PROPAGATION AND QUALITY ASSESSMENT FOR THE INTRODUCTION OF *GREYIA RADLKOFERI* INTO COMMERCIALIZATION

by

NOLUYOLO NOGEMANE

Submitted in accordance with the requirements for
the degree of

Doctor of Philosophy

in the subject

Agriculture

at the

UNIVERSITY OF SOUTH AFRICA

SUPERVISOR: Dr G. Prinsloo
CO-SUPERVISOR: Prof N. Lall

15 June 2017

DECLARATION

Name: _____

Student number: _____

Degree: _____

PROPAGATION AND QUALITY ASSESSMENT FOR THE INTRODUCTION OF *GREYIA RADLKOFERI* INTO COMMERCIALIZATION

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.

_____ _____

SIGNATURE DATE

PREFACE

A section of the research study presented in this thesis has been accepted for publication in a peer-reviewed journal and parts of this research study were presented at conferences.

Scientific publication in peer-reviewed journal:

Nogemane N. and Prinsloo G., 2017. Seed Germination and Vegetative production of *Greyia radlkoferi*, an Indigenous Tree of Cosmeceutical Importance. Acta Horticulturae: In press

Scientific publications in preparation:

Nogemane N., Lall N., Prinsloo G., 2017. Metabolomics profile and anti-tyrosinase activity of *Greyia radlkoferi* leaf extracts in response to seasonal changes.

Nogemane N., Lall N., Prinsloo G., 2017.Chemical composition and biological activities of *Greyia radlkoferi* leaf extracts processed by different drying methods using ^{1}H-NMR based metabolomic approach as a quality control tool.

International conferences:

Nogemane N. and Prinsloo G., 2016. ^{1}H-NMR based metabolomics approach to understanding the influence of seasons on the metabolomics profile of *Greyia radlkoferi.* 12th Annual Conference of the Metabolomics Society, Dublin, Ireland. Poster presentation.

Nogemane N. and Prinsloo G., 2016. Effects of temperature on seed germination of *Greyia radlkoferi*, an indigenous tree of cosmeceutical importance. 7th International Symposium for Seed Transplant and Stand Establishment of Horticultural Crops (SEST, seed Indaba Africa). University of Pretoria, Gauteng, South Africa. Oral presentation.

Local conferences:

Nogemane N. and Prinsloo G., 2016. ^{1}H-NMR-based metabolomics approach to understanding the drying effects on the secondary metabolites of *Greyia radlkoferi.* South African Association of Botanists (SAAB). University of Free State, Bloemfontein, South Africa. Oral presentation.

Nogemane N. and Prinsloo G., 2016. ^{1}H-NMR based metabolomics approach to understanding the influence of seasons on the metabolomics profile of *Greyia radlkoferi*. Conference for Southern African Biochemistry and Informatics for Natural Products (SABINA) 2016. Protea Kruger Gate, Mpumalanga, South Africa. Poster presentation.

ACKNOWLEDGEMENTS

My heartfelt gratitude goes to my mother, Kholeka Nogemane, for all her love and support throughout the duration of my studies. I would like to acknowledge my son Lingomso Nogemane, who has brought me so much joy and happiness. Many thanks also goes to my late father, GV Mabusela for being there for me until his departure- 'I know you would have been proud'.

To my supervisors, Dr Prinsloo and Prof. Lall, I am very grateful for your supervision and guidance during the course of my studies. I have become a better researcher because of your constant positive criticism.

My appreciation also goes to the members of the Medicinal Plant Research Group under the leadership of Dr Prinsloo in the Department of Agriculture and Animal Health of the University of South Africa. Your comments and suggestions were most valuable to see me through this study. A special 'thanks' goes to Cynthia Marokane for assistance with laboratory techniques and some data collection. Your help is appreciated.

Lastly, I would like to acknowledge the University of South Africa for the AQIP grant, which did not only fund the study but offered me time off work in order to undertake my PhD study on full-time basis. The Department of Science and Technology is also acknowledged for partly funding this study. Without funding, this work would not have been possible.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

APPENDICES

ABSTRACT

Greyia radlkoferi is a South African indigenous tree, which has recently been discovered to be a source of extracts that have a potential in the development of cosmeceutical herbal products having the ability to treat hyperpigmentation disorders. For product development however, *G. radlkoferi* would need to be available in a commercial scale. *Greyia radlkoferi* grows naturally in the wild and is often available for cultivation as an ornamental plant. In order to establish this plant into cultivation, suitable propagation techniques must be established for rapid multiplication of trees and thus a sustainable leaf production. For consistent and improved leaf supply to the market, agronomic practices that will enhance leaf production were investigated in the current study. Furthermore, in order to meet market demand in terms of good quality extracts with guaranteed therapeutic efficiency, pre-harvest and post-harvest factors that affect the chemical composition of the extracts were investigated. Recently developed biotechnology techniques such as metabolomics using ^{1}H-NMR and multivariate data analysis offered a platform to study the chemical variation of extracts. Therefore, the current study was aimed at understanding the requirements for propagation and optimum leaf production as well as conditions that favour optimum production of secondary metabolite of *G. radlkoferi* plant material (at pre and post-harvest) and thus assess its commercial viability.

To understand the effects of temperature on seed germination of *G. radlkoferi*, seeds were exposed to five temperatures (10°C, 15°C, 20°C, 25°C and 30°C) in the incubators in the laboratory. Germination of *G. radlkoferi* by seeds was discovered to be temperature dependent. The optimum germination temperature of 81% was obtained at 25°C. In the case of vegetative propagation by stem cuttings, the effect of cutting position (basal or apical), exogenous rooting hormone (Seradix1, Seradix 2, 0.1% IBA, 0.3% IBA and 0.8% IBA) and cutting position were investigated in the glasshouse. The cutting position had a significant effect on rooting of *G. radlkoferi* cuttings with basal cuttings exhibiting 35% rooting as compared to 6% rooting attained for the apical cuttings. A clear trend in rooting response to application of rooting hormones was observed, with 0.1% Indole butyric acid (IBA) showing the highest rooting percentage of 63%. Considering the outcomes of the propagation studies as well as the limited

material for vegetative propagation, seed propagation appears to be the most suitable technique for large-scale multiplication of *G. radlkoferi*.

The effect of different pruning techniques as well as harvesting frequencies on fresh and dry weights of *G. radlkoferi* leaves were evaluated. Factors considered were four pruning treatments ('pruned but not tipped', 'tipped but not pruned', 'not pruned nor tipped' as well as 'pruned and tipped') and three harvesting periods (monthly, bimonthly and once–off). Bimonthly harvests highly increased leaf production compared to trees that were harvested monthly and once-off with higher leaf fresh weight yield of 238 g per tree or 2.38 tons/per hectare as well as dry weight yield of 83 g per tree or 0.830 tons/hectare. This outcomes of this study further suggested that a suitable pruning practice for *G. radlkoferi* would be to either 'prune only' or 'cut back the main stem' rather than a combination of the two treatments.

The influence of seasons (summer, autumn, winter and spring) on the anti-tyrosinase activity and metabolomics profile of *G. radlkoferi* leaf extracts were investigated. Seasons significantly influenced the chemical composition and the efficacy of the plant extracts. Tyrosinase enzyme inhibition was investigated against monophenolase (tyrosine) with kojic acid as positive control. The highest tyrosinase inhibition concentration with IC_{50} (50% tyrosinase inhibition concentration) value of 30.3±1.8 µg/ml were obtained in winter harvested leaves compared to the other seasons. The lowest IC_{50} values were obtained in spring. Metabolomics analysis using orthogonal partial least square discriminant analysis (OPLS-DA) provided a clear class separation according to the harvest season. Extracts from winter harvested leaves contained sucrose, acetamide, alanine and a compound of the catechin group (gallocatechin-(4 alpha->8)-epigallocatechin) as revealed by ^{1}H-NMR metabolomics with assistance of LC-MS. Since compounds of the catechin group are well-known tyrosinase inhibitors, the high tyrosinase activity exhibited in extracts of winter harvested *G. radlkoferi* leaves could be ascribed to the presence of gallocatechin-(4 alpha->8)-epigallocatechin. Based on the outcomes of the seasonal study, we suggest that in order to obtain extracts with high bioactivity, the best suitable time for harvesting leaf samples is in late autumn-early winter.

Processing leaf material using three different drying methods (sun, oven and air drying) significantly influenced chemical composition and the efficacy of the plant extracts. Extracts prepared from air-dried leaf material showed the highest tyrosinase inhibition with IC_{50} value of 17.80 µg/ml compared to extracts of the other drying methods. Extracts of leaves processed with air drying preserved most metabolites during processing while extracts of sun-dried and oven-dried leaves clearly depleted some metabolites especially amino acids and some aromatic compounds. ^{1}H-NMR metabolomics approach with the assistance of LC-MS data successfully determined a positive association of alanine, acetamide, sucrose and gallocatechin-(4 alpha->8)-epigallocatechin as the chemical constituents contributing to the variation in the air-dried leaves compared to the oven-dried leaves. A positive association of valine, alanine, leucine, isoleucine, gallocatechin-(4 alpha->8)-epigallocatechin and glucose contributed to the variation in air-dried group, compared to the sun-dried group. The highest tyrosinase inhibitory activity exhibited in air-dried samples compared to the other drying methods was associated with the presence of gallocatechin-(4 alpha->8)-epigallocatechin. Because air drying preserved most leaf metabolites compared to sun and oven drying, it was regarded as the most suitable method for processing *G. radlkoferi* leaf material.

This study is the first scientific account that provides guidelines and recommendations to (1) establish *G. radlkoferi* as a cultivated plant for commercialization, (2) optimize leaf production for sustainable supply to the commercial markets and (3) optimize medicinal content of *G. radlkoferi* related to harvesting time and post-harvest processing (drying), for enhanced quality of extracts and its products.

