technique in the emerging area of metabolomics studies. So far ^{1}H- NMR has been successfully used in the area of toxicology and clinical diagnostics (Chan., 2009). Although ^{1}H- NMR is a very powerful tool in metabolic analysis, it has several disadvantages due to its relatively low resolution and the congestion of signals in certain parts of the spectra when applied to the analyses of a mixture.

Genetic or environmental conditions bring about major changes in metabolites of a plant. Metabolomics is a convenient tool to assist in understanding of such responses (Bhatia *et al*., 2014). Metabolite profiling of a species provides thorough understanding of phenotypic expression which in turn assists in plant metabolite engineering, functional genomics and physiology apart from integrating plant sciences with nutrition and human health (Trethewey., 2004). It is important to determine the entire range of metabolites of medicinal plants so as to identify bioactive

molecules. Lack of complete information of the phyto-constituents limits its application for cosmeceutical application.

Chapter 3

The effect of drying and storage on the quality of *Greyia flanaganii* leaf samples

3.1 Introduction

Greyia flanaganii is an evergreen shrub endemic to Eastern Cape, one of the three closely related species of the family Greyiaceae. This plant is very frost tolerant as it remains evergreen even in areas exposed to frost in winter. It is traditionally believed to have the ability to ward off sickness (Mbambezeli., 2005). During a study done by Bohm and Chan. (1992) it was shown to contain a mixture of flavonoid compounds (an example of known tyrosinase inhibitors).

Greyia flanaganii is one out of the 117 medicinal plant species that has been scientifically validated for skin hyperpigmentation problems (Lall *et al.*, 2014). Again out of the 50 plants belonging to different families tested for their activity on tyrosinase using L-tyrosine as substrate, ethanolic leaf extract of *G.flanaganii* showed best inhibitory activity with an IC_{50} of 32.62 ug/ml (Mapunya *et al*., 2009). It was further tested on tyrosinase using L-DOPA as the substrate exhibiting 24% inhibition of the enzyme at 200 µg/ml and further investigated for its toxicity an effect on melanin production by melanocytes *in vitro*. Tyrosinase inhibitors have become increasingly important for medicinal and cosmetic products that may be used to prevent or treat pigmentation disorders. In a previous study, bioassay guided fractionation of *G. flanaganii* extract led to the isolation of 7 compounds and the compound $2^{'}$,$4^{'}$,$6^{'}$-trihydroxydihydrochalcone exhibited significant ($P<0.005$) anti-tyrosinase activity exhibiting the IC_{50} of 69.15 µm (Mapunya *et al.*, 2011). Based on the results obtained on the leaf extract of *G. flanaganii*, it can be considered as a possible anti-tyrosinase agent and for the treatment of dermatological disorders (Mapunya *et al*., 2009).

There is therefore need for commercial production of the species to be used for hyperpigmentation solutions. However the pre-processing conditions including drying and storage of the leaf extract may cause alterations in the chemical profiles and hence the need to optimise these conditions to maintain quality of the extract with respect to anti-tyrosinase activity. Studies were carried out on four different drying methods viz oven drying, air drying (on the bench), sun drying (in a greenhouse) and freeze drying, stored in three different conditions (fridge stored, freezer stored and shade stored) and stored over a period of six months. A ^{1}H-NMR based metabolomics analysis

was conducted to determine the changes in the chemical profile of the plants during the different storage and drying conditions.

3.2 Materials and method

The objective was to note any metabolite changes and monitor anti-tyrosinase activity on leaf samples exposed to four different drying conditions and three different storage conditions. Samples were oven dried for three days at 50°C, air dried for two weeks at room temperatures on a bench in the laboratory, sun dried for one week on a bench in the greenhouse with average temperatures of 37°C and freeze dried for three days. The leaf extracts were stored in freezer, fridge and shade conditions.

3.2.1 Collection of materials

The leaves of *G. flanaganii* were harvested from two sites, University of Pretoria (GPS coordinates S25°45^{1} 2" E28°13^{1} 5"), and Mothong African Heritage site (S25° 42^{1}14" E28°20^{1} 43" on 18 December 2015 and identified at H.G.W.J Schweickerdt Herbarium (UP).*L sericea* leaves were harvested only from the University of Pretoria garden. Healthy fresh, undamaged and non-infested leaves were randomly harvested by hand from the whole plant and put in brown paper bags.

3.2.2 Sample preparation

Drying of the leaf material was stopped when a constant mass of material was reached and the duration taken is stated for each drying condition used. Commonly used drying methods in medicinal plants (sun drying, oven drying, shade drying and freeze drying) were investigated. For freeze drying, leaf samples were lyophilised in a vacuum freeze drier (LABCONCO VACUTEC) 0.010 mBar for 3 days until a constant weight was reached. For oven drying, leaves were evenly spread out in the laboratory oven (EcoTherm Labotech) at 50°C for 72 hours. The selected temperature was based on recommendations of low temperatures of between 30^{0} C and 50^{0} C (Muller and Heindl., 2006). For air drying the leaves were spread out on the bench in a well-ventilated laboratory, protected from direct sunlight, with room temperature ranging from 20°C – 22°C for two weeks. Same procedure used by Mapunya *et al.* (2009:2011) on *G flanagann*i and Sharma *et al.* (2014) on *L sericea* was adopted. For the sun dried sample, the leaves were spread on a bench in a greenhouse, protected from direct sunlight, with a temperature range of 35^{0}C - 37^{0}C for one week. For air and sun drying methods the leaves were occasionally turned to ensure

even drying. The dried leaf samples were grinded and sieved through a sieve of 0.70 mm particle size using a hand held grinder.

3.2.3 Storage

Fifty milligrams of each sample differently dried was taken for NMR analysis and bioassay analysis before storage. The dried material was put in brown paper bags and stored in three different conditions namely fridge storage at 4°C , freezer storage at -80°C, adapted from the recommendations of the freezer temperatures of between -70^0 C to -80^0 C (Bhatia *et al*.,2014) and shade storage (in a closed cupboard) at room temperature 20^0 C – 22°C. Fridge and shade storage conditions are commonly used by commercial farmers due to their low cost to preserve their plant material before selling or further processing. Although freezer storage at -80^0 C is expensive and can only be used for high value materials, it has been found to better preserve chemicals in plant materials hence it was used in this study. From each storage condition samples were taken after 3 months and 6 months and an NMR analysis, anti-tyrosinase, and anti-bacterial analysis done.

3.2.4. Extract preparation for NMR analysis

A direct extraction method for general metabolomics using water and methanol in the ratio 1:1 was used. Fifty milligrams of dried sample powder was weighed and transferred to a 2ml Eppendorff tube. To each sample, 0.75 ml of deuterated methanol and 0.75 ml of KH_2PO_4 buffer in D_2O (pH 6, 0) containing 0.1 % (w/w) TSP was added to the sample. The sample was vortexed for 2 minutes using a vortex shaker (LAB –smart MX-S). It was then ultrasonicated for 20 minutes at room temperature using a BRAXTON 1800 sonicator and then centrifuged (LABOTEC, SCIE –PLAS) for 20 minutes at room temperature at 10 000 rpm. For each sample, 650 µl of the supernatant was transferred into a 5 mm NMR tube (Norell) and subjected to ^{1}H –NMR analysis. Two replicates of each sample were done. The ^{1}H-NMR measurements were performed using a 600 MHz Varian INOVA NMR spectrometer (CSIR, Pretoria, South Africa) maintained at 26°C. The acquisition time of each spectrum was 4 minutes which consisted of 32 scans.

3.2.5 Pre-processing of data

The NMR files were pre-processed using MestReNova 10.0. Phase correction, baseline correction (Whittaker smoother) and normalisation was done. The samples were referenced to the 0.1 % TSP added to each sample. The filter and smooth factors kept constant throughout the process. Binning was done from 0.000 to 10.000 ppm with 0.4 ppm bins and saved as an ASCII file.

3.2.6 Data analysis

Multivariate data analysis software SIMCA 14.1 (MKS UMETRICS) was used to analyse the results using principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA) following Pareto scaling method. Water and methanol peaks were excluded 4.6-5.0 ppm and 3.18-3.22 ppm. PCA, an unsupervised analysis provides an overview of all observations of the sample, revealing how the observations are clustered in the metabolomic data based on the factor at hand. Scatter plots of the PCA scores compared samples between and within treatments and thus identify groupings, trends and strong outliers. In the absence of a distinct discrimination pattern in the metabolic fingerprints, subsequent analysis was carried out using OPLS-DA, a supervised pattern recognition method. This method separates the systematic variation in the X-matrix into two parts with one part linearly related to the Y-matrix and one is unrelated to the Y-matrix in order to see the differences or similarities between the samples by the factors measured. In the case where groupings, trends or patterns were noted, contribution plots were used to determine the NMR regions responsible for the grouping of the samples. In this study compounds responsible for the variations were not identified.

3.3 Results and discussion

The effect of drying and storage on the quality of *Greyia flanaganii* leaf samples

3.3.1 Impact of drying on quality of leaf material

The PCA scores plot **(fig 3.1)** did not show noticeable and clear separation into groups implying that there is no significant differences ($P<0.05$) in chemical metabolites of samples subjected to different drying techniques. Even after the six months of storage the different drying techniques did not influence the preservation of the chemicals in the samples as indicated in the PCA scores plot **(fig 3.2),** R2X (cum) 0.5036, Q^2 (cum) 0.202. The PCA scores plot failed to separate the samples into groups. Comparison of the ^{1}H-NMR spectra of the four differently dried materials **(fig 3.3)** shows no differences ($P<0.05$) in the metabolites profile. The metabolites were stable and not affected by the different drying conditions used (high temperatures, 55°C in oven dried, 35°C in sun drying, room temperatures, 25°C in shade storage and low temperatures in freeze dried). This can probably be explained, because the temperatures were not high enough to destroy the volatile and heat sensitive bioactives in the extract especially the flavonoids and chalcones which are the major polyphenols in the species. These results contrast with findings of Nogemane and Prinsloo. (2016) on a related *Greyia* species (*Greyia radlkoferi*) and a study by Mediani *et al*.

(2012) on a different species *Cosmos caudatus*, which showed differences in metabolite profile dried in different conditions -80°C, 44.5°C and 25°C .However the results are in agreement with studies on olive leaf extract by Magarita *et al.* (2015), who observed no remarkable differences between air dried samples at 120°C and vacuum dried at 55°C in terms of phenolic characterisation.