Keywords: *Greyia radlkoferi*, seed germination, temperature, IBA, cutting propagation, pruning, harvesting frequencies,^{1}H-NMR metabolomics, anti-tyrosinase, Gallocatechin-(4 alpha->8)-epigallocatechin.

CHAPTER 1: GENERAL INTRODUCTION

1.1 BACKGROUND

It is estimated that 80% of the world's population, mostly from developing countries depend on traditional medicine for primary health care and particularly around 27 million South Africans depend on traditional medicine for their primary health care needs (Mander 1998). Plants are used in African medicine to treat various ailments such as fever, asthma, constipation, hypertension, skin diseases as well as for cosmetic purposes (Dold & Cocks 2005; Van Wyk 2008).

The traditional use of plants against skin diseases and especially for cosmetic purposes has been a common practice in the traditional medicine use of many cultures for many years. Plants have once been the main source and foundation of all cosmetics, before other methods of synthesizing substances with similar properties were discovered (Aburjai & Natsheh 2003). The earliest known use of cosmetics derived from plants were reported with Egyptians as far back as 3100-2907 BC. These include olive oil and its plant extract which were used to keep the skin supple and for cleansing and beautifying skin (Dold & Cocks 2005).

The traditional use of plants for cosmetic purposes continue to be a common practice in rural South Africa despite the modern and new cosmetic products that are massively available in the market. Some of the medicinal plants that are still being used for cosmetic purposes particularly in the Eastern Cape province include *Cassiipourea flanaganii* traditionally known as ummemezi for a desirable complexion, *Spirotachys africana* commonly known as Tamboti for baby rash and *Hydnora africana* for skin blemishes (Dold & Cocks 2005). These plants are used to either enhance beauty, for health purposes or well-being. Ethnobotanical literature which is the information often gathered from indigenous people or traditional healers has also recorded a substantial number of medicinal plants that are still traditionally used to treat various dermatological disorders in Southern Africa (Rabe & Van Staden 1997; Mapunya *et al*. 2012; Mabona *et al*. 2013; Mabona & Van Vuuren 2013; Lall & Kishore N 2014). The dermatological disorders listed in the literature include wound healing, infectious diseases, necrotizing skin

ailments, abnormalities of the epidermis, anti-inflammatory disorders, burns, surface abrasions and other unspecified skin ailments.

Interest in traditional systems of medicine and, in particular, herbal-derived medicines, has increased substantially in the markets of both developed and developing countries over the past few decades. This is no different for the personal care industry. Recently, the use of herbal derived products in the form of botanical actives, extracts or antioxidants in the production of cosmetics has increased tremendously in the cosmetic industry. According to the "Global Cosmeceuticals Market Outlook 2020" report; the global cosmeceuticals market is anticipated to reach US$ 61 Billion by 2020 (Research and Markets 2015). The interest in these products is majorly driven by mounting desire of consumers to maintain healthy skin without using chemicals. Herbal cosmetics are regarded as (i) safer-often without side effects, (ii) cheaper, (iii) easily accessible (i.e. available over the counter) (Rahimi *et al*. 2012; Fisk *et al*. 2014) (iv) have positive effects in the skin, (v) an ancient legacy of natural healing of herbal medicines as well as (vi) increase in wariness towards chemicals contained in some commercial products. Furthermore cosmetic regulating agents have become aware of adverse effects associated with certain commercial available cosmetic creams, especially those used for the treatment of skin hyperpigmentation (Ladizinski *et al*. 2011). Examples of such compounds include hydroquinone and corticosteroids and are derived from chemical and fungal agents (Draelos 2007). These compounds have been discovered to have chronic, cytotoxic and mutagenic effect on humans (Nerya *et al*. 2003). Thus, there is a call for safer hyperpigmentation treatment agents as opposed to bleaching compounds. Herbal medicine furthermore have an ability to enhance beauty through ingredients that provide an additional health-related function or benefit (Dureja *et al*. 2005).

Even though information on medicinal plants traditionally used for dermatological purposes in Southern Africa is well documented, only a few of these plants have been exploited for product development and commercialization. In an attempt to keep-up with the new developments in the cosmeceutical industry, medicinal plant research is recently progressing towards exploring the possibility of product development and commercialization of cosmeceutical products from indigenous plants. Researchers are turning their attention to natural products to look for new leads to develop safer cosmetics. This task is undertaken by some South African Universities,

Science/research councils with the support of the Department of Science and Technology and knowledge holders such as traditional healer organizations. Several authors in the country have already done and published some work relating to cosmeceutical research (Abdillahi *et al*. 2005; Momtaz 2007; Momtaz *et al*. 2008; Mapunya 2009; De Cahna *et al*. 2015; Mapunya *et al*. 2012; Mabona *et al*. 2013; Mabona & Van Vuuren 2013; Lall & Kishore 2014; Lall *et al.* 2016).

South African medicinal plants used for dermatological purposes are gaining more value in the cosmetic industry, as many skincare products are now being supplemented with plant extracts. Some of the few successful cosmetic products available in the market include, the African Extracts™ Rooibos (*Aspalathus linearis*) body care products (http://www.africanextracts.com), Alcare aloe® skin care products which contain *Aloe ferox* extracts (http://www.aloe.co.za), Sausage Tree Cream® from *Kigelia africana* extracts which used to treat eczema, psoriasis and other skin ailments (http:www.faithful-to-nature.co.za/Sausage-Tree-Cream-African-Kigelia-p-92.html) as well as Puremedy® (containing *Hypericum perforatum*) which is used to treat a variety of skin ailments including wounds and skin infection (http://www.puremedy.com/calendula_stjohns.html).

Generally, herbal product development is faced with many challenges such as issues of quality and quantity standard demands from the herbal markets. Production of a finished product of good quality partly depends on the quality of raw medicinal plant material supplied. The market requires standardized plant material both in qualitative and in quantitative terms. Biomass production and the concentration of the bioactive compound in the tissues need to be standardized i.e. should not be only as high as possible but also as constant or uniform as possible (Maggini *et al*. 2014).

Bioactive compounds which are often secondary metabolites are known to vary in plants due to several factors classified as intrinsic (genetic) or extrinsic (environment factors, cultivation practices, harvesting methods and time, post-harvest processing, transport and storage practices) factors. This variation in secondary metabolite production compromises the quality of the raw medicinal plant material, consequently affecting the quality of the finished products. To ensure that the quality and quantity of extracts are uniform and the biomass production is improved, the

best quality control measures such as monitoring metabolite production and the optimization of leaf production techniques need to be considered. However, a vast supply of medicinal plants to the market is wild collected. Wild collected medicinal plants are not a good plant material for product development as they present more concerns in addition to issues of metabolite variation. These concerns include contamination by other species or plant parts through misidentification, accidental contamination or intentional adulteration, all of which may be unsafe and therefore compromise the quality of the raw plant material. Furthermore, bioactive compounds and biomass productions of wild collected medicinal plants would prove to be difficult to standardise and optimize as these plants vary in many factors. Introducing the medicinal plant of interest into cultivation provides a solution to improve quality and quantity standards as it will provide a platform to control the production of leaf material and bioactive compound production. The majority of companies, including the mass-market, over-the-counter pharmaceutical companies, as well as the larger herbal companies prefer cultivated plant material (Laird and Pierce, 2002). From their perspective cultivated medicinal plants guarantee reliable botanical identification, certified organic/biodynamic, steady supply of raw materials, standardized volume and prices agreed upon between growers and the market, controlled post-harvest handling and therefore assured quality control and product standards that can be adjusted to regulation and consumer preference (Schippmann *et al. 2002;* Schippmann *et al.* 2006). However, introducing medicinal plants into cultivation may not be easy. Indigenous plants grow naturally in the field and are adapted to the natural conditions of their growing environment. The requirement of propagation of most of these plants is usually not known. Determination of propagation techniques suitable not just for multiplication of the plant, but for enhancing the commercial production in terms of shortening the germination time need to be investigated. Furthermore, best agricultural practices for optimum leaf production also need to be determined.

Once the plant is established in the field, monitoring of the metabolite changes in response to extrinsic factors would be easily applicable. This would be achieved by means of recently developed biotechnology techniques such as metabolomics. Metabolomics is a biotechnology tool used for chemical screening and subsequent detailed comparison of the primary and secondary metabolomes in different samples, and thus revealing the differences or similarities among groups and further reveal metabolites responsible for the detected differences. Plant

metabolomics becomes an ideal technique for monitoring plant metabolite variation as it can provide information on conditions that best produce appropriate and high metabolite contents in the extracts.

1.2 AIM AND OBJECTIVES

Currently, there is no scientific research that has been reported on the cultivation and metabolomics analysis of *Greyia radlkoferi,* a tree or shrub with a potential in the treatment of hyperpigmentation disorders. Therefore, the main aim of the present study was to assess the commercial viability of *G. radlkoferi* by establishing suitable agronomic practices for optimum leaf production and to monitor its medicinal content in response to pre- and post-harvest factors.

The objectives of this study were to:

- Enhance production of *G. radlkoferi* plants through rapid seed propagation.
- Generate a protocol for vegetative production of *G. radlkoferi.*
- Evaluate the effect of pruning on leaf yield.
- Determine the frequency of leaf harvesting without compromising growth of the plant.
- Examine the response of the metabolomic profile of *G. radlkoferi* to seasonal changes.
- Examine the response of the metabolomic profile of *G. radlkoferi* to different drying methods.
- To determine changes in tyrosinase activity of leaf extracts of *G. radlkoferi* in response to varying seasons and drying methods.

1.3 STRUCTURE OF THE THESIS

The thesis consists of an introductory section and literature review, followed by two experimental chapters on agronomic aspects and another two experimental chapters on plant metabolomics and bioactivity studies.