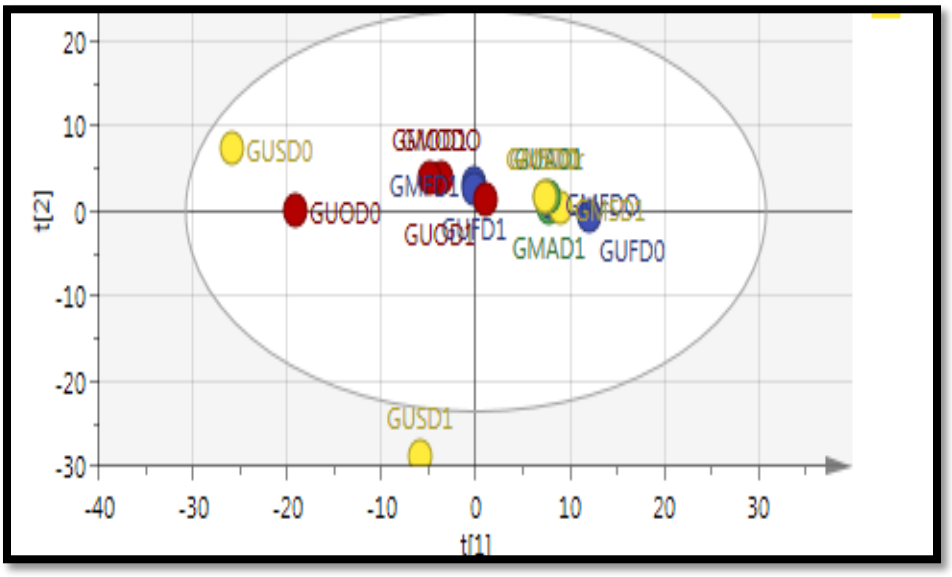

Fig 3.1.PCA score showing *G. flanaganii* samples from two locations (GM and GU) dried in four different conditions before storage where green = air dried (AD), blue= freeze dried (FD), red = oven dried (OD) and yellow = sun dried (SD).Two replicates for each drying condition. R2X (cum) = 0.8754 and Q^2 (cum) = 0.731

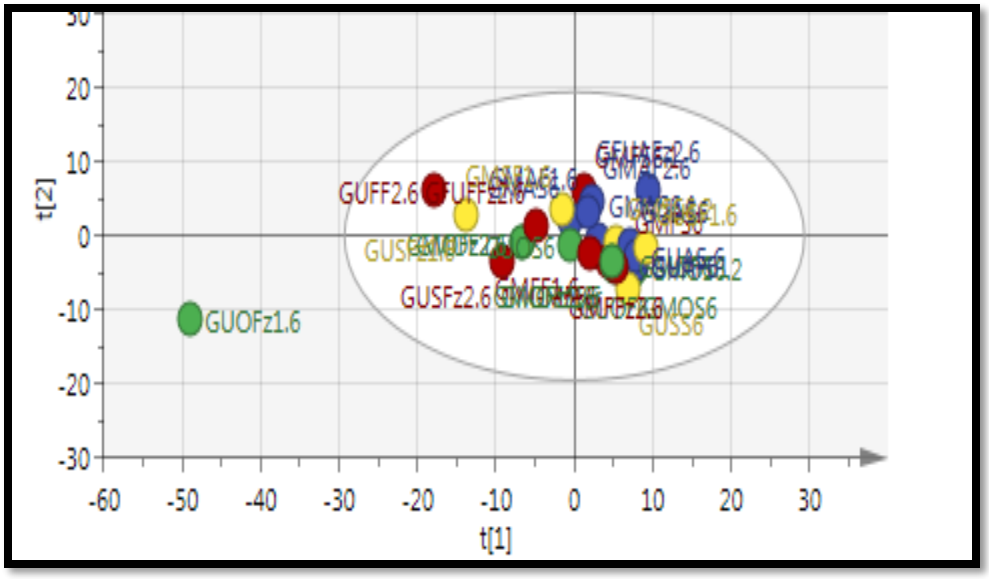

Fig 3.2. PCA score plot showing samples dried in different conditions after six months of storage where green = oven dried, blue = air dried, red = freeze dried, and yellow = sun dried. Two replicates in each storage condition of Freezer (Fz), Shade (S) and Fridge (F). R2X (cum) = 0.5036 and Q^2 (cum) = 0.202

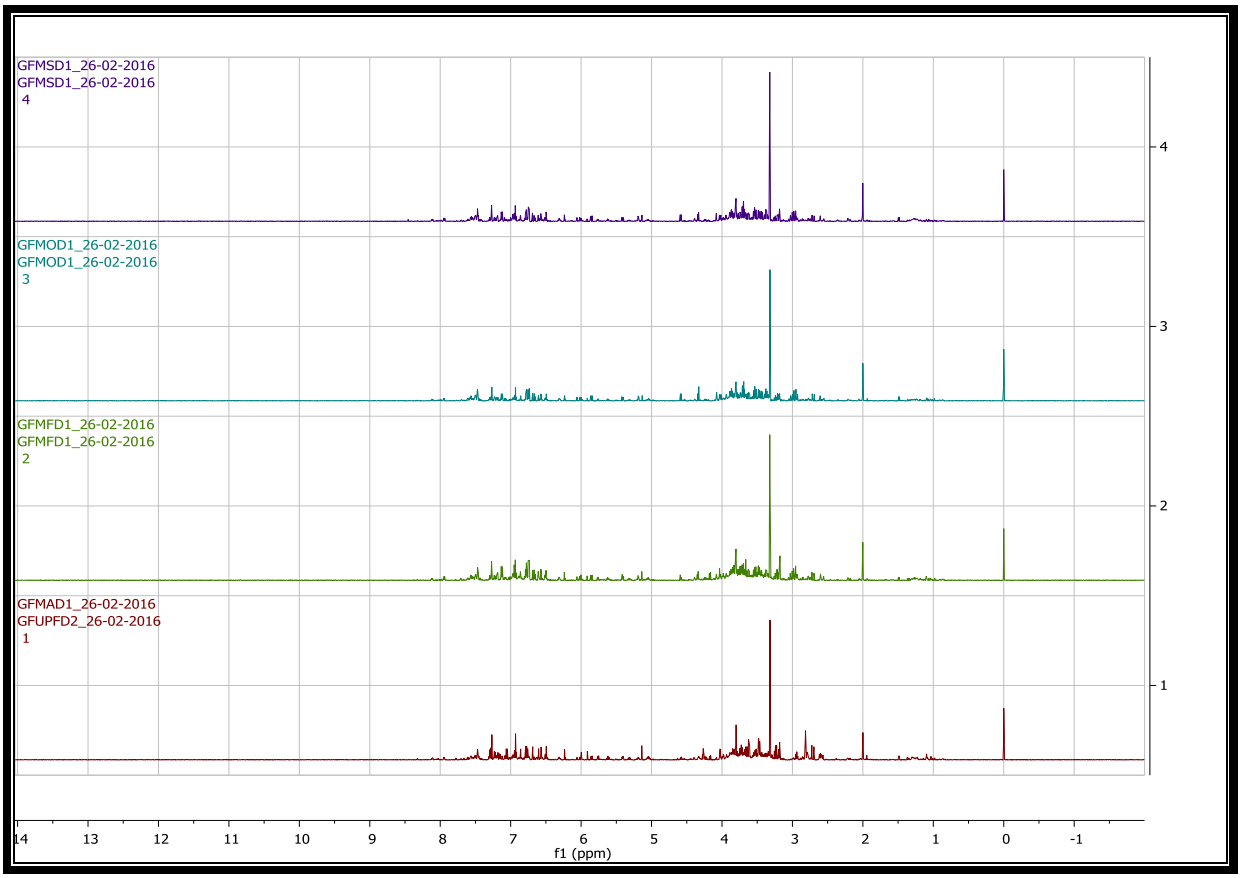

Fig 3.3. NMR spectra of one sample (*G.flanaganii* from Mothong Heritage site) dried in four different conditions and stored for six months in three different conditions. (1 = air dried, 2 = freeze dried, 3 = oven dried and 4 = sun dried).Each sample represents all the different storage conditions

3.3.2 Impact of Storage on chemical composition of the leaf materials

Storage condition PCA score **(fig 3.4)** and OPLS - DA score **(Fig 3.5)** did not cause any significant change ($P<0.05$) in the metabolite profile of the dried leaf samples over the six months of study. The PCA score shows no separation into groups. The powdered dried leaf sample metabolites are very stable over time and not easily altered by storage conditions in shade, fridge and freezer conditions. Even the initial dehydration techniques did not significantly affect the bioactive composition, and bioactive stability in six months of storage in the different environments. The results agree with findings of Magarita *et al*. (2015), on olive leaf extract where the storage condition (refrigeration, room temperature, hot environments) did not influence the content of the

major polyphenolic compounds quantified in the extract. Similarly, extracts of *Schotia brachypetala* Sond (Fabaceae) leaves did not show changes in chemical composition when stored for 18 months at -15°C (McGaw., 2001). However different results were obtained on other plant species. Bioactive composition (total phenolic and total flavonoids) content of *Moringa oleifera* leaf extract decreased after six months of storage at 25°C and 40°C (Vongsak *et al.*, 2013). Similarly, Laher *et al*. (2013) observed changes in chemical composition of tested plants (*Ocimum basilicum, Senna petersiana, and Hypoxis hemerocallidea*) over storage time. Flores *et al*. (2014) studied the storage of spray dried blueberry pomace extract for 42 days at different temperatures, observed an increase in the total phenolic and AC of the extracts. After the *in vitro* biological activity and chemical composition study of nine frequently used South African medicinal plants, Stafford *et al.* (2004), observed changes in the TLC fingerprints of material after storage. This confirms that storage has an effect on certain plant material.

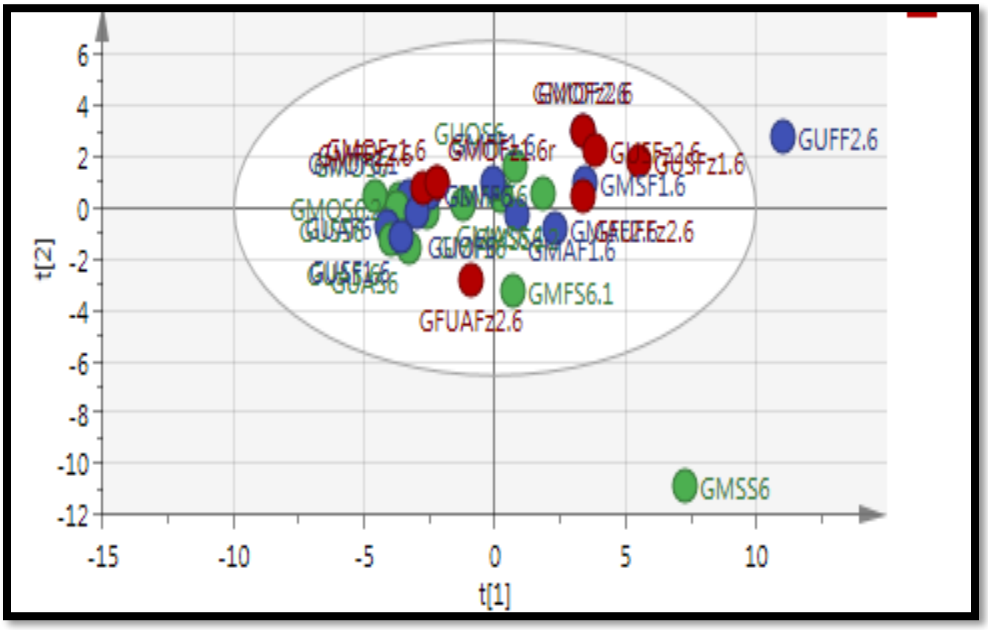

Fig 3.4. PCA scores plot showing all *G.flanaganii* samples differently dried and stored for six months in three different storage conditions (green = shade stored, blue = fridge stored, red = freezer stored). Two replicates of each sample in each storage condition.R2X (cum) = 0.9732 and Q^2 (cum) = 0.894

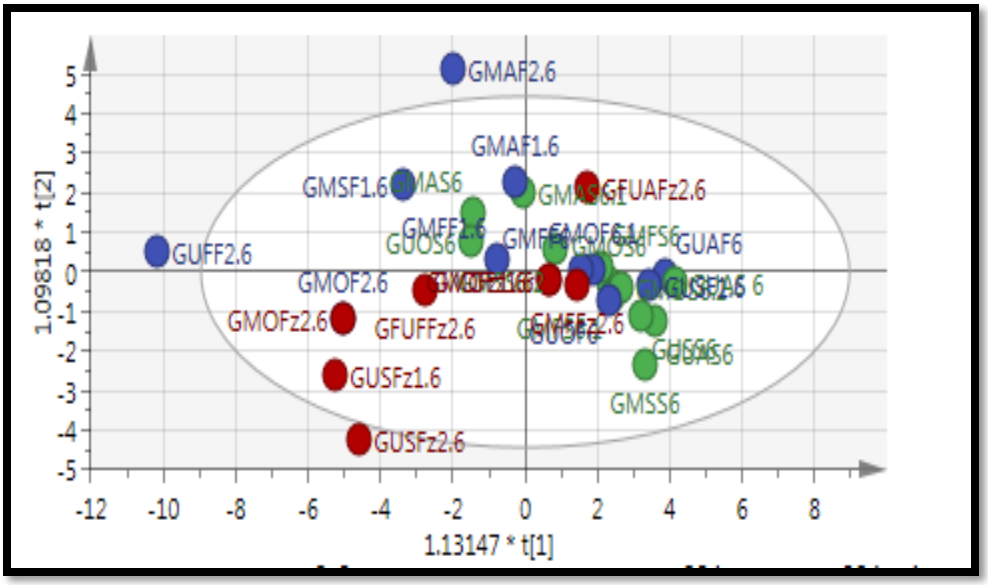

Fig 3.5.OPLS –DA scores plot of all *G.flanaganii* samples differently dried, showing no significant difference (P<0.05), after six months of storage in the three different storage conditions (green = shade stored, blue = fridge stored and red = freezer stored).Two replicates for each sample.R2X (cum) = 0.935 and Q^2 (cum) = 0.608

3.3.3 Impact of storage duration on chemical composition

The PCA scores plot **(fig 3.6)** shows samples after three and six months of storage grouping together, although not clearly, separately from samples before storage, R2X (cum) 0.866, Q^2 (cum) 0.8466. Freshly dried samples slightly differed from stored dried samples implying that time of storage caused some minor chemical variations in the samples. However a contribution plot did not show chemicals responsible for the minor variations in samples suggesting that the differences are not significant. The PCA scores plot shows no separation of samples stored for three and six months **(fig 3.7)**. From three months to six months the chemicals in the samples are significantly the same. This means that minor chemical variations in extracts occur in the first three months of storage after which they remain stable. In another study extracts of *Schotia brachypetala* Sond (Fabaceae) leaves stored for 18 months at -15°C did not show changes in chemical composition (McGaw., 2001) and on *Combretum erythrophyllum* (Burch) (Ellof., 1999). However, different results were obtained on other plant species. Bioactive composition (total phenolic and total flavonoids) content of *Moringa oleifera* leaf extract decreased after six months of storage at 25°C and 40°C (Vongsak *et al*., 2013). Laher *et al*. (2013) observed changes in chemical composition

of tested plants (*Ocimum basillicum*, *Senna petersiana* and *Hypoxis hemerocallidea*) over storage time. Flores *et al*. (2014) studied the storage of spray dried blueberry pomace extract for 42 days at different temperatures, and observed an increase in the total phenolic of the extracts.

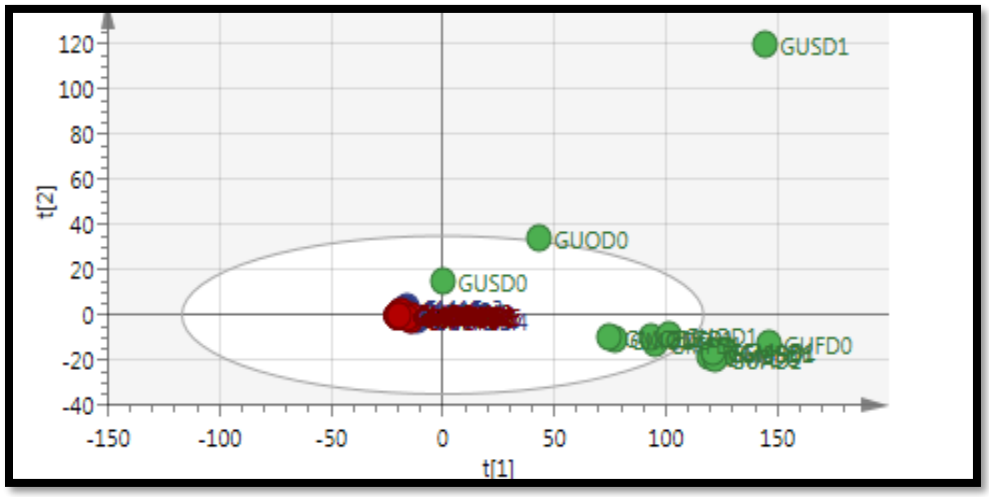

Fig 3.6.PCA scores plot of *G.flanaganii* samples differently dried before storage, stored for three and six months stored in three different storage durations (green = before storage, blue = after 3 months and red = after 6 months of storage).R2X (cum) 0.866, Q^2 (cum) 0.8466.

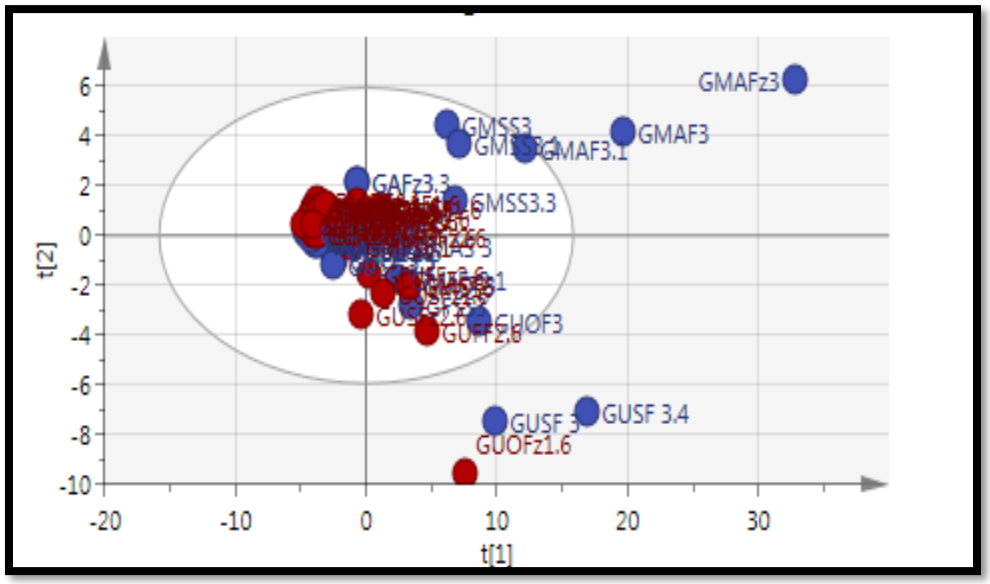

Fig 3.7.PCA scores plot showing no significant difference in differently dried *G.flanaganii* samples stored in different conditions for three months (blue) and six months (red).R2X (cum) = 0.936 and Q^2 (cum) = 0.847

3.3.4. Impact of different locations on the chemical composition of the leaf materials

The OPLS – DA scores plot **(fig 3.8)** shows samples from Mothong heritage site grouping separately from samples harvested from the University of Pretoria when stored for six months. The chemical composition in the samples from these two locations is different. The longer the duration of storage the more observable variations in the samples from the two areas. Since both locations are in the same agro-ecological region, the difference can be attributed to the different microclimate and geographical aspect of the two areas. Mothong Heritage site is located on a mountain site whereas University of Pretoria is on relatively flat lowland. Another cause of difference maybe the possibility of hybrid plant material of closely related species in the genus at Mothong Heritage site.

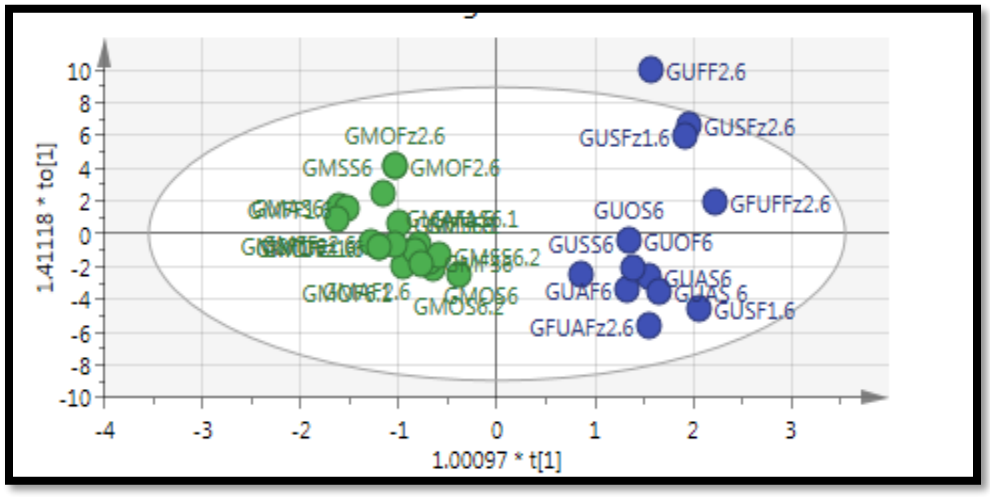

Fig 3.8. OPLS-DA score showing differently dried *G.flanaganii* samples from Mothong Heritage site (GM) in green grouping separately from samples from the University of Pretoria garden (GU) in blue after six months of storage in different conditions (Fz=freezer, F=fridge, S=shade).Two replicates for each sample.R2X (cum)= 0.7182 and Q^2 (cum)= 0.9548.

To show the chemicals significantly different in the extracts from the two locations a contribution plot was done **(fig 3.9).** The contribution plot shows the NMR regions positively associated with Mothong African Heritage site (above the line) and the NMR regions negatively associated (below the line). These peaks indicate the NMR regions which are linked to specific compounds.

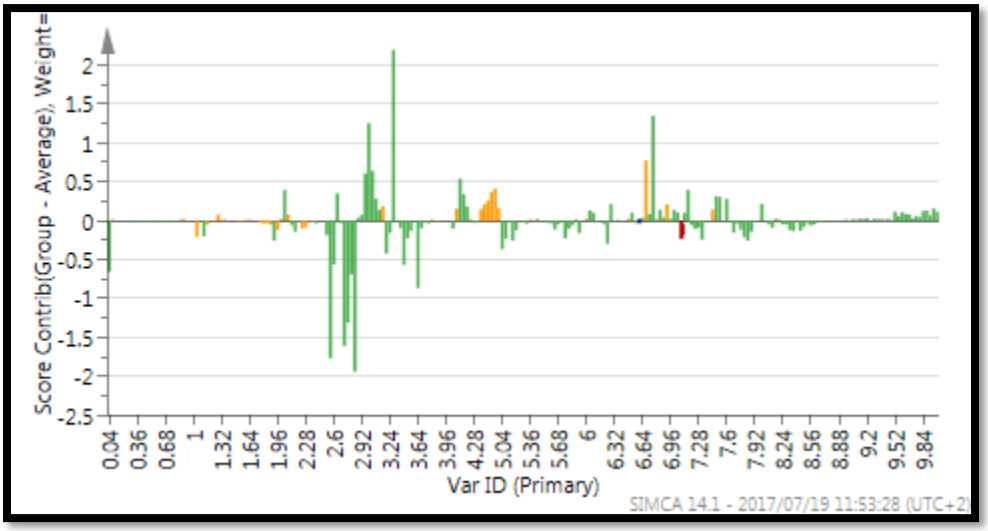

Fig 3.9.Contribution plot showing metabolites responsible for separating dried samples harvested from Mothong and University of Pretoria after six months of storage.

3.4. Conclusion

Drying condition and storage condition has no effect on the metabolite content of *G.flanaganii*, but the location of the species has an effect on the metabolite content. The longer the samples from different locations are stored, the more differences in the metabolite content could be observed. The cultivation site and uniformity of planting material is very important since it affects the chemical composition of the species. Therefore quality assessment of samples is crucial before commercial cultivation of the species for commercial production. Evaluation of anti-tyrosinase activity of samples will confirm if the difference in chemical composition significantly affect the species as a hyperpigmentation agent.