Chapter 1 This chapter looked at the background of traditional medicinal use, the rationale and the objectives of the current study.

Chapter 2 This chapter reviews the literature on distribution, botanical description, phytochemical constituents and significance of *G. radlkoferi* as a cosmeceutical plant. The study further reviews quality determinants of raw medicinal plants, cultivation as a possible solution to raw medicinal plant quality and metabolomics as a possible quality control tool. The study also discusses the status of medicinal plant cultivation in South Africa and also reviews various plant propagation techniques.

Chapter 3 This chapter investigates and discusses propagation of *G. radlkoferi* using seeds and stem cuttings.

Chapter 4 This chapter investigates and discusses the pruning and harvesting frequency effects on leaf production of *Greyia radlkoferi.*

Chapter 5 This chapter investigates the chemical variation of cultivated *Greyia radlkoferi* in response to different seasons, namely summer, autumn, winter and spring using NMR analysis coupled with multivariate analysis. The inhibitory effect of leaf extracts on tyrosinase activity for each season has also been discussed.

Chapter 6 This chapter provide the chemical variation of cultivated *Greyia radlkoferi* in response to different drying methods using NMR analysis coupled with multivariate analysis. The inhibitory effect of leaf extracts on tyrosinase activity for each drying method is also discussed.

Chapter 7 The general discussion and conclusions are presented and provide a synthesis and a coherent overview of the results obtained in this study.

Chapter 8 Appendices

1.4 REFERENCES

Abdillahi, H.S., Finnie, J.F. & Van Staden, J., 2011. Anti-inflammatory, antioxidant, anti-tyrosinase and phenolic contents of four Podocarpus species used in traditional medicine in South Africa. *Journal of Ethnopharmacology*, 136(3), pp.496–503. Available at: http://dx.doi.org/10.1016/j.jep.2010.07.019.

Aburjai, T. & Natsheh, F.M., 2003. Plants Used in Cosmetics. Phytother Res, 1000(April), pp.987–1000.

Brook, B.W., Sodhi, N.S. & Bradshaw, C.J. a, 2008. Synergies among extinction drivers under global change. *Trends in Ecology and Evolution*, 23(8), pp.453–460. Available at: http://dx.doi.org/10.1016/j.det.2010.08.010.

De Cahna, M.N. *et al.*, 2015. Extract of Greyia radlkoferi and use thereof. Available at: www.google.com/patents/WO2013171720A1?cl=en.

Dold, A.P. & Cocks, M.L., 2005. Imbhola yesiXhosa: Traditional Xhosa cosmetics. *Veld & Flora*, 91(3), pp.123–125.

Draelos, Z.D., 2007. Skin lightening preparations and the hydroquinone controversy. *Dermatologic Therapy*, 20(5), pp.308–313.

Dureja H, Kaushik D, Gupta M, Kumar V, L. V, 2005. Cosmeceuticals : An emerging concept. , (3), p.7613.

Fisk, W. a. *et al.*, 2014. The use of botanically derived agents for hyperpigmentation: A systematic review. *Journal of the American Academy of Dermatology*, 70(2), pp.352–365. Available at: http://dx.doi.org/10.1016/j.jaad.2013.09.048.

Ladizinski, B., Mistry, N. & Kundu, R. V., 2011. Widespread use of toxic skin lightening compounds: Medical and psychosocial aspects. *Dermatologic Clinics*, 29(1), pp.111–123. Available at: http://dx.doi.org/10.1016/j.det.2010.08.010.

Laird SA, Pierce AR (2002). Promoting sustainable and ethical botanicals. Strategies to improve commercial raw material sourcing. Results from the sustainable botanicals pilot project. Industry surveys, case studies and standards collection. – New York, Rainforest Alliance (www.rainforestalliance.org/news/archives/news/news44.html).

Lall, N. & Kishore N, 2014. Are plants used for skin care in South Africa fully explored? *Journal of Ethnopharmacology*, 153, pp.61–84. Available at: http://www.scopus.com/inward/record.url?eid=2-s2.0-

84897114922&partnerID=40&md5=64a75d4a9d61ea2e90fb7f389acfd83b.

Lall, N., Mogapi, E., Canha, M.N. De, et al., 2016. Insights into tyrosinase inhibition by compounds isolated from Greyia radlkoferi Syzsyl using biological activity, molecular docking and gene expression analysis. *Bioorganic & Medicinal Chemistry*. Available at: http://dx.doi.org/10.1016/j.bmc.2016.09.054.

Mabona, U. *et al*., 2013. Antimicrobial activity of southern African medicinal plants with dermatological relevance: From an ethnopharmacological screening approach, to combination studies and the isolation of a bioactive compound. *Journal of Ethnopharmacology*, 148(1), pp.45–55. Available at: http://dx.doi.org/10.1016/j.jep.2013.03.056.

Mabona, U. & Van Vuuren, S.F., 2013. Southern African medicinal plants used to treat skin diseases. *South African Journal of Botany*, 87, pp.175–193. Available at: http://www.sciencedirect.com/science/article/pii/S0254629913002433.

Maggini, R., Kiferle, C. & Pardossi, A., 2014. Hydroponic production of medicinal plants. In D. alexandre M. Pereira, ed. *Medicinal Plants: Antioxidant Properties, Traditional Uses and Conservation Strategies*. Nova Science Publishers, pp. 91–116. Available at: https://www.novapublishers.com/catalog/product_info.php?products_id=46118.

Mander, M., 1998. Marketing of indigenous medicinal plants in South Africa - a case study in Kwazulu-Natal. *FAO - Food and Agriculture Organization of the United Nations.*, pp.1–158. Available at: www.fao.org/docrep/W9195E/w9195e00.htm.

Mapunya, B.M., 2009. Mushroom tyrosinase activity of novel and known phenolic compounds isolated from *Greyia fanaganii (bolus) Manyatja Brenda Mapunya Master of Science Option : Medicinal Plant Science*.

Mapunya, M.B., Nikolova, R.V. & Lall, N., 2012. Melanogenesis and antityrosinase activity of selected South African plants. *Evidence-based Complementary and Alternative Medicine*, 2012.

Momtaz, S. *et al*., 2008. Tyrosinase inhibition by extracts and constituents of Sideroxylon inerme L. stem bark, used in South Africa for skin lightening. *Journal of Ethnopharmacology*, 119(3), pp.507–512.

Momtaz, S., 2007. *Tyrosinase inhibitors isolated from Ceratonia siliqua (L .) and Sideroxylon inerme (L .)*, MSc dissertation, University of Pretoria, Pretoria, viewed 2017.08.29 <

http://hdl.handle.net/2263/26846 >

Nerya, O. *et al*., 2003. Glabrene and isoliquiritigenin as tyrosinase inhibitors from licorice roots. *Journal of Agricultural and Food Chemistry*, 51(5), pp.1201–1207.

Rabe, T. & Van Staden, J., 1997. Antibacterial activity of South African plants used for medicinal purposes. *Journal of Ethnopharmacology*, 56(1), pp.81–87.

Research and Markets, 2015. Global Cancer Immunotherapy Market Outlook 2020 - Research and Markets. *Global Cosmeceuticals Market Outlook 2020*. Available at: http://www.researchandmarkets.com/reports/3274870/global-cancer-immunotherapy-market-outlook-2020#relb1 [Accessed February 23, 2017].

Schippmann, U., *et al*., 2002. Impact of culitvation and gathering of medicinal plants on biodiversity: Global Trends and Issues. , pp.1–22.

Schippmann, U., Leaman, D.J. & Cunningham, a B., 2006. A comparison of cultivation and wild collection of medicinal and aromatic plants under sustainability aspects. *Medicianl and Aromatic Plants: Agricultural, Commercial, Ecological, Legal, Pharmacological and Social Aspects*, pp.75–95. Available at: http://apps.webofknowledge.com/full_record.do?product=UA&search_mode=GeneralSearch&qid=18&SID=U1qIUqUgJUXJIBbZynZ&page=1&doc=1.

Van Wyk, B.E., 2008. A broad review of commercially important southern African medicinal plants. *Journal of Ethnopharmacology*, 119(3), pp.342–355.

CHAPTER 2: LITERATURE REVIEW

2.1 GREYIA RADLKOFERI

2.1.1 Botanical description

Greyia radlkoferi, commonly known as the mountain bottlebrush is a deciduous tree or a shrub which is indigenous to South Africa (Palgrave & Palgrave 2002). The genus *Greyia* is named after Sir George Grey (1812-1898), explorer and governor of South Australia, the Cape colony and New Zealand while the species name is derived from Ludwig Radlkofer (1829-1927), Professor of Botany and Director of the Botanical Museum in Munich (de la Cruz 2004). *G. radlkoferi* is a member of the *Melianthaceae* family and according to Coates Palgrave *et al*., (1985), the *Melianthaceae* family consists of a single genus, *Greyia* and 3 species, namely *G. radlkoferi*, *G. flanaganii* and *G. sutherlandii*.

Greyia radlkoferi grows as a shrub to a small tree of about 2-5m in height (Figure 2.1A). The main stem of *G. radlkoferi* is crooked with a very rough, fissured and dark grey bark and young stems are yellowish brown and smooth.

Figure 2.1: *Greyia radlkoferi (*a) tree (b) leaf (c) flower

The leaves are oval shaped and are clustered towards the ends of branchlets (Figure 2.1B). They are glossy green in colour with sparse hair above and thick almost fleshy dense velvety white hairs beneath. The hairy character of this tree distinguishes it from its related species, *G. flanaganii* and *G. sutherlandii*. The petioles of *G. radlkoferi* are also very hairy and longest of all

the three species. This plant drops its leaves when the cold weather arrives and it loses most of its leaves with age. It produces a dense cluster of bell shaped flowers blooming in upright racemes with deep scarlet petals (Figure 2.1C). The racemes develop at the end of the stem before or with young leaves in July to October. The fruit capsules are cylindrical and they split open at one end to release seeds in spring to summer. The shrub is mostly used as a decorative tree for inland gardens (de la Cruz 2004) and is popular as an ornamental plant (Palgrave & Palgrave 2002).