Chapter 4

The effect of drying and storage on the quality of *Leucosidea sericea* leaf samples

4.1 Introduction

Leucosidea sericea which is commonly known as Oldwood is the only species in the genus (Nair *et al*., 2012). It has a narrow global distribution and is mainly found in Southern Africa where it occurs abundantly in South Africa and Lesotho and to a lesser extent Swaziland and Zimbabwe. In South Africa, *L. sericea* occurs in seven provinces namely Eastern Cape, North West, KwaZulu-Natal, Free State, Gauteng, Mpumalanga and Limpopo (Nair *et al*., 2012).

Through different chromatographic (eg TLC) and spectroscopic techniques (eg NMR, LC –MS, GC –MS, FTIR) several compounds have been extracted and isolated. These include phenolics and associated compounds including flavonoids, gallotannins and condensed tannins (Aremu *et al.*, 2011; 2010): long chain fatty alcohols (phytol acetate, triacontanol, phytol and (E)-3, 7, 11, 15-tetramethylheptadec-2-ene-1, 17-diol) and one phloroglucinol derivative (alpha kosin), (Sharma and Lall., 2014) and essential oils (Pitso and Ashafa., 2015). These isolated compounds exhibited different biological activities including anti-microbial, anti-inflammatory, cytotoxic and anti-oxidant properties. The antibacterial activity of ethanol extract of *L. sericea* inhibited bacterial growth and exhibited a noteworthy MIC value of 15.6 µg/ml against *Propionibacterium acnes*. Alpha kosin exhibited the best anti-bacterial activity (MIC = 1.9 µg/ml) against *P. acnes* as compared to tetracycline (positive control) with a MIC value of 3.1 µg/ml (Sharma and Lall., 2014). Even though triacontanol and phytol had no significant inhibitory effect against *P. acnes* (Sharma and Lall., 2014), their antibacterial potential against other microbes such as *Mycobacterium tuberculosis, M. avium* (Rugutt and Rugutt., 2012), *Escherichia coli, Staphylococcus aureus, Bacillus cereus* and *Lactobacillus acidophilus, (*Upadhay *et al*., 2010), have been documented.

Research has been carried out by Sehlakwge and Prinsloo. (2016), on the best propagation method and effect of seasons on anti-acne activity for the species to be grown commercially for use as a solution for acne. This study investigated the effect of drying and storage methods on the bioactive profile and subsequent anti-bacterial activity (*Staphylococcus aureus*) of the leaf extract of the species. Recommendations on the optimum drying method and storage conditions will be made if the species is to be commercialised

4.2 Materials and methods

4.2.1 Collection of materials

Leaves of *L. sericea* were harvested from the University of Pretoria on 18 December 2015 and identified at H.G.W.J Schweilckerdt Herbarium. Healthy leaves were randomly harvested by hand from the plant.

4.2.2 Sample preparation

The same procedure was followed as in the previous chapter **3.2.2**

4.2.3 Storage

The same procedure was followed as in the previous chapter **3.2.3**

4.2.4. Extract preparation for NMR

The same procedure was followed as in the previous chapter **3.2.4**

4.2.5 Pre-processing of data

The same procedure was followed as in in the previous chapter **3.2.5**

4.2.6 Analysis of data

The same procedure was followed as in the previous chapter **3.2.6**

4.3 Results and discussion

4.3.1 Effect of drying on quality of *Leucosidea sericea* leaf materials

The PCA scores plot **(Fig 4.1)** did not show any significant difference ($P<0.05$) between the different drying methods investigated (sun drying, air drying, oven drying and freeze drying).The composition and concentration of metabolites were not affected by the drying conditions. The bioactives are therefore stable in the conditions investigated. These results disagree with Pitso and Ashafa. (2015) on the effect of air drying, sun drying, oven drying and microwave drying on the composition of essential oils from same species. They concluded that sun drying resulted in highest yield of compounds compared to other methods. The findings of this study concurs with results from Sefidkon *et a*l. (2006) where no significant difference was observed between oil yield of oven dried, shade dried and sun dried samples of *Satureja hortensis*. In other studies the observation was that drying method had no significant effect on chemical components (total compounds) but

on proportion of various components (Omidbaigi *et al.*, 2004), on Roman chamomile and on *Helichrysum odoratissimum* (Asekun *et al*., 2007).

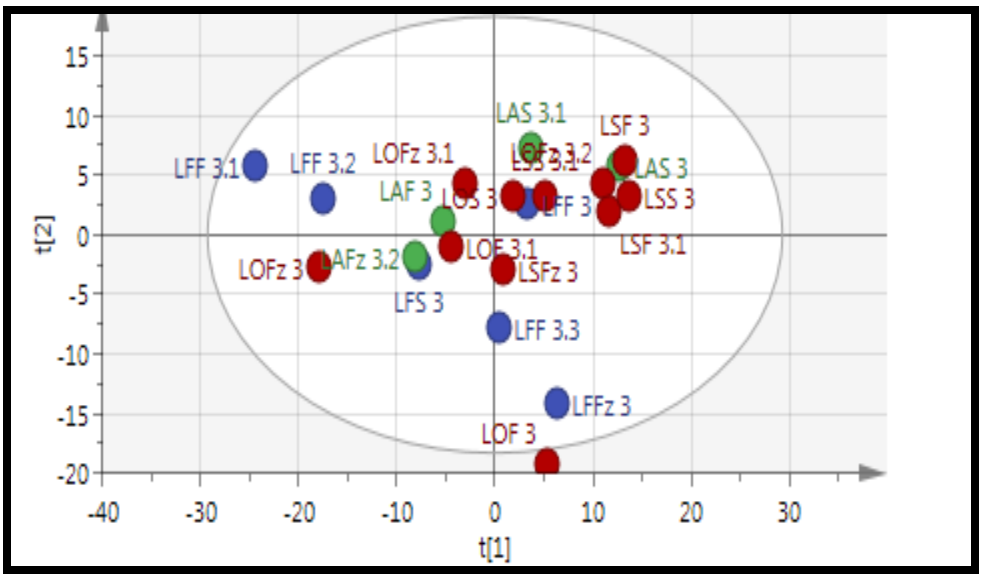

Fig 4.1. PCA scores plot showing no significant difference (P<0.05) in *L.sericea* samples differently dried (green = air dried, blue = freeze dried and red = oven dried) and stored for three months in different conditions. Two replicates for each sample. R2X (cum) = 0.987 and Q^2 (cum) = 0.759

4.3.2 Impact of storage duration on quality of *Leucosidea sericea* leaf material

The PCA **(fig 4.2)** and OPLS-DA scores plots **(fig 4.3)** shows the samples after three months and six months of storage (irrespective of the storage condition) grouping together separate from the samples before storage. This shows that storage duration caused some changes in the metabolite composition compared to the freshly dried samples. The phytochemicals in the dried leaf samples slightly changed in the first three months of storage after which there was no alterations up to six months of storage. **Fig 4.4** shows no significant difference (P<0.05) between samples stored for three and six months. The metabolites encountered initial metabolite alterations and then remained stable. Drying samples removes moisture, reduces enzymatic and biological activity which cause changes in the phytochemicals hence the differences noted for the first three months. The NMR metabolite spectra **(fig 4.5)**, shows the samples before storage and storage after six months. There are notable changes in the profiles of the two samples concurring with the clear clustering that was

obtained in the scores plots. The spectra show peaks at same regions suggesting they contain same compounds but shows differences in concentrations.

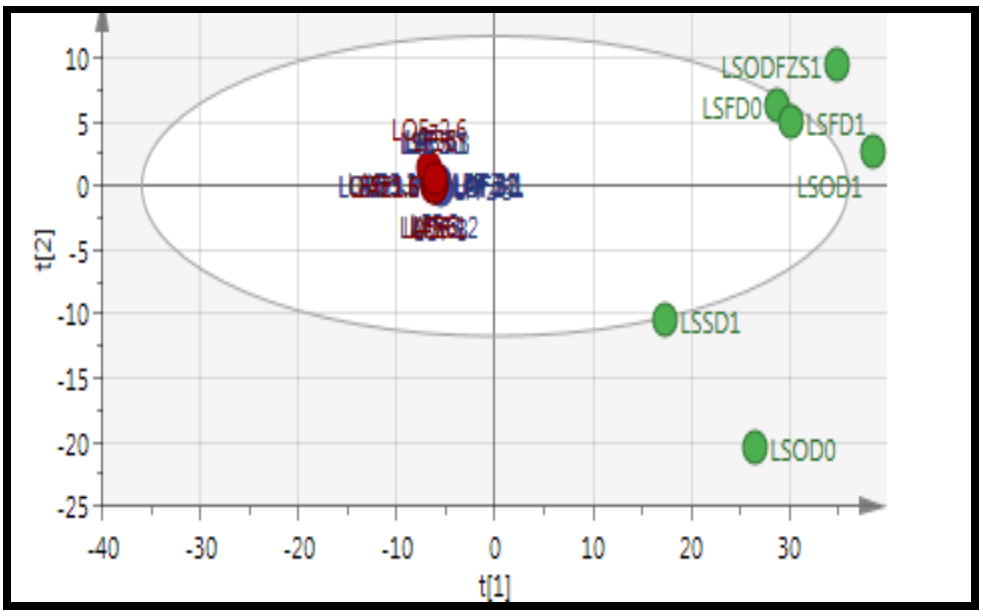

Fig 4.2. PCA scores plot showing significant differences (P<0.05) in samples before storage (green) and samples after storage (blue = three months of storage and red = six months of storage).Three (blue) and six months (red) stored samples grouped together separately from freshly dried sample (green). R2X (cum) = 0.657 and Q^2 (cum) = 0.809

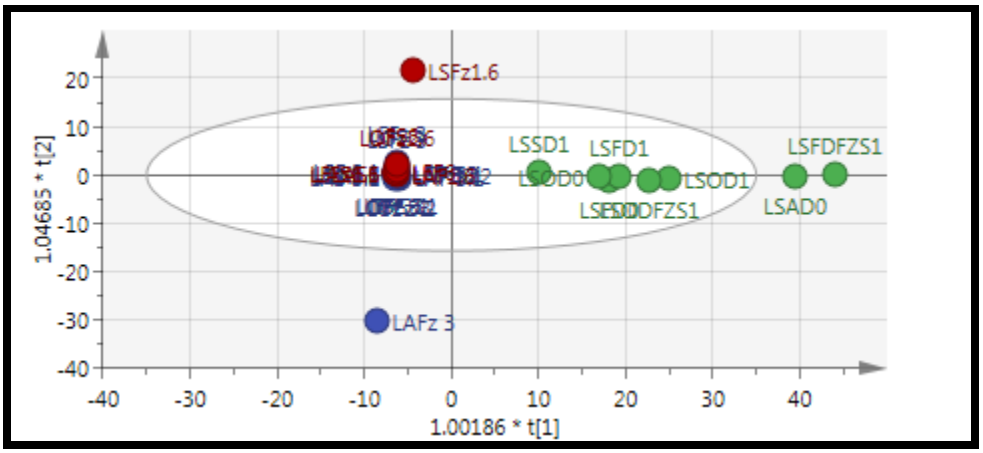

Fig 4.3. OPLS-DA scores plot showing *L. sericea* samples (green = before storage, blue = three months of storage and red = six months of storage).R2X (cum) = 0.831 and Q^2 (cum) = 0.9492

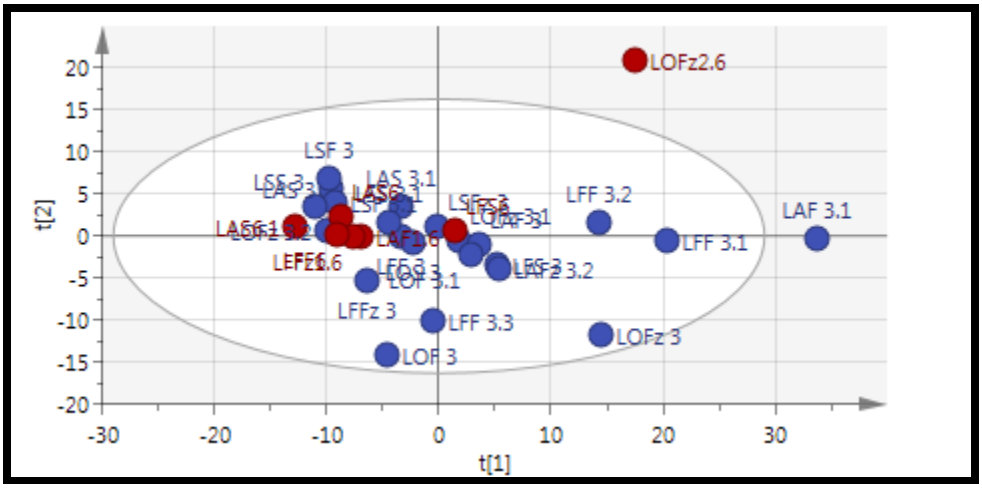

Fig 4.4. PCA scores plot showing no significant differences (P<0.05) between *L.sericea* samples differently dried and stored in different conditions for three months in blue and six months in red. R2X (cum) = 0.988 and Q^2 (cum) = 0.827

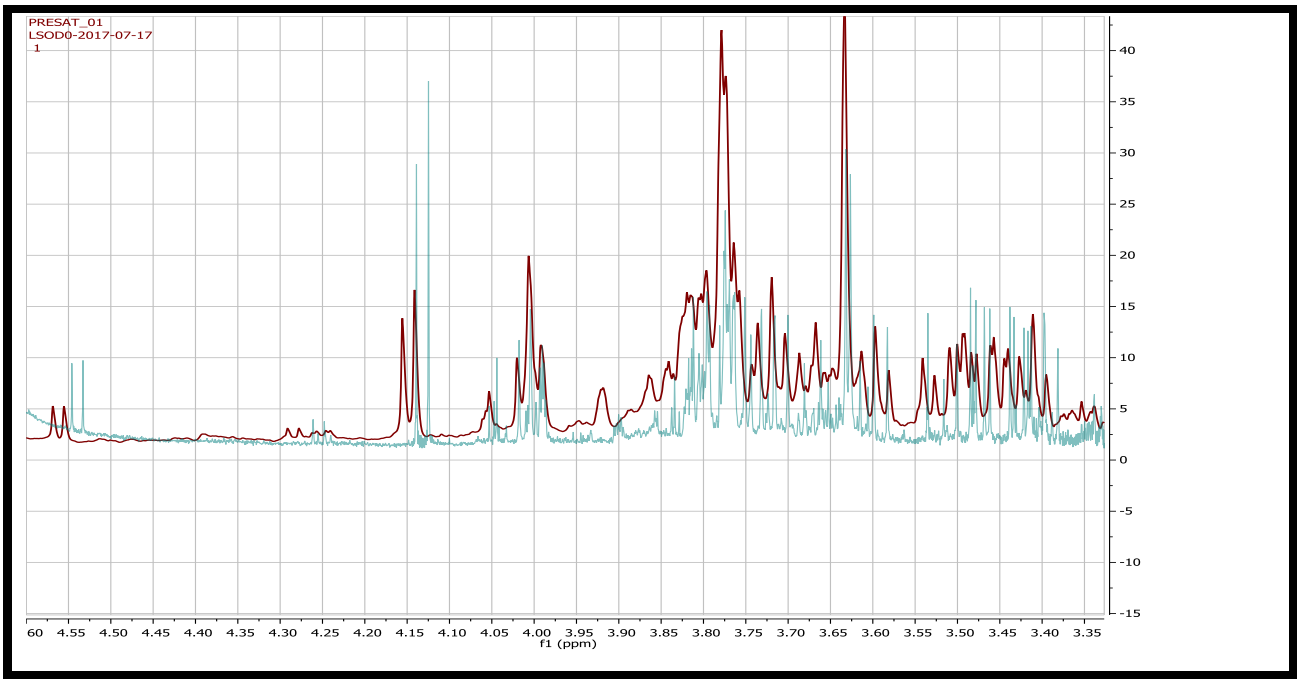

Fig 4.5. NMR metabolite spectra of samples before and after six months of storage. Red = fresh oven dried and blue = oven dried and fridge stored for six months. Each spectra is from samples representing the others.

4.3.3 Effect of storage conditions on quality of leaf materials

The study investigated different storage conditions on the quality of *L. sericea* dried leaf samples. The PCA scores plot **(fig 4.5)** did not separate into distinct groups indicating that there is no significant effect (P<0.05) of storage conditions on the metabolites in the leaf samples up to a period of six months, R2X = 0.825, Q^2 = 0.638. The PCA scores plot results suggests that the dried samples can be stored for six months in any of these conditions and still remain chemically stable. The metabolites in the dried stored samples remained the same as that of freshly dried samples confirming the stability of the metabolites in the different storage conditions.

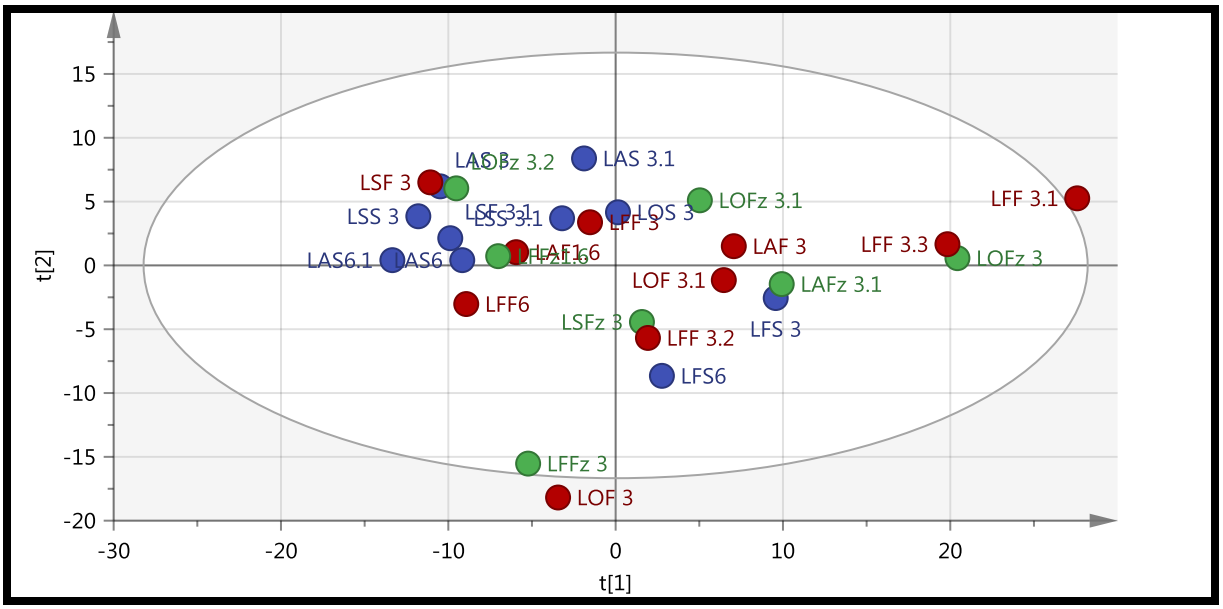

Fig 4.6. PCA scores plot showing no significant difference (P<0.05) in samples stored in different conditions (green = freeze storage, blue = shade storage and red = fridge storage).R2X (cum) = 0.825 and Q^2 (cum) = 0.638

4.4 Conclusion

The drying methods and storage conditions did not result in a significant difference (P<0.05) in the metabolites in *L. sericea* leaf extracts. The metabolites are stable and can be preserved in different drying and storage conditions. Storage duration has a significant effect (P<0.05) on the

metabolites of the species. There is a significant difference in metabolite concentrations between freshly dried and leaf samples stored for three months. Generally freshly dried samples contains higher concentrations of the same metabolites than stored dried leaf samples. Leaf samples stored for three and six months contain the same metabolites composition and concentration. The anti-bacterial activity will confirm the quality of the leaf extract after exposure to the different drying, storage condition and durations. It is important to check the activity of samples after three and six months of storage as the profiles clearly changed.

Chapter 5

The effect of drying and storage on the anti-bacterial activity of *Leucosidea sericea* plant leaves

5.1 Introduction

Staphylococcus aureus, also known as golden staph, is a gram positive, round shaped bacterium frequently found in the nose, respiratory tract and on the skin. Although *S. aureus* is not always pathogenic and commonly found as a commensal, it is a common cause of skin infections including abscesses. Pathogenic strains often promote infections by producing virulence factors such as protein toxins and the expression of a cell surface protein that binds and inactivate antibodies (Maeda., 2008).

Staphylococcus aureus can cause a range of illnesses, minor skin infections such as pimples, impetigo, boils, cellulitis, folliculitis, carbuncles, scalded skin syndrome and abscesses (Maeda *et al*., 2008). *Staphylococcus aureus* is extremely prevalent in persons with atopic dermatitis, a form of the common skin disease eczema. Atopic dermatitis has a well-established association with skin colonisation or infection by *S. aureus*, which can exacerbate the disease (Meylan *et al*., 2017). It promotes skin inflammation through multiple pathways. Increased colonisation of the skin surface by *S.aureus* leads to the secretion of virulence factors including phenol-soluble modulins and proteases .These can stimulate mast cell degranulation, IL -4 release, increased IgE levels and increased skin inflammation (Meylan *et al* .,2017). *Staphylococcus aureus* is mostly found in fertile, active places, including the armpits, hair and scalp. Large pimples that appear in those areas may exacerbate the infection if lacerated. This can lead to staphylococcal scalded skin syndrome, a severe form of which can be seen in newborns. The opportunistic pathogen *S. aureus* is a common coloniser of the human skin but once overcoming the skin barrier it may cause a variety of pyogenic and systemic infections (Karsten ., 2017).

Leucosidea sericea (the sole species in this genus) is a tree found in Southern Africa and possesses several therapeutic effects against infection diseases in humans and livestock. The species contain diverse phytochemical composition which includes a rich source of essential oils and different classes of phytochemicals (phenolics, phloroglucinols, cholestane, triterpenoids and saponins). Polyphenols are known for their anti-inflammatory and anti-oxidant activity as well as anti-bacterial properties. Documented biological activities which were mainly observed under *in vitro*

systems include anti-microbial, anti-parasitic, acetylcholinesterase inhibition and anti-inflammatory properties (Mafole *et al*., 2017). A quantitative pharmacological screen of both leaves and stems of *L. sericea* (Aremu *et al.*, 2010, 2011) uncovering its biological activity has been done using different solvents which are petroleum ether, dichloromethane, ethanol and water .

Extracts with MIC values less than 1.0 mg/ml were considered as having good anti-microbial activity quoted by Mafole *et al*. (2017). Petroleum ether, dichloromethane and ethanol leaf extracts of *L. sericea* showed a significant antimicrobial activity with an MIC value of 0.098 mg/ml, 0.025 mg/ml and 0.195 mg/ml respectively against *S. aureus* bacteria. In another study a total of 21 different bacterial (gram positive and gram negative) strains have been tested using microplate minimum inhibitory concentration (MIC) determination and disc diffusion assays. *Leucosidea sericea* showed a noteworthy inhibitory activity of 0.02 mg/ml and 0.08 mg/ml against *S. aureus* and *Pseudomonas aeruginosa* respectively (Aremu *et al*., 2010).

Anti-bacterial assays were performed to determine the effect of storage conditions, drying conditions and storage time on the biological activity of the samples.

5.2 Materials and methods

The leaf material was harvested, dried and stored as explained in chapter **3** section **3.2.1, 3.2.2** and **3.2.3** respectively.