2.1.2 Distribution and Habitat

Greyia radlkoferi is found in Northern KwaZulu-Natal (previously known as Zululand) bordering the country of Swaziland (Figure 2.2). It can also be found in eastern Mpumalanga and the Gauteng area. It is usually found in varying habitats such as on forest margins, along rivers, among rocks in grasslands and on rocky-mountains or cliff edges.

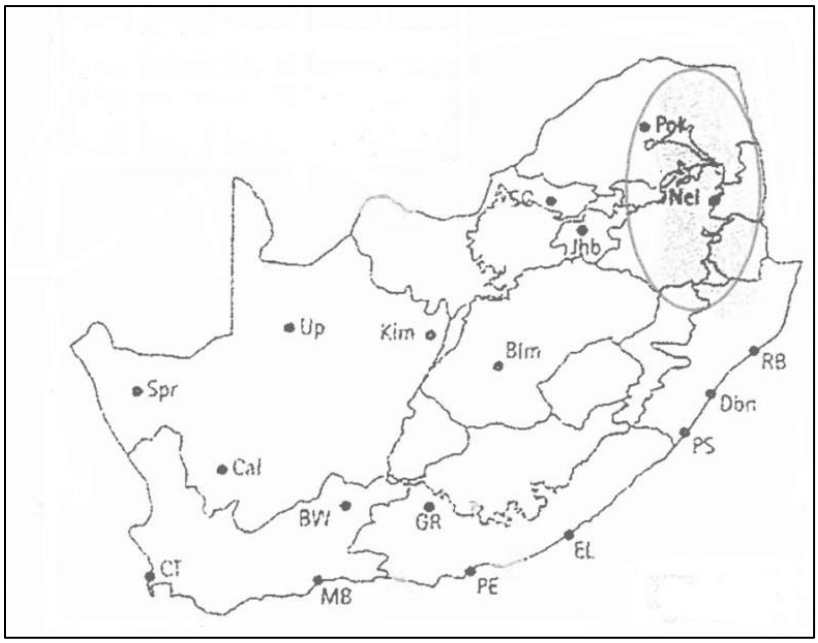

Figure 2.2: Geographical distribution of *Greyia radlkoferi* in South Africa

2.1.3 Ethnobotanical and Phytochemical studies

A recent study reported that leaf extracts and compounds isolated from *G. radlkoferi* have the ability to reduce melanin production responsible for skin hyperpigmentation at enzyme level and in in-vitro cell studies (De Cahna *et al*. 2015; Lall *et al*. 2016). The ethanolic leaf extracts of *G. radlkoferi* revealed significant tyrosinase inhibition by exhibiting a 50% inhibitory concentration (IC_{50}) ranging from 17.96-32.62 µg/ml when L-tyrosine was used as a substrate. These results displayed by leaf extracts of *G. radlkoferi* were comparable to those of kojic acid, isoliquirtigenin and arbutin which are well-known effective melanin inhibitors commonly used in the treatment of skin hyperpigmentation. The leaf extracts of *G. radlkoferi* further showed a 50% melanin reduction in melanocytes during in-vitro studies. The results of the in-vitro studies were also comparable to those of arbutin. Subsequent clinical studies showed that gel cream formulation obtained from leaf extracts of *G. radlkoferi* were able to overcome mild complications such as skin irritation as well as serious effects such as cytotoxicity, mutagenicity and carcinogenicity associated with certain hyperpigmentation agents (De Cahna *et al*. 2015). Furthermore, microbial and heavy metal analysis investigation of the cosmeceutical actives of *G. radlkoferi* reported an absence of microbial contamination and heavy metals such as lead, arsenic and mercury.

Phytochemical studies using bioassay guided fractionation allowed for the isolation of five phenolic compounds from the leaf extracts of *G. radlkoferi*, namely 5,7-dihidroxyflavone[(2S)-pinocembrin]; 2,6'-dihydroxy-4'-methoxydihydrochalcone; 2', 4', 6' trihydroxydihydrochalcone; 3,5,7- trihydroxyflavone (Galangin) and 4',5',7-trihydroxyisoflavone (Genistein). These compounds exhibited differing levels of tyrosinase inhibition when used in isolation, with 2', 4', 6'- trihydroxydihydrochalcone showing the highest anti-tyrosinase activity of all the five compounds. Furthermore, a synergistic action in the inhibition of tyrosinase was also observed among these compounds (De Cahna *et al*. 2015).

The isolated phenolic compounds were identified as belonging to two sub-groups of flavonoids, namely calchones and flavonols. These compounds are well known substances that have been previously isolated and identified from leaves of other plants including those of other Greyiaceae species. 2', 4', 6' trihydroxydihydrochalcone has previously been isolated from *Lindera umbellate, Boesenbergia pandurate* and *Greyia flanaganii*. Galangin (3, 5, 7-trihydroxyflavone)

has previously been isolated from *Helichrysum aureonitens, Alpina officinarum* and the rhizomes of *Alpina galangal*. Genistein (4', 5, 7 –trihydroxyisoflavone) has been reported in *Genista tinctoria*. Pinocembrin (5, 7-dihydroxyflavanone) is reported to have been isolated in *Turnera diffusa*, *Glycyrrhiza glabra* L. and *Greyia flanaganii*. 2',6'-dihydroxy-4'-methoxydihydrochalcone has been isolated from *Greyia flanaganii* and from a number of Piper species such as *P. hostmannianum, P.dennisii* and *P.aduncum*. This information is in accordance to citations obtained in Lall *et al*. (2016).

Flavonoids are polyphenolic compounds that are abundant in plants and are categorized, according to chemical structure into flavonols, flavones, flavanones, isoflavones, catechins, anthocyanidins and chalcones. Over 4,000 flavonoids have been identified in plants. Flavonoids have stimulated a lot of interest recently because of their potential beneficial effects on human health. They have been reported to have biological properties such as antiviral, antiallergic, antiplatelet, anti-inflammatory, antitumor and antioxidant activities. In the form of plant extracts, flavonoids have been used in dermatology and cosmetics for a long time (Arct & Pytkowska 2008).

2.2 INTRODUCING MEDICINAL PLANTS INTO CULTIVATION

The presence of useful compounds within medicinal plants has attracted a lot of research interest. Research and development with the support of the government and the industry is currently focused on bioprospecting and pharmacological investigations of plants with the intention of developing commercial products. However, before product development can commence, the plant of interest must be available on a commercial scale through cultivation. The first step to cultivation of medicinal plant is mainly propagation to prove the reproducibility of the plant and to determine the propagation technique that best shortens the germination time of the seeds. Determination of such factors is important for commercial producers as it will increase yields leading to more availability of the plants to the market and thus increased profits. Because medicinal plants grow naturally in the wild and their regeneration and growth is dependent on the conditions to which they have become adapted to in their growing habitat, introducing medicinal plants to new habitats may not be technically easy. Information about specific requirements for germination and growth is often lacking as these plant are newly introduced

into cultivation. Successful propagation of a new plant is dependent on understanding some basic principles of plant growth and various methods of manipulating these plants based on propagation principles. Plant propagation may take place by means of two methods, namely, through seeds (sexual propagation) and through vegetative propagation (asexual) means (Hartmann *et al*. 2011).

2.2.1 Seed germination

Seed germination is a stage of plant development, which commences with the uptake of water by the dry seed (imbibition) and is completed when a part of the embryo, usually the radicle emerges from the seed covering, leading to a seedling. The germinability of the seeds is a critical event in determining the success of a plant species (Teketay 1996). For germination to be initiated, three conditions must be fulfilled. Firstly, the seed must be viable i.e. the embryo must be alive and capable of germination; the seed must be subjected to appropriate environmental conditions such as proper temperature, availability of moisture, photoperiod (light and dark), air supply (oxygen and carbon dioxide) and the primary dormancy present within the seed must be overcome (Hartmann *et al.* 2011). The requirements of these environmental conditions by seeds vary from species to species (Hartmann & Kester 1983) and manipulating these conditions according to the needs of each species often results in successful germination. Scientific studies undertaken by various authors on different plants successfully showed the influence of one or more of these factors on seed germination. Some of the authors whose studies payed attention to germination of seeds under temperature and light response include Araya, (2005) on *Athrixia phylicoides* seeds, Kulkarni *et al*., (2007a) in *Dioscorea dregeana* and Kambizi *et al*., (2006) on *Withania somnifera*. In an attempt to break seed dormancy, several studies have been undertaken by Mbangcolo, (2008) in *Cyclopia* species, Kulkarni *et al*., (2007b) on *Acacia hebeclada* and *A. mearnsii* seeds and Olmez *et al*., (2006) on *Capparis ovata.* Studies involving growth regulators and biostimulants were undertaken by Abdelgadir *et al*., (2012) on *Jatropha curcas*. All of these studies showed varying germination response to these factors depending on the germination needs of each plant.

2.2.2 Vegetative production

Vegetative production involves the multiplication of plants from any vegetative part through cuttings, grafting, budding and micro-propagation (Hartmann *et al*. 1997). Among these vegetative propagation methods, cutting is reported to be the most preferred method as it captures and conserve the genetic diversity of the plant. A cutting is defined as any vegetative part of the plant which, when detached from the parent plant and placed under conditions favourable for regeneration, will develop into a complete plant similar in all characteristics to the parent plant (Hartmann & Kester 1983). Cuttings can be classified based on the part of the plant taken namely, stem cuttings, root cuttings, divisions or leaf cuttings and leaf bud cuttings (Hartmann *et al*. 1997). Adventitious root formation is a pre-requisite to successful cutting propagation (Hartmann *et al*. 2011). The development of adventitious roots is influenced by different factors such as cutting type, rooting hormone, rooting medium, environmental and physical factors (Wilson 1993).