5.2.1 Bioassay extract preparation

The dried leaf samples (2 g), was mixed with 200 ml of solvent (absolute ethanol). The mixture was put in a shaker for 24 hours at room temperature to allow compounds to leach into the solvent. The extract was then filtered through filter paper (Fisher brand 90mm). The filtrate was concentrated under vacuum (BUCHI, rotavapor, R-200) to remove the solvent and yield the dry extracts.

5.2.2 Determination of anti-bacterial activity

The extract of the plant samples were tested against *S. aureus* by determining the minimum inhibitory concentration (MIC) values obtained by a micro dilution method. This assay was done using the methods as described by Mapunya *et al*. (2011), with slight modifications. The ethanol extracts were dissolved in 10% DMSO to obtain a stock solution of 2 mg/ml. The positive control

(tetracycline) was dissolved in sterile distilled water to obtain a stock solution of 0.2 mg/ml. The 96 well plates were prepared by dispensing 100 µl of the nutrient broth into each well. Hundred micro litres (100 µl) of the plant stock samples and positive control were added to the first row of wells in triplicates. Two-fold serial dilutions were made in broth to give concentrations of 500 to 3.9 µg/ml and 50 to 0.3 µg/ml for the plant extracts and positive control, respectively. The 72 h culture of bacteria was dissolved in nutrient broth and the suspensions were adjusted to 0.5 McFarland standard turbidity at 550 nm. About 100 µl of this bacterial inoculum with 105—106 CFU/ml was then added to all the wells. The wells with 2.5% DMSO and bacterial suspension without samples served as the solvent and negative controls, respectively. The plates were then incubated at 37°C for 24 h under anaerobic conditions. The MIC value was determined by observing the colour change in the wells after the addition of presto blue (defined as the lowest concentration that showed no bacterial growth).

5.3 Results and discussion

5.3.1 Effect of drying on anti-bacterial activity of *Leucosidea sericea* leaf materials

Drying conditions significantly affected the microbial activity of the samples as shown by the MIC values in the **table 5.1.**

Table 5.1. MIC values in (µg/ml) of differently dried plant samples of *L. sericea* against *S. aureus*

DRYING CONDITION	OVEN DRIED	SUN DRIED	AIR DRIED	FREEZE DRIED
MIC (mg/ml)	0.25	0.5	0.5	0.125

Dried leaf samples from all the drying methods investigated showed a good anti-bacterial activity according to Mafole *et al*. (2017), who concluded that samples with MIC value less than 1 show a good anti-bacterial activity. Freeze drying conditions resulted in samples showing the highest microbial activity (MIC 0.125 µg/ml) whilst sun and air dried samples both showed lower microbial activity (MIC 0.5 µg/ml). Freeze dried samples (MIC 0.125 µg/ml) showed better microbial activity compared to the results of Mafole *et al*. (2017) where an MIC 0.195 µg/ml was obtained using the same species. However, Aremu *et al*. (2010) obtained a better MIC (0.025 µg/ml) using leaf extract of the same species. The results suggest that there are changes in

concentration of compounds which better inhibit the bacteria in freeze and oven dried samples compared to compounds in the samples dried in other conditions. The high temperatures (in oven dried) and low temperatures (in freeze drying) caused stress on cell walls due to cell damage by temperature and ice crystals. In both cases drying makes the release of compounds easier due to cell wall breakdown related to water removal (Hossain *et al.*, 2010). Again the low temperatures might have avoided the degradation or volatisation of some phytochemicals during freeze drying hence the highest anti-bacterial activity. The shorter drying time in oven dried and freeze dried samples also contributes to the higher anti-microbial activity. Garcia Viguera *et al*. (2010), observed that short drying times at high temperatures seem to preserve the phenol content better than long drying processes at mild temperatures. In another study on daylily flowers by Mao *et al.* (2006) concluded that total phenolic compounds was higher in freeze dried samples than hot air dried samples. Contrary to these findings, in another study by Magarita *et al.* (2013), freeze drying was the worst dehydration method for obtaining olive leaf extracts, providing both the lowest anti-oxidant activity and total phenolic compounds.

5.3.2 Impact of storage condition on anti-bacterial activity of *L.sericea* leaf material

Drying the raw material not only influenced the initial extract composition but also the bioactive potential evolution during storage. From previous studies, the impact of storage condition on extract constituents (including essential oils and total phenolic content) and biological activity (anti-bacterial, anti-fungal, anti-oxidant) showed varying results according to the species investigated. In this study, the effect of storage condition indicated that freeze dried samples had the best activity but the extract should be used fresh. The effect of storage duration was varied and mainly influenced by the pre-processing technique before storage and the storage conditions.

Table 5.2. MIC values (µg/ml) of samples after 3 and 6 months of storage in different conditions (fridge storage, shade storage and freezer storage)

Duration of storage		**3 months**			**6 months**		
	Before Storage	**Shade storage**	**Fridge storage**	**Freezer storage**	**Shade storage**	**Fridge storage**	**Freezer storage**
OVEN DRIED	0.25	0.5	0.25	0.5	0.5	0.125	0.25
AIR DRIED	0.5	0.5	0.125	0.5	0.5	0.25	>0.5
SUN DRIED	0.5	0.25	0.25	0.5	0.125	0.125	0.5
FREEZE DRIED	0.125	0.25	0.5	0.5	0.5	>0.5	0.5

In the current study, generally fridge storage conditions showed better anti-bacterial activity after three and six months for all the samples except for the freeze dried samples. The anti-bacterial activity in fridge storage conditions over the six months period increased when compared to the freshly dried samples except for the freeze dried sample which encountered a lower anti-bacterial activity. The low temperatures (4°C) in fridge conditions are able to reduce enzymatic activity of polyphenols oxidase and peroxidase resulting in better preservation of bioactives. Again the low temperatures allowed slow chemical activity that resulted in an increase in the phytochemicals responsible for increasing anti-bacterial activity. In fridge conditions relevant chemicals are probably converted into more active compounds or increased in concentration, during the storage period.

Interestingly shade storage conditions lowered the anti-bacterial activity of the samples over the time of storage except for the sun dried samples that improved the anti-bacterial activity and the air dried that maintained the activity. The room temperature in shade storage allowed some

chemical degradation in the samples leading to reduced anti-bacterial activity. This refutes the conclusion made by Laher *et al*.,(2013) that plants stored in the dark at room temperature should be taken as a guideline after the investigation on impact of storage on three species (*S. petersiana, O. basilicum* and *H. hemerocallidea*).

Storage in freezer conditions maintained the anti-bacterial activity of oven dried, air dried and sun dried samples. This agrees with research on the influence of storage condition on the essential oil composition of *Thymus daenensis* Celak, Rowshan *et al* .(2013), where the essential oils stored in a freezer -20°C kept its primary quality in comparison to fridge and room temperature storage condition for three months. Again the investigation of impact of storage conditions on *Hypericum perforatum* dried total extracts showed lowest decay at -20°C and highest decay in 40°C (Koyu *et al* .,2015). Very low temperatures – 70°C in freezer storage inactivated enzymatic reactions and stopped chemical reactions thus the initial bioactives were stable hence the anti-microbial activity was maintained. However the MIC for freeze dried samples increased meaning that the anti-bacterial activity was lowered.

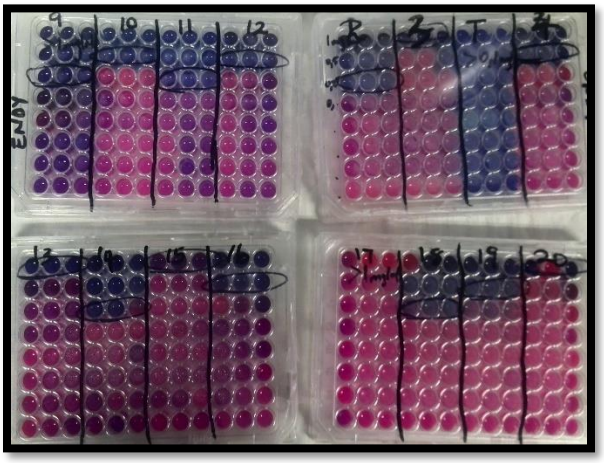

Fig 5.1 Images of plates of the colorimetric assay on the 96 well plates showing *L.sericea* extracts against *S aureus* bacteria. The blue colour shows absence of bacterial growth while the red colour indicates presence of bacterial growth.

5.4 Conclusion

Drying condition affected the microbial activity of the samples. Lyophilised samples produced best results freshly after drying and should not be stored. Storage condition and duration also affected the anti-bacterial activity. Thermal drying conditions (oven, sun and air drying) gives a

lower anti-bacterial activity immediately after drying, but increases the anti-bacterial activity when fridge stored for three to six months. Thermally dried samples require fridge and freezer storage temperatures for preservation and enhancing the bio-activity of the samples. The best storage conditions are: oven dried samples, fridge stored for six months; air dried samples fridge stored for three months; sun dried samples stored for six months under both shade and fridge conditions. Storage condition of temperature was the only factor investigated in the study. There is a need to investigate all the storage conditions including light and humidity which are known to influence chemical components in samples.

Chapter 6

Anti-tyrosinase activity of *Greyia flanaganii* leaf extracts

6.1 Introduction

Men and women, increasingly pay more attention to their skin and to take good care of skin. Skin is the largest organ in human body with a surface area of 1, 5 – 2, 0 m^2 and approximately 15 % of weight in the whole organism (Xie *et al*., 2017). Generally people are subjected to lots of skin problems i.e. freckles, wrinkles, photo aging, inflammation, comedo and skin cancer due to an abnormal intrinsic metabolism or extrinsic UV radiation of excessiveness (Sharma *et al*.,2013). Some synthetic compounds such as TiO_2, nano ZnO or kojic acid can be added to cosmetics in order to enhance physical sunscreen and lightening efficiency. These man made substances are unsafe for skin health due to some damages to the skin follicles (Sharma *et al*., 2013), skin corrosion, cytotoxicity and intensive allergy. Kojic acid has been used for decades in the cosmetic industry as an anti-melagenic agent. However, two major drawbacks of the compound are cytotoxicity and instability during storage (Mapunya *et al*., 2009). There are increasing demands for natural plant extracts to be taken into cosmetics for medicinal properties. Also compared with synthetic products, herbal products are mild and biodegradable and have low toxicity.

Tyrosinase is an oxygen oxidoreductase or a polyphenol oxidase and a multifunctional copper containing enzyme that is very critical for melanin pigment production and regulates melanogenesis within melanocytes in human (Xie *et al*., 2017). Among all the tyrosinase inhibitors and skin enlightening agents is a medicinal plant, *Greyia flanaganii*, one of the three species in the genus. The Greyiaceae family is rich in flavonoid compounds (Bohm and Chan., 1992). These secondary metabolites have been shown to inhibit the tyrosinase enzyme *in vitro*.

According to Xie *et al*. (2017), the tyrosinase inhibitory mechanism *in vitro* is related to a phenomenon of competitive inhibition with L-DOPA in natural phenolics. These substances have similar chemical structures to L-DOPA, and thus are likely to be catalysed by tyrosinase accompanying with oxygen participation. Secondly, because phenolic acids have a strong electron withdrawing ability, they can inhibit the substitution reaction between the oxygen atom of tyrosinase in oxidation state and aromatic ring due to the bonding of phenolic acid with the enzyme. Thirdly, the polyphenolic chemical structure is very similar to L-DOPA and tyrosine as a substrate catalysed by tyrosinase. They thus have a competition with these to bond with

tyrosinase so as to recede this enzyme action. Scientific evidence demonstrated that *G.flanaganii* among other species such as *Aloe ferox*, *Aspalathus linearis, Calendula officinalis* , *Crocus sativus*, *Kigelia africana*, *Sideroxylon inerme* etc. possess significant biological properties and can actively restore, heal and protect the skin (Chan *et al*., 2014).