Outcomes of various studies have successfully confirmed the influence of various factors on propagation of stem cuttings of different plant species. Indole-butyric acid (IBA) improved the rooting of stem cuttings of *Milicia excels* (Ofori *et al*. 1996), *Cordiu alliodora* (Mesén *et al*. 1997), *Athrixia phylicoides* (Araya 2005), *cyclopia* species (*C. genistoides* and *C. intermedia*) (Mbangcolo 2008), *Warburgia ugandensis* (Akwatulira *et al*. 2011) and *Tinospora crispa* (Aminah *et al.* 2015). Cutting type (basal or apical) is another factor that has also been proven to influence rooting. In some plants, the proportion of cuttings that developed roots were higher among cuttings obtained from median and lower parts of the plant than apical parts (Lebrun *et al*. 1998; Tchinda *et al*. 2013; Amri *et al*. 2010). In other plants, rooting percentage was higher in cuttings obtained from the apex of the plant than the basal (Aminah *et al*. 2006; Araya 2005; Mbangcolo, 2008). Some plants show no consistent trend in percentage rooting response to cutting position (Mesén *et al*. 1997). Studies in *Jatropha curcas* (Severino *et al.* 2011) and *Tinospora crispa* (Aminah *et al.* 2015) showed an influence of the length of the cutting on rooting ability. These studies show that the longer the stem cutting, the better the rooting ability. The age of the donor plant (mother stalk plant) also influences the rooting ability of the cuttings. Cuttings of *Dalbergia melanoxylon* obtained from juvenile donor plants rooted better than the ones obtained from mature donor plants (Amri *et al*. 2010).

Rooting of cuttings can also be significantly improved by using appropriate growing media. Growing media such as sand, gravel, sawdust, pine bark, top forest soil and compost influenced the rooting ability of *Milicia excels* (Ofori *et al*. 1996), *Cordiu alliodora* (Mesén *et al*. 1997), *Athrixia phylicoides* (Araya 2005) and *Warburgia ugandensis* (Akwatulira *et al*. 2011). Some of these studies have shown that the influence of growing media on rooting ability varied with the hormone treatment used. Mbangcolo (2008) (in *cyclopia* species) showed that stem cuttings of bush tea root better in summer while Araya (2005) (in *Athrixia phylicoides*) showed a better rooting in spring and in autumn. It becomes clear from the above studies that the influence of factors that affect the development of adventitious roots is species specific.

2.3 QUALITY DETERMINANTS OF RAW MEDICINAL PLANTS AND THEIR PRODUCTS

Commercial marketed traditional herbal medicines derived from medicinal plants are often assessed for quality to ensure their safety (Govindaraghavan & Sucher 2015). There is a need, however for quality assessment measures that do not only address safety, but efficacy as well. Quality of herbal finished products in terms of efficacy and safety are directly dependent on the quality and chemistry of raw medicinal material, specifically bioactive substances. Bioactive substances are secondary plant metabolites that have proven beneficial effects on human health (Maggini *et al*. 2014). However, the chemical profiles of the medicinal raw material qualitatively and quantitatively varies due to factors that may be classified as intrinsic (genetic) or extrinsic (environment, collection methods, cultivation, harvest, post-harvest processing, transport and storage) (Mendonça-Filho 2006). These factors bring about major changes in phytochemical metabolites of a plant thus making metabolic products of medicinal plants diverse in category and content. Therefore, it is important to understand factors that affect secondary metabolite changes for consistent chemical profiles and thus biological activities.

Secondary metabolites are plant chemicals biosynthetically derived from primary metabolites (Balandrin *et al*. 1985). Secondary metabolites are limited in their distribution in the plant kingdom, being restricted to particular taxonomic groups i.e. they can be found within species, genus, family or closely related group of families (Balandrin *et al*. 1985). They play an ecological role within plants as pollinator attractants, they represent chemical adaptations to

environmental stresses and serve as chemical defense against micro-organisms, insects and higher predators and even other plants (allelochemicals). Scientific investigations have confirmed that plant secondary metabolites are synthesized in a response to various conditions that deviate from the optimal such as abiotic stresses (low/high temperature, light, UV radiation, drought/water stress, high salinity, nutrient deficiency) (Lavola 1998; Zobayed *et al*. 2005; Zobayed *et al*. 2007; Pavarini *et al*. 2012; Ncube *et al*. 2012; Sellami *et al*. 2013). Similarly, biological factors such as, herbivory, pathogens or microbial infestation and insect attacks causes variation in secondary metabolite synthesis (Andrew *et al.* 2007; Manish *et al*. 2014; Dambolena *et al*. 2010). Different cultivation practices such as soil characteristics, fertilizer application, harvesting practices (time and techniques), and post-harvest processing such as drying and storage practices also cause fluctuations in the production of plant secondary metabolites and therefore causing notable changes in the chemical profile of any plant material (Dubey *et al*. 2004; Harbourne *et al*. 2009; Isolabella *et al*. 2010; Mhamdi *et al*. 2010; Dartora *et al*. 2011; Pavarini *et al*. 2012; Zhao *et al*. 2013; Adeyemi *et al*. 2014; Routray & Orsat 2014; Booker *et al*. 2014; de Matos Nunes *et al*. 2014; Ogundele & Animasaun 2015; Dhami & Mishra 2015; Potisate *et al*. 2015). In addition, heavy metals and uncontrolled use of pesticides in cultivated medicinal plants alters the chemical profile of the plant material (Murch *et al*. 2003; Dogheim *et al*. 2004). These deviations are often responsible for losses in productivity and for spatial (geographical) and temporal (growing season) variations (Southwell & Bourke 2001; Kim *et al*. 2006; De Cerqueira *et al*. 2007; Chamorro *et al*. 2008; Demuner *et al*. 2011; Ncube *et al*. 2011; Kadu *et al*. 2012; Lubbe *et al*. 2013; Kim *et al*. 2014; Scognamiglio *et al*. 2014). Genetic factors and the developmental stage of the plant also causes remarkable variation in the secondary metabolite production (Sato *et al*. 2004; Bapela *et al*. 2007; Baraldi *et al*. 2008; Hosni *et al*. 2011; Farag *et al.* 2012; Bagdonaite *et al*. 2012; Kwon, Ahn, *et al*. 2014).

Since medicinal plants derive their therapeutic effects from secondary metabolites, the driving force behind the production and accumulation of these compounds will play a major role in dictating the quality of the raw medicinal plant material and its extract. The nature of the secondary metabolites produced, their concentrations and bioactivity will strictly be determined by the response to the environmental, biological and the cultivation factors mentioned above. Several studies undertaken on various plants proved that plant age, seasonal variation and

geographical deviation in harvest site contributes towards variation in biological activity of some medicinal plants (Taylor & Van Staden 2001; Buwa & Van Staden 2007; De Cerqueira *et al*. 2007; Van Vuuren *et al*. 2007; Demuner *et al*. 2011; Yang 2012; Kim *et al*. 2015; Lemos *et al*. 2015). A correlation in bioactivity with secondary metabolite changes has been established using metabolomics. In a study seeking to understand the effect of seasonal variations of metabolome and tyrosinase inhibitory activity of *Lespedeza maximowiczii* during growth periods, Kim and co-authors were able to show a correlation between secondary metabolite production and anti-tyrosinase activity using Pearson's correlation coefficients (Kim *et al*. 2015). The tyrosinase inhibitory activity of this plant was the highest in the samples 6 months after germination. Of all the secondary metabolites identified from six growth periods, only secondary metabolites that were positively correlated with tyrosinase inhibition activity were present in the largest quantities 6 months after germination. In another study, Lee and co-authors were able to correlate metabolite profile of *Neptunia oleracea* processed with different drying methods with antioxidant and α-glucosidase inhibitory activities using ^{1}H-NMR based metabolomics (Lee *et al*. 2016). A bi-plot obtained from multivariate data analysis showed that freeze dried samples that had higher antioxidant and α-glucosidase inhibitory activities compared to oven dried and air dried samples, were strongly correlated with increased concentration of a few identified secondary metabolites. Similarly in a most recent publication, the oven dried group in *Uraria crinite* exhibited significantly higher (anti-oxidative, anti-inflammatory etc.) activities than those of the shade group, and the sun group showed the highest increase in concentrations of two flavonoids, spatholosineside A and triterpenoids (Chao *et al*. 2017). Therefore, composition, concentration, availability and bioactivity are some of the important factors deciding the application of a biological/commercial product. Variation in these factors in raw medicinal plant material causes routine complications for the validation of its medicinal efficacy and safety.

2.4 CULTIVATION OF MEDICINAL PLANTS: A POSSIBLE SOLUTION TO QUALITY PRODUCTS

Cultivation of medicinal plants is a commercially attractive option to industries as it could offer them greater control over their needs (Hamilton 2004). From the perspective of the market, cultivation provides certain advantages over wild harvested plants. Cultivation provides reliable botanical identity, guarantees the steady supply of raw material, allows controlled post-harvest

handling, allows for observation of product standards according to regulations and consumer preferences, allow an easy crop certification procedure (certified organic) and renders possible agreement between wholesalers and pharmaceutical companies in volumes and prices over time with the grower (Máthé & Máthé 2008). These advantages offer the opportunity to overcome problems that are inherent in herbal extracts such as misidentification of plants, genotypic and phenotypic variability, extract variability and instability, toxic components and contaminants (Canter *et al*. 2005). The market requires standardised plant material both in qualitative and in quantitative terms, that is the biomass production and the concentration of the bioactive compound in the tissues should be not only as high as possible, but also as constant as possible (Maggini *et al*. 2014). To ensure reproducible quality of any herbal remedy, proper control of starting material is of utmost importance. Variations that often result in the uncertainty in the quality and quantity standards of the bioactive compound and thus the therapeutic benefit of the product of medicinal plant can be controlled through cultivated plants.

Production of medicinal plants by cultivation allows the opportunity to monitor the production of secondary metabolites and thus determine the conditions that favour optimum accumulation of the desired metabolite. This may provide the best season, time of the day for harvesting or the best geographical/altitude location that produce medicinal plants with increased phytochemical compounds, resulting in high quality supply of plant material. In addition, cultivation may be a viable alternative that offers the opportunity to overcome the problems of spatial and cultivation variation, leading to yield optimization and a uniform high quality product. For example, plants can be grown in areas of similar climate, similar or improved soil conditions, can be irrigated to increase yields, can be harvested at the right time and proper storage and processing techniques can be applied. Good Agricultural Practices (GAP) for medicinal plants should be the first step in quality assurance (Govindaraghavan & Sucher 2015) since they are meant to provide defined and reproducible production conditions for medicinal and aromatic plant producers. The introduction of defined production protocols could be expected to contribute to the production of quality phytomedicines.

2.5 BRIEF HISTORY INTO CULTIVATION OF SOUTH AFRICAN INDIGENOUS PLANTS FOR COMMERCIALIZATION

The increased interest in commercial products from indigenous crops as well as the need to protect plant biodiversity creates an opportunity for farmers to produce medicinal plants. In South Africa, cultivation of new and alternative crops (medicinal, cosmetic, ornamental and indigenous vegetables) offers a potential new niche for new rural farmers or small scale farmers who would otherwise not be able to compete commercially with the existing traditional crop farmers (Reinten & Coetzee 2002). These crops receive a higher price in the market if grown as certified organic and have a higher value per hectare than agricultural crops (Makunga *et al*. 2008).

In South Africa, several research projects have been conducted on the cultivation of medicinal plants by a wide variety of organisations, Universities and government bodies. Research institutes such as Agricultural Research Council (ARC) have taken wide strides in terms of research into cultivation and commercialization of indigenous plant material and thus establishment of new alternative crops. Crops that have been researched for commercialization purposes and are either currently in the market or have a great potential in the market are in the fields of floriculture, medicinal plants, indigenous vegetables and beverages. Currently commercialized flora plants include Fynbos plants such as *Proteaceae, Ericaceae, Rutaceae* and *Restionaceae* as well as bulbous plants such as *Gladiolus*. Beverages such as rooibos (*Aspalathus linearis*) and honeybush (*Cyclopia spp.*) have been explored for tea purposes. Examples of medicinal plants partly or fully developed as commercial crops with established plantations include *Agathosma betulina*, *Aloe ferox*, *Artemisia afra*, *Aspalathus linearis*, *Bulbine frutescens*, *Cyclopia genistoides*, *Harpagophytum procumbens*, *Hoodia gordonii*, *Hypoxis hemerocallidea*, *Lippia javanica*, *Mesembryanthemum tortuosum* (=*Sceletium tortuosum*), *Pelargonium sidoides*, *Siphonochilus aethiopicus*, *Sutherlandia frutescens* (=*Lessertia frutescens*), *Warburgia salutaris* and *Xysmalobium undulatum* (Van Wyk 2011). Indigenous vegetables include *Amaranthus*, *Cleome*, *Dovyalis*, *Plectranthus*, and *Vigna* (Reinten & Coetzee 2002). Some of these plants are only in demand in small niche markets while others have a potential to become new products for consumers. For example, rooibos and honeybush tea industries are expanding with a world

market and have been transformed to a commercial viable agribusiness, while *Proteaceae* flowers have already established themselves in the international flower trade.

2.6 PLANT METABOLOMICS AS A QUALITY CONTROL TOOL FOR MEDICINAL PLANTS

The quality of medicinal plant material plays a fundamental role in guaranteeing the quality of herbal preparations. The purpose of quality control of raw medicinal plant material is to monitor the efficacy, toxicity substances and variation in the production process. However, the herbal industry and regulatory agencies have more interest in the chemical composition of raw material, as it is the main factor determining product quality and authenticity. The variation of the chemical constituents in the extract due to genetic and environmental conditions in plants is a well-known factor. This difference does not only occur in the main bioactive compounds but also in the low-concentration compounds in the extract. In herbal preparations the content of all or low bioactive compounds is as valuable as that of highly bioactive compounds, as some compounds have a tendency to function in synergy. "Synergy" simply means that the effect of the combination is greater than the sum of the individual effects (Rasoanaivo *et al.* 2011). Thus crude extracts have often been reported to have higher activity than pure industrially produced drugs or drugs isolated from the plant. A significant synergysm has been demonstrated in many studies including a study between *Cinchona* bark alkaloids active against *Plasmodium falciparumin* invitro and some, which are not. In the study, a combination of a least potent of the alkaloids; quinine (IC_{50}=280ng/ml) with those with low 50% inhibitory activity; quinidine (IC_{50}=80ng/ml) and cinchonine (IC_{50}=80ng/ml), was 2-10 times more effective invitro against quinine-resistant strains with (IC_{50}=25ng/ml) than individual alkaloids. Furthermore, the mixture of alkaloids showed a more consistent effect than any of the alkaloids used singly (Druilhe *et al*. 1987). Thus new approaches/strategies such as metabolomics are believed to have a potential in providing suitable solutions to variation in chemical constituents and efficancy. Metabolomics is concerned with the comprehensive analysis of metabolites as a whole in a given sample and offer a way of assessing the chemical variability in a biological system.

2.6.1 The metabolomics approach

Metabolomics is the omics technology, which was developed in the 1990's, and has become an important field in botany. Currently, research in metabolomics has become increasingly widespread. The study of metabolomics is generally defined as a holistic qualitative and quantitative analysis of all metabolites present within a biological system under specific conditions (Verpoorte *et al*. 2007). It has been applied in many different fields including microbial metabolomics, animal metabolomics, medical metabolomics, plant metabolomics and metabolomics of food and herbal products (Putri *et al*. 2013). In the context of plants, the goal of metabolomics is to measure all the metabolites in a cell or tissue type of a plant, both qualitatively and quantitatively. The intention is to provide a clear metabolic picture of metabolites of that cell or tissue type during different growth stages or under certain stimuli conditions such as environmental stress and physical stimulations such as processing (Gang *et al*. 2010). In simple terms, metabolomics analysis provide a snapshot (a point-in-time-chemistry) of a biological system (cell, tissue or whole organism), showing which metabolites are present and their levels at a given time point under specific physiological conditions (Verpoorte *et al*. 2007). This provides an opportunity for a comprehensive understanding of the metabolite structure, its regularity, and the regulatory mechanism of the metabolite in response to changing factors. Understanding these factors would be of great significance for either increasing or timing periods for increased content of active ingredients and thus ensuring traditional medicine of best quality.

A plant metabolomics analysis comprises three main experimental stages. A reliable sample preparation procedure that is simple, rapid and at the same time reproducible and suitable for high throughput analysis; data acquisition using advanced analytical methods and data mining using chemometrics that are suitable for handling huge datasets usually/normally generated by metabolomics analysis (Kim, Choi, *et al*. 2010; Tugizimana *et al*. 2013). These include multivariate data analysis such as principal component analysis (PCA) and partial least squares-discriminant analysis (PLS-DA) which are employed routinely to extract information from very large datasets. A detailed flow diagram for a plant metabolomics approach is outlined in Figure 2.3.

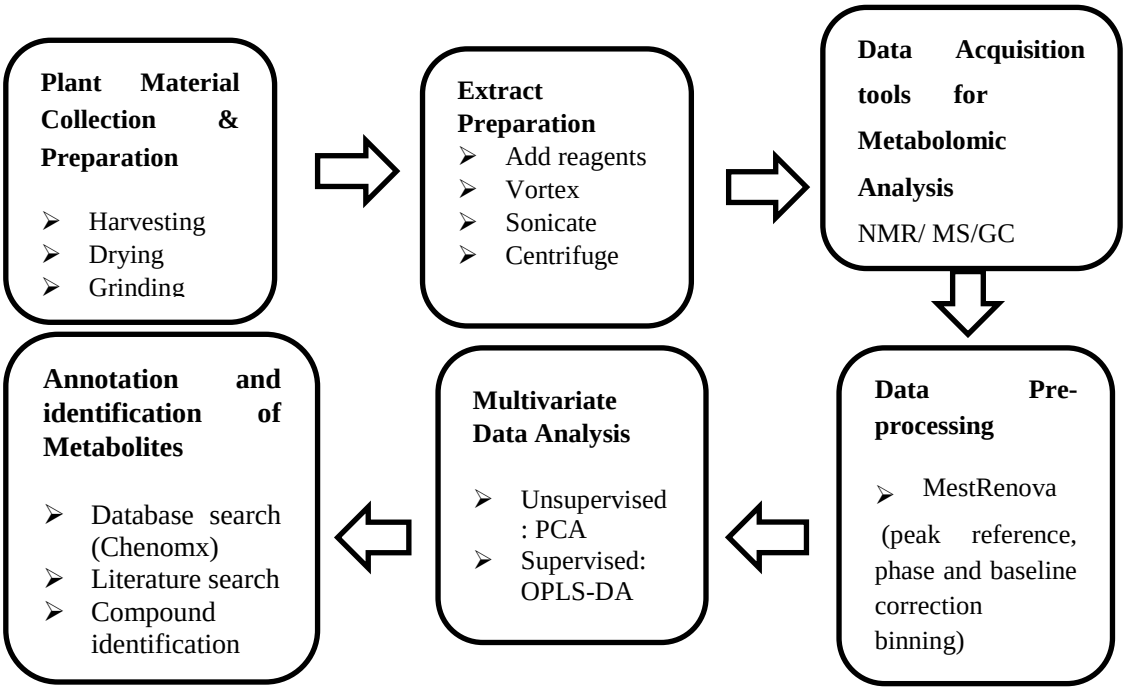

Figure 2.3 Flow chart representing experimental stages of metabolomics analysis adapted from Tugizimana *et al*. (2013).