About 50 plants belonging to different families were tested for their activity on tyrosinase using L-tyrosine as a substrate (Mapunya *et al*., 2009). An ethanolic leaf extract of *G. flanaganii* showed the best inhibitory activity (anti-tyrosinase) exhibiting an IC_{50} of 32.62 µg/ml using L-tyrosine as the substrate. It was further tested on tyrosinase using L-DOPA as the substrate exhibiting 24 % inhibition of the enzyme at 200 µg/ml. *Greyia flanaganii* was further investigated for its toxicity and effect on melanin production by melanocytes in vitro. The extract exhibited 9.89 % reduction of melanin content at IC_{50} of 6.25 µg/ml and on melanocytes was found to be 400 µg/ml (Mapunya *et al*., 2009). Isolated compounds showed good radical scavenging activity and low toxicity of the cells with reduction of melanin content of the cells (Mapunya *et al*., 2011). The plant extract has shown good anti-tyrosinase activity, anti-inflammatory activity and anti-oxidant activity and these activities are attributed to skin care ethno-botanicals. The compounds isolated from the plant are: 3S -4- hydroxyphenethyl 3-hydroxy-5-phenylpentanoate, 2', 4', 6'-trihydroxy-dihydrochalcone, 2', 6', 4'-trihydroxy-4'-methoxydihydrochalcone, 2', 6'-dihydroxy-4''-methoxydihydrochalcone, 5, 7-dihydroxyflavanone [(2S)-pinocembrin], 2', 6'-dihydroxy-4', 4'-dimethoxy dihydrochalcone and (2R, 3R)-3, 5, 7-trihydroxy-3-0-acetylflavanone.

6.2 Materials and methods

The leaf material was harvested, dried and stored as explained in section **3.2.1, 3.2.2** and **3.2.3** respectively.

6.2.1 Bioassay extract preparation

The dried leaf sample, (2 g), was mixed with 200 ml of solvent (absolute ethanol). The mixture was put on a shaker for 24 hours at room temperature to allow compounds to leach into the solvent. The extract was then filtered through filter paper (Fisher, 90mm). The filtrate was concentrated under vacuum (BUCHI, rotavapor, R-200) to remove the solvent and yield the dry extracts.

6.2.2 Anti-tyrosinase activity

The method described by Momtaz *et al.* (2008) was used with few modifications. For each drying method, 2 mg of dry extract was dissolved in 100 µl dimethyl sulfoxide (DMSO) to make a stock

solution. For every sample, 30 µl of the stock solution was added to 970 µl of 6.5 pH potassium phosphate buffer in the first well of a 24 well plate. This was then serially diluted to make eight wells of subsequent half concentrations, from the highest concentration (600µl/ml) to the lowest (37.5 µg/ml) after dilutions. Kojic acid was used as a positive control and DMSO as a negative control.

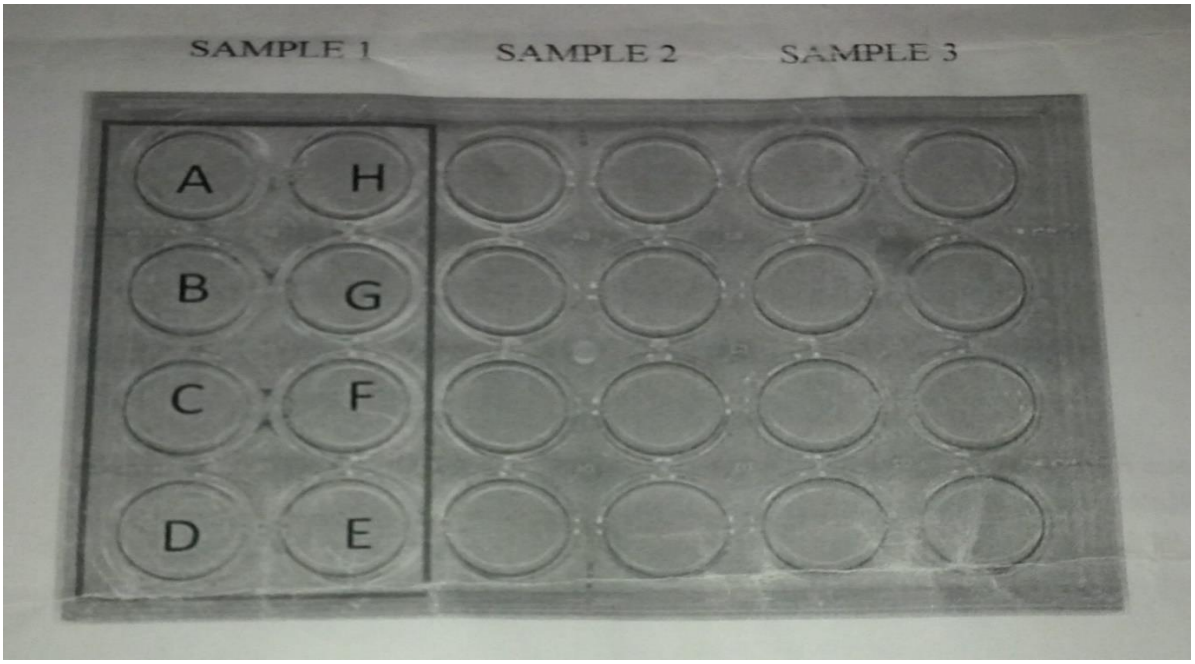

Fig 6.1. Representation of plant extract dilutions from the highest concentrations (A=600µg/ml) to the lowest concentration (H= 37.5 µg/ml) using a phosphate buffer (pH 6.5)

In a 96 well microliter plate, 70 µl of each sample solution of different concentrations were combined with 30 µl of tyrosinase (333 units/ml in phosphate buffer, pH 6.5) in triplicate. After incubation at room temperature for 5 minutes, 30 µl of substrate 2 Mm L- tyrosine was added to each well.

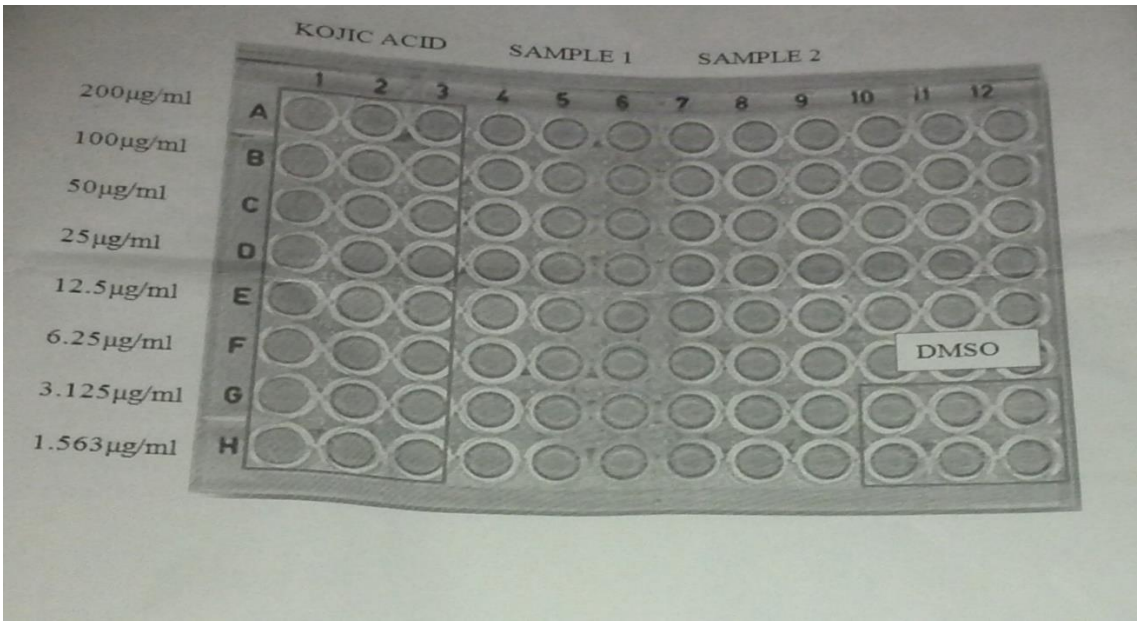

Fig 6.2. A 96-well plate representation of the tyrosinase inhibition assay showing the concentrations from the highest (top) to the lowest (bottom). Positive control (Kojic acid), DMSO (negative control) and plant extracts were tested in triplicate.

The microtitre plates were incubated for 30 minutes at room temperature. Optical densities of the wells were then determined at 492 nm with the BIO-TEK power wave XS multi –well plate reader. The fifty percent inhibitory concentration was calculated.

6.2.3 Calculation of IC_{50} values

The readings from the well plate reader were exported to excel programme, the average and standard deviation calculated for each sample using the value of DMSO as control. The formula below was used to calculate the % inhibition.

% inhibition =100-((average of sample/Average of control) x 100)

A scatter graph was plotted using the concentrations on the X-axis and % inhibition on the Y-axis, trendline inserted and the equation displayed on the chart. Using the equation on the chart with R^2 from 0.95 to 1, y intercept = 50, the IC_{50} was obtained.

6.3 Results and discussion

6.3.1 Effect of different locations of *G. flanaganii* leaf extracts on anti-tyrosinase activities

Table 6.1 showing the IC_{50} values of differently dried leaf extract samples harvested from two different locations. The IC_{50} values are the means of the three replicates. A high IC_{50} value represents a low inhibitory activity and a low IC_{50} value represents a high inhibitory activity.

Table 6.1. Anti-tyrosinase activity of *G.flanaganii* leaf extracts from different localities and exposed to different drying methods. Results are presented as the mean ±standard deviations

Drying condition	**IC_{50} (µg/ml)**			
	University of Pretoria garden	**R^2 values**	**Mothong Heritage site**	**R^2 values**
Sun dried	33.08±0.78	0.98	134.07±4.56	0.922
Oven dried	15.73±0.85	0.953	25.9±0.96	0.947
Air dried	36.86±2.01	0.971	61.9±4.85	0.957
Freeze dried	16.8±0.69	0.903	97.42±2.75	0.938
Kojic acid	24.23±0.523			0.981

Plant leaf extracts processed with different drying methods showed good anti-tyrosinase activity irrespective of the drying method and plant material source **(table 6.1)**. Dried leaf extracts harvested from the University of Pretoria garden generally showed better tyrosinase inhibiting activities compared to leaf extracts harvested from Mothong African Heritage site across all the different drying methods. In both locations oven drying produced extracts with the best IC_{50} values of 15.73 µg/ml and 25.9 µg/ml for University of Pretoria garden and Mothong African Heritage site respectively.

6.3.2 Effect of different drying methods on the anti-tyrosinase activity

All leaf extracts dried using the different techniques exhibited significant anti-tyrosinase activities using L- tyrosine substrate, **(table 6.1).** These results confirm that *G.flanaganii* leaf extracts have tyrosinase inhibiting properties as previously reported by Mapunya *et al*. (2009 & 2011). Again the same trend where all the differently dried samples showed good anti-tyrosinase activity was reported by Nogemane *et al*. (2017) on *Greyia radlkoferi* comparing three drying methods (oven,

air and shade drying) on anti-tyrosinase activity. However, the inhibition activity of the extracts varied with the drying method employed. During the drying process, some changes in the structure and bioactivity of certain metabolites might occur.