Metabolomics sets itself apart from targeted phytochemical analysis methods by being a data-driven approach with predictive power that aims to assess all measurable metabolites without pre-selection or pre-conception (Tugizimana *et al*. 2013). In order to achieve this aim, advanced analytical tools that provide high degrees of sensitivity, selectivity and reproducibility are required. A range of analytical techniques employed in plant metabolomics include gas chromatography-mass spectrometry (GC-MS), liquid chromatography-mass spectrometry (LC-MS), nuclear magnetic resonance (NMR) spectroscopy and Fourier-transform infrared (FT-IR) (Jiang *et al*. 2006; Van der Kooy *et al.* 2008; Rusanov *et al*. 2011; Mediani *et al*. 2012; Albrecht *et al*. 2012; Farag *et al*. 2012; Heyman & Meyer 2012; Lee *et al*. 2013; Sellami *et al.* 2013; Zhao *et al.* 2013; Booker *et al*. 2014; Fan *et al*. 2014; Farag *et al.* 2014; Kwon, Ahn, *et al.* 2014; Kwon, Bong, *et al*. 2014; Nkomo *et al*. 2014; Mahrous & Farag 2015; Heyman *et al*. 2015; Ordoudi *et al*. 2015; Zahmanov *et al.* 2015). Amongst these analytical tools LC-MS and NMR are the most commonly used.

2.6.2 NMR as a metabolomic tool

As a tool for metabolomics, NMR has some unique advantages over MS-based methods. NMR is an unbiased, rapid, non-destructive technique with a relatively simple sample preparation and thus lessens the chance of sample loss or the introduction of variability into the preparation (Verpoorte *et al*. 2007; Kim, Choi, *et al.* 2010). NMR allows the simultaneous detection of diverse groups of secondary metabolites (flavonoids, alkaloids, terpenoids) besides abundant primary metabolites (sugars, organic acids, amino acids) (Kim, Choi, *et al*. 2010) thus providing a general view of all the metabolites present in an organism. Furthermore, NMR spectra offers the opportunity to make the direct comparison of concentrations of all compounds possible, with signals reflecting the real molar concentrations or levels of metabolites present in a plant without the need for calibration curves of each individual compound. In addition, NMR is a very useful technique for structure elucidation and using various two-dimensional NMR measurements, many signals can be identified often without the need for further fractionation of the extract. NMR spectroscopy has very high reproducibility as it is based on the physical characteristics of compounds (Van Der Kooy *et al.* 2009). Despite its disadvantage of low sensitivity (Kim, Choi, *et al*. 2010) NMR spectroscopy is still being favoured for metabolomic analysis due to the robustness of data and the ability to cover a broad range of metabolites (Van Der Kooy *et al*. 2009). On the other hand, the challenge of low sensitivity is being addressed by new developments in NMR hardware such as the development of low temperature probes such as the CryoProbe (Bruker Biospin GmbH, Rheinstetten, Germany) or Cold Probe (Varian, Palo Alto, CA, USA).

2.6.3 Use of metabolomics in traditional medicine

Plant metabolomics has been successfully applied in many fields of traditional medicine. Many studies have widely used spectroscopy methods coupled with chemometric methods to either classify, authenticate and characterize different species of medicinal plants or to investigate the influence of seasons, geographic origin/location/sites, growth environment, developmental stages, harvesting, drying, processing and storage on the phytochemical composition and thus quality of traditional medicinal plants (Jiang *et al*. 2006; Fan *et al.* 2014; Farag *et al*. 2014; Kim, *et al*. 2010; Kwon, *et al*. 2014; Farag *et al*. 2012; Kim *et al.* 2014; Wang *et al*. 2004; Zahmanov *et al.* 2015; Kim *et al.* 2005; Scognamiglio *et al*. 2014; Duan *et al*. 2012; Lee *et al*. 2013; Nkomo

et al. 2014; Sellami *et al.* 2013; Kim & Verpoorte 2010; Mediani *et al*. 2012; Ordoudi *et al*. 2015; Zhao *et al*. 2013; Scognamiglio *et al*. 2015; Ma *et al*. 2008) Plant metabolomics has also been used as a quick method for drug discovery and in the quality control or evaluation of herbal products (commercial prepared extracts) (Rasmussen *et al*. 2006; Van der Kooy *et al*. 2008; Van Der Kooy *et al.* 2009; Agnolet *et al*. 2010; Heyman & Meyer 2012; Heyman *et al.* 2015).

2.6.3.1 Classification and characterization of species

Metabolomic profiling has been used in the classification and characterization of different *Swertia* species using NMR (Fan *et al*. 2014). NMR based metabolomics was found to be reliable and effective for metabolomic profiling and discrimination of the two *Swertia* species (*Swertia mussotii* and *Swertia chirayita*). Significantly higher contents of gentiopicrin, isoorientin, glucose, loganic acid and choline were found in *Swertia mussotii* and *Swertia chirayita* exhibited higher levels of swertiamarin, oleanolic acid, valine and fatty acids. Another example would be that of characterization of 6 *Nigella* species using UPLC-qTOF-MS and GC–MS coupled to chemometrics (Farag *et al*. 2014). Large scale profiling resulted in annotation of 52 metabolites. Major peaks contributing to the discrimination among species were assigned as kaempferol glycosidic conjugates with kaempferol-3-O-[glucopyranosyl-(1-2)-galactopyranosyle-(1-2) glucopyranoside identified as potential taxonomic marker for one of the species, *Nigella sativa*. Metabolomic profiling was also used to investigate the chemical diversity within ginger species and between ginger and closely related species in the genus *Zingiber* (Zingiberaceae) using GC/MS-based metabolic profiling. The metabolomic profile of other Zingiber species studied was very different both qualitatively and qualitatively, when compared to *Zingiber officinale* and to each other. Because these species were exposed to the same identical conditions, at the same time in the same greenhouse, variation in metabolites composition was therefore attributed to genetic variation rather than environmental factors.

2.6.3.2 Seasonal influence

Plant metabolomics using NMR combined with PCA was used to determine seasonal metabolic changes in *Phillyrea augustifolia* (Scognamiglio *et al*. 2014). Collected leaves were assessed for each month from April 2011 to March 2012. The results of PCA showed clustering according to seasons in which leaves were collected. For example, July and August were clustered together

representing the summer season and the December to March cluster represented the winter season. Oleuropein and dialdehydic form of decarboxymethyloleuropein (DHPEA-EDA) were identified as the main metabolites present in all extracts. Sugar signals, mainly glucose, proline and organic acids dominating in the summer season while phenolic compounds were absent. The sample collected in April was dominated by oleuropein while aldehydic metabolite dominating the sample collected in June and oleuropein and dialdehydic form of decarboxymethyloleuropein (DHPEA-EDA) dominating the spectrum of September. In general, plants collected in winter showed the highest amount of oleuropein.

2.6.3.3 Influence of geographic origin

In order to investigate the effects of geographic origin in *Astragalus membranaceous* and *Paeonia albiflora* originating from China and Korea, NMR and ICP-AES/ICP-MS coupled with integrated multivariate analysis was used (Kwon, Bong, *et al*. 2014). Metabolomics were effective in determining the geographical origins of the two species allowing diverse environmental factors such as climate and geology to be considered. Another example would be that of classification of *Anemarrhena asphodeloides* from Korea and China, using UPLC-QTOF MS and multivariate analysis. PCA successfully clustered 21 samples of plant material into two groups according to the place of origin (Korea or China).

Metabolomic profiling using LC-MS coupled with PCA and OPLS-DA was also used to assess the metabolite content of seed pods of *Sutherlandia frutescens* growing in 15 different geographical environments, 13 wild growing and 2 cultivated sites (Albrecht *et al*. 2012). PCA analysis revealed five distinct clusters with cultivated/garden plants forming a distinct cluster from wild collected plants. The garden grown plants had unique metabolites. Focusing on populations from only two regions, Karoo and coastal Gansbaai area, OPLS-DA separated the groupings into two clusters according to the area of origin. Sutherlandioside, the anticancer compounds and its derivatives was detected in plants harvested in the Karoo while those harvested in Gansbaai area did not show any sutherlandin traces suggesting that for increased sutherlandioside, Karoo would be the best area to harvest. In-vitro grown plants showed a similar metabolite profile to wild collected plants. These studies validate the potential of this

metabolomic technique as an important tool to identify and determine the origins of medicinal plants.

2.6.3.4 Influence of growth environment

HPLC-ESI/IT/MS and HPLC-ESI/QqQ/MS/MS analysis were used to qualitatively characterize and to quantify secondary metabolites in umbels of *Ammi visnaga* plants grown under different conditions viz. in the field, hydroponically controlled and contrasted by salinity and/or hypoxia (Sellami *et al*. 2013). The study paid attention to two groups of bioactive compounds namely the furanochromes and pyranocoumarins. The results of the study showed that growing conditions do influence the qualitative and quantitative profile of *A. visnaga* secondary metabolites. Increased levels of bioactive metabolites were produced in the hydroponic cultured *A. visnaga* plants.