In the current study sun and air dried leaf extracts exhibited an IC_{50} value of 33.08 µg/ml and 36.86 µg/ml respectively which is comparable to the IC_{50} of 32.62 ug/ml of air dried samples obtained by Mapunya *et al*. (2011) using L-DOPA as the substrate. Oven and freeze dried ethanol leaf extracts showed best tyrosinase inhibiting activities of 15.73 µg/ml and 16.8 µg/ml respectively which is two times better when compared to the other drying methods (sun and air drying). The activity is even higher than the inhibitory activity of kojic acid (IC_{50} = 24.23 µg/ml) which was used as the positive control. This suggests that oven dried and freeze dried samples contained more phytochemicals with tyrosinase inhibiting properties. In oven and freeze drying all the storage conditions of temperature, light, air flow, and humidity are better controlled resulting in better preservation of metabolites as compared to the sun and shade dried samples. Also coupled with this, is the short drying period in oven and freeze drying, reducing the time exposure to physical contamination, chemical and enzymatic degradation of metabolites. Freeze drying is known to have high extraction efficiency because ice crystals formed within the plant matrix can rapture cell structure, which allows exit of cellular components and access of solvent, consequently better extraction. In a study by Chan *et al*. (2008), the HPLC chromatogram of leaves of *Etlingera elatior* showed greater amounts of minor compounds following freeze drying, which supports these findings. Increases in total phenolic compounds and anti-oxidant properties following thermal treatment has been attributed to the release of bound phenolic compounds brought about by the breakdown of cellular constituents and the formation of new compounds with enhanced anti-oxidant properties (Chan *et al*., 2008). The current findings contradict results of Nogemane *et al*. (2017) on *G.radlkoferi* where air dried sample exhibited the highest inhibitory activity (IC_{50} of 17.80 ug/ml) while the least activity was exhibited in oven dried samples with IC_{50} value of 32.45 µg/ml.

6.3.3 Effect of storage condition and duration on the anti-tyrosinase inhibiting activities

The drying method determines the storage method and the duration of storage in order to preserve or enhance the inhibitory activity of the leaf extracts. The stored dried samples responded differently to storage condition and duration. This agrees with the results of Koyu and

Haznedaroglu. (2015), on the impact of storage conditions on *Hypericum perforatum* where the stability of plant extract constituents differs with storage conditions of temperature, humidity and light. Freshly oven and air dried leaf samples from both locations and freeze dried from the University of Pretoria garden exhibited better inhibitory activity than the stored samples (**table 6.2),** irrespective of storage condition and duration**.** In general, storage for three and six months all resulted in a lower activity than the fresh material, with only a few exceptions. Cold storage conditions (freezer, -80°C and fridge, 4°C) could not completely preserve the unstable phytochemicals produced by these drying methods, except for some samples at six months with better activity than the fresh samples. Phytochemicals responsible for inhibitory activity suffered chemical and enzymatic degradation and become unavailable when stored. Different results were obtained by Koyu and Haznedaroglu. (2015) on *H. perforatum.* In their investigation a large number of compounds were more stable at -20°C in the sixth month.

Freeze dried leaf extracts harvested from Mothong African Heritage site showed better inhibition activity for all conditions after three months of storage and after storage for six months in fridge and room temperature conditions (highlighted in green). However cold freezer conditions could not keep the extracts stable for six months. The best inhibitory activity, (IC_{50} value of 16.8 µg/ml and 15.73 µg/ml), was obtained from the freeze and oven dried leaf extracts harvested from University of Pretoria garden respectively.

Table 6.2. IC_{50} (µg/ml) values of *G. flanaganii* leaf samples harvested from two locations (UPG = university of Pretoria garden; MHS = Mothong African Heritage site), differently dried and stored under three conditions (shade, fridge and freezer) analysed before storage, after three and six months. Tests were carried out in triplicates and the results are presented as the mean ± standard deviation and the R^2 values are between 0.95 to 1.

		STORAGE DURATION						
		BEFORE STORAGE	3 MONTHS STORAGE			6 MONTHS STORAGE		
DRYING METHOD	LOCATION		SHADE	FRIDGE	FREEZER	SHADE	FRIDGE	FREEZER
OVEN DRIED	UPG	15.73±2.374	19.24±0.860	87.07±5.475	100.17±2.77	44.7±1.422	52.85±0.433	38.82±0.853
	MHS	25.9±0.539	119.07±7.60	60.85±0.169	108.48±4.24	55.24±0.857	40.44±0.045	155.93±3.31
SUN DRIED	UPG	33.08±0.796	103.01±2.15	61.88±0.789	66.51±0..853	447.5±6.97	77.38±3.20	61.8±0.11
	MHS	134.07±3.85	146.73±3.85	74.21±1.7	73.95±3.791	108.63±5.49	63.72±1.85	69.1±2.991
FREEZE DRIED	UPG	16.8±0.169	78.17±0.543	105.07±4.701	85.63±2.56	71.89±2.57	67.12±4.14	60.91±0.73
	MHS	97.42±1.42	69.7±3.761	19.41±0.506	25.7±0.753	19.67±0.492	56.5±2.33	155.93±7.82

AIR DRIED	**UPG**	36.86±0.896	47.83±0.155	89.94±4.18	197.39±7.451	39.61±0.786	54.19±3.467	312.53±10.8
	MHS	61.9±0.169	102.41±1.68	141.38±1.07	163.83±4.18	143.22±8.09	121.67±1.74	173.12±9.53

6.4 Conclusion

Storage condition is not as important as the storage duration in the sample metabolite and activity. The activity cannot be preserved or improved by storage in any of the storage condition used. Location is significantly affecting the activity of the extracts. Based on the results obtained, oven and freeze drying plant leaf extracts from the University of Pretoria gives the best anti-tyrosinase activity and can be used in skin hyperpigmentation solutions. However, depending on available resources and efficiency of the commercial production chain, the options are to oven, freeze or air dry the samples from The University of Pretoria garden without storage. Further investigation are required controlling storage temperature, light and humidity

Chapter 7

General conclusion and Recommendations

7.1 *Greyia flanaganii* metabolomics

The multivariate chemometric analysis (PCA and OPLS-DA) showed that drying conditions cause no significant effect on the metabolite composition of G. *flanaganii* leaf extracts. The ^{1}H-NMR spectra of the extracts showed peaks at the same values indicating presence of same chemical constituents. Storage condition did not show any significant differences in the metabolite composition. However, freezer storage slightly separated from other storage conditions although not clearly. Samples stored for three and six months grouped together slightly separate from the fresh (control) samples.

7.2 Location of the *G.flanaganii*

The extracts from plants harvested from University of Pretoria showed differences in chemical constituents from extracts harvested from Mothong African Heritage site. The multivariate analysis (PCA scores plot) identified some significant differences in the metabolite composition of the samples from the two locations. However compounds responsible for the differences were not identified. These differences in chemical composition has caused the different anti-tyrosinase activities observed between the leaf extracts from the two locations. Extracts from the University of Pretoria exhibited a better inhibiting activity than the extracts from Mothong African Heritage site. The plant leaf extract harvested from the University of Pretoria are predicted to contain a higher concentration of polyphenols, chalcones and flavonoids, such as the catechins which have been previously reported to have anti-tyrosinase inhibiting activities (Nogemane *et al*., 2017) since compound identification was not done in this study.

7.3 Greyia flanaganii anti-tyrosinase activity

Assessing the anti-tyrosinase activity of *G. flanaganii* dried samples, oven dried and freeze dried extracts gave the best activity double the activity of air and sun dried extracts. The slight differences in the concentration of chemicals in the extract separated the drying techniques into two groups, (air and sun dried) exhibited comparatively the same activity different from the activity exhibited by freeze and oven dried extracts. This is in contrast with the metabolomics analysis where drying conditions showed no significant differences. The better activity was

however only observed before storage and explain therefore that no clustering was observed in the metabolomic analysis.

Generally *G. flanaganii* freshly dried leaf extracts showed better anti-tyrosinase inhibiting activities than stored dried extracts. This is supported by the significant differences noted between fresh and stored sample metabolites. Only sun dried samples showed better activity after six months. This was not observed in metabolomics analysis.

7.4 *Leucosidea sericea* metabolomics

Different drying and storage methods showed no significant difference on the metabolite concentration. The metabolites are stable and their composition and concentration remained significantly unchanged. Significant differences were noted between the metabolite spectra of fresh samples and stored samples indicating that storage duration affected the metabolite profile. Freshly dried samples contained higher metabolite concentration than stored dried samples irrespective of the storage condition. However samples stored for three and six months grouped together showing that they have same metabolite composition.

7.5 *Leucosidea sericea* anti-bacterial activity

Although no differences were observed in the metabolites of differently dried samples, the anti-bacterial inhibiting activity showed that freeze dried and oven dried extracts showed good anti-bacterial activity with freeze drying giving the best activity (MIC 0.125 µg/ml). Sun and air dried exhibited the same anti-bacterial activity of 0.5 µg/ml. The drying conditions can be put into two different groups according to their effect on biological activity which is the same trend with *G. flanaganii* (air and sun dried) versus (freeze and oven dried).

Storage duration differently affected the bacterial activity depending on the initial drying and storage condition used. Fridge storage showed better activity after six months of storage except for freeze dried samples. This difference was not observed in the metabolomics analysis. Freeze dried extracts showed best activity before storage but dropped after storage in all the investigated storage conditions. Sun, oven and air dried extracts showed better anti-bacterial activity after storage than freshly dried extracts, the trend which was also observed in the metabolomics analysis. Sun and oven dried extracts are best stored for six months while air dried extracts can be best stored for three months.

Metabolomic analysis showed no significant differences on drying and storage methods maybe because storage time has a major impact on the chemical composition. Differences in metabolites as a result of drying and storage conditions is less than with storage time.

7.6 Comparison of results between two species.

Location of species showed significant differences ($P<0.05$) in *G.flanaganii* species, but not investigated in *L.sericea*. However, differences of *G.flanaganii* samples from the two different sites may not necessarily be an effect of location, but because the plant material were from different sources. It is important to make sure that plant material is correctly identified and propagated. Both species showed differences in metabolomics when fresh extracts are compared to three and six months stored extracts. Generally *L.sericea* exhibited better activity after storage while *G. flanaganii* generally showed lower activity after storage.

7.7 Shortcomings of the study.

Due to the difficulties in culturing of *P acnes*, the anti-bacterial activity of *L sericea* leaf extract was tested on *S aureus* which also causes some skin problems. However the colorimetric assay plates showed some degree of contamination. Studies have showed that *P acnes* cause serious problems in skin hyperpigmentation and I therefore recommend that bioassay tests canbe done against *P.acnes*. In another study light, UV radiation and humidity conditions canbe better controlled during storage of samples since these conditions are also known to affect the chemical composition and concentration of plant material.

7.8 Recommendations.

Bioassay results do not always reflect the metabolomics results. Storage time is a major factor influencing the activity and metabolomics analysis of species. In *G. flanaganii* the strong clustering in location indicates that location is a very important factor influencing the metabolomics and activity of the species. Therefore the main determinants of change in material in this study are location and storage time for *G.flanaganii* and storage time for *L.sericea.* It is important to note that care should be taken when storing material to maintain the quality.

Based on the findings of this study, it is recommended to freeze or oven dry *G. flanaganii* plant leaf extracts and then use for hyper-pigmentation solutions freshly after drying. Assessing the plant source material is crucial before commercial cultivation of the species. Plant material from the University of Pretoria garden is better than that from Mothong African Heritage site in terms

of the anti-tyrosinase inhibiting ability. It is therefore also recommended that marker compounds responsible for biological activity be identified to assist in quality assessment of material from different locations and from different sourced material. This will assist in identifying the quality and uniformity of the plant material at Mothong African Heritage site plantation. For *L. sericea* extracts the best is to freeze or oven dry and use before storage. Alternatively, material should be sun and oven dried and then be stored for six months while air dried extracts can be best stored for three months. These conditions resulted in similar or even better activity than the freshly dried material.

Overall, it can be concluded from this study that there is no general rule that govern drying method, storage conditions or storage time to preserve biologically active compounds. In this study it was observed that the plant species, source of material and all pros-harvest handling methods investigated have an effect on the biological activity of the material. Each plant should therefore be separately investigated to determine the effect of post-harvest handling on the metabolite composition and biological activity before commercial production is attempted.

Future research can be done on isolation of compounds from both species and testing them on different storage conditions. *Greyia flanaganii* fingerprinting can be carried out to ensure correct material.

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