2.6.3.5 Quality control of herbal products or commercial prepared extracts

NMR based metabolomics combined with HPLC-PDA-MS-SPE-NMR and PCA analysis was used to investigate standardized *Ginkgo biloba* preparations (Agnolet *et al*. 2010). Standardized *G. biloba* leaf extracts were assessed for metabolite content as well as identity of the flavonoid glycoside and trirpene trilactones (TTLs) (3.1% of ginkgolides and 2.9% bilobalide) which are known to be constituted in the leaf extract. The results of the study showed a global composition of standardized *G. biloba* preparations. Because NMR is non-selective, simultaneous assessment of TTLs and flavonoid glycoside were achieved. Cases of non-conformity to standard ratio between ginkgolides and bilobalide and detection of the potentially harmful components were discovered.

In another study NMR metabolomics was combined with PCA to assess *Artemisia* plant material formulation sold by a herbal company, which claims that the capsule contain artemisinin and its derivatives and therefore able to fight against malaria (Van der Kooy *et al*. 2008). These authors also aimed at assessing whether the artemisinin used in the capsules was indeed obtained from *A. afra* as the company claimed and not from *A. annua*, which is known to produce the compound. The results of the study showed that the *A. afra* extract does not contain any artemisinin and even if the company had obtained artemisinin from *A. annua*, the concentrations claimed in the

capsules are far less than the ones obtained in this study. These studies suggest that metabolomics would be a valuable and rapid tool in the quality control of herbal products.

2.6.3.6 Influence of plant developmental stages

GC and GC-MS coupled with PCA and PLS-DA was used to investigate metabolites of *Artemisia annua* L. at five stages of development viz. tender seedling stage, adult seedling stage, pre-flower budding stage, flower budding stage and full flowering stage (Ma *et al*. 2008). The PCA results showed separation into 5 groups according to the developmental stage. However, there was no clear separation of groupings between tender seedling stage and adult seedling stage indicating an absence of difference in metabolites between these two stages. On the other hand, a clear difference of metabolites was evident at pre-flower budding stage, flower budding stage and full flowering. The concentration of artemisinic acid and dihydroartemisinic acid slightly increased from pre-flower budding stage to flowering stage for one of the samples while artemisinin showed no significant increase, but arteannuin B increased significantly at flowering stage. The pre-flower budding stage was determined as the best sampling or harvest stage for the production of artemesinin and its derivatives. This study showed that it is possible to discriminate different developmental stages and to determine the best harvesting developmental stage using metabolomics.

2.6.3.7 Influence of harvesting, processing methods including drying and storage

NMR combined with PCA and PLS was used to distinguish variations among *Cosmo caudatas* plant material that was processed with various drying methods viz. freeze, air and oven drying (Mediani *et al*. 2012). Freeze and air drying contained higher levels of flavonoids and sugars compared to the oven dried plant material. Most of the identified metabolites were present in plant material exposed to the three drying methods. However, there was a marked variation in the concentrations of the constituents present. The results obtained from this study suggests that freeze and air drying were the best drying method that yielded potential valuable products in terms of antioxidant activity.

NMR metabolomics coupled with PCA was employed to assess the effects of harvesting time on *Cannabis sativa* (unpublished data cited by Kim and Verpoorte (2010). Samples were harvested

at different times of the day, in the morning or evening and the harvesting time for the third sample is not known. PCA results showed a clear separation with samples clustered according to the collection time of the day. The levels of cannabinoids varied with the harvesting time and therefore contributed to the separation.

LC-MS and PCA were used to assess the contribution of postharvest processing to phytochemical variation of *Corydalis rhizoma* and rhizome of *Corydalis yanhusuo* (Zhao *et al*. 2013). The harvested rhizomes were grouped according to three commonly used traditional processing methods i.e. left raw, boiled or raw plants fumigated with sulphur before they were exposed to drying and storage. The PCA results showed a clear separation between the three treatments demonstrating a variation in the phytochemical profile. Water boiling showed an increase in most compounds while sulphur-fumigation showed a decrease in most compounds. Protopine, coptisine and palmatine were identified as the most variable components in the processing of these plants. Water boiling was the best processing method that yielded increased phytochemical contents.

To study the effect of storage on metabolites using metabolomics, NMR combined with PCA and PLS-DA were employed in a study by Ordoudi and co-authors in 2015. Metabolite changes were assessed in plant material of saffron that was exposed to two storage periods, namely freeze and air drying; 0-4 years and from 5-15 years after processing (Ordoudi *et al*. 2015). The PCA showed a clear separation of samples into two groups according to the storage period. The metabolites responsible for the discrimination were identified as sugars bound to crocetin, glucose in picrocrosin, free sugars and fatty acids. The results obtained in this study showed that saffron appropriately stored retained valuable products at least after four years of storage. The results suggested that NMR was a valuable metabolomic tool to show quality deterioration of saffron upon storage period.

2.6.3.8 Investigating the use of metabolomics as an efficient tool for drug discovery

NMR-based metabolomics guided fractionation was used to identify anti-HIV active dicaffeoylquinic and tricaffeoylquinic acid in *Helichrysum populifolium* (Heyman *et al*. 2015). Out of 64 plant extracts from 30 *Helichrysum* species, only 22 extracts from 5 species were able

to inhibit HIV activity in a cell-based assay. NMR-based metabolomics was able to identify 5 specific chemical shift areas that could be linked to anti-HIV activity of the extracts. OPLS-DA showed a separation of active and non-active extracts and it became possible to correlate phytochemical composition with the biological activity of the extracts. The activity profile was then used to guide the fractionation process by narrowing down and focusing the fractionation and purification process to speed up the putative identification of the 5 compounds with anti-HIV activity in the most active species. Five new anti-HIV compounds were identified from *H. populifolium* of which three of those compounds were dicaffeoylquinic acid derivatives viz. 3,4-dicaffeoylquinic acid or 3,5-dicaffeoylquinic acid and 4.5 dicaffeoylquinic acid as well as two tricaffeoylquinic acid derivatives viz. 1,3,5-tricaffeoylquinic acid and either 5-malonyl-1,3,4-tricaffeoylquinic acid or 3-malonyl-1,4,5-tricaffeoylquinic acid. NMR-based metabolomics guided fractionation showed a potential to speed up drug discovery with anti-HIV activity by reducing the number of bioassay runs as well as directing the purification and isolation process in a more focused manner in searching only for the identified anti-HIV characteristic in the plant extract of interest.

2.7 REFERENCES

Abdelgadir, H. a. et al., 2012. Enhancing seedling growth of Jatropha curcas-A potential oil seed crop for biodiesel. *South African Journal of Botany*, 78, pp.88–95. Available at: http://dx.doi.org/10.1016/j.sajb.2011.05.007.

Adeyemi, SB., Ogundele, KO., Animasaun, A.M., 2014. Influence of drying methods on the proximate and phytochemical composition of Moringa oleifera Lam. *Global journal of medicinal plant research*, 2(1), pp.1–5.

Agnolet, S. et al., 2010. 1H NMR-based metabolomics combined with HPLC-PDA-MSSPE-NMR for investigation of standardized Ginkgo biloba preparations. *Metabolomics*, 6(2), pp.292–302.

Akwatulira, F. et al., 2011. Influence of rooting media and indole-3-butyric acid (IBA) concentration on rooting and shoot formation of Warburgia ugandensis stem cuttings. *African Journal of Plant Science*, 5(8), pp.421–429.

Albrecht, C.F. et al., 2012. LC-MS-based metabolomics assists with quality assessment and traceability of wild and cultivated plants of Sutherlandia frutescens (Fabaceae). *South African Journal of Botany*, 82, pp.33–45. Available at: http://dx.doi.org/10.1016/j.sajb.2012.07.018.

Aminah, H. et al., 2015. Effect of Hormone and Cutting Length on the Rooting of Tinospora Crispa. , 5(3), pp.3–6.

Aminah, H., Nor Hasnita, R.M.N. & Hamzah, M., 2006. Effects of indole butyric acid concentrations and media on rooting of leafy stem cuttings of shorea parvifolia and shorea macroptera. *Journal of Tropical Forest Science*, 18(1), pp.1–7.

Amri, E. et al., 2010. Vegetative propagation of African Blackwood (Dalbergia melanoxylon Guill. & Perr.): effects of age of donor plant, IBA treatment and cutting position on rooting ability of stem cuttings. *New Forests*, 39(2), pp.183–194. Available at: http://link.springer.com/10.1007/s11056-009-9163-6.

Andrew, R.L. et al., 2007. Heritable variation in the foliar secondary metabolite sideroxylonal in Eucalyptus confers cross-resistance to herbivores. *Oecologia*, 153(4), pp.891–901.

Araya, H. T., 2005. Seed germination and vegetative propagation of bush tea (*Athrixia phylicoides*) By Hintsa Tesfamicael Araya. *Submitted in partial fulfilment of the requirements for the degree MSc (Agric) Horticulture Department of Plant Production*

and Soil Science In t. University of Pretoria.

Arct, J. & Pytkowska, K., 2008. Flavonoids as components of biologically active cosmeceuticals. *Clinics in Dermatology*, 26(4), pp.347–357.

Bagdonaite, E. et al., 2012. Variation in concentrations of major bioactive compounds in Hypericum perforatum L. from Lithuania. *Industrial Crops and Products*, 35(1), pp.302–308.

Balandrin, M.F. et al., 1985. Natural plant chemicals: sources of industrial and medicinal materials. *Science (New York, N.Y.)*, 228(4704), pp.1154–1160.

Bapela, M.J. et al., 2007. Variation in the content of naphthoquinones in seeds and seedlings of Euclea natalensis. *South African Journal of Botany*, 73(4), pp.606–610. Available at: http://www.sciencedirect.com/science/article/pii/S0254629907003201 [Accessed July 7, 2015].

Baraldi, R. et al., 2008. Distribution of artemisinin and bioactive flavonoids from Artemisia annua L. during plant growth. *Biochemical Systematics and Ecology*, 36(5–6), pp.340–348. Available at: http://www.sciencedirect.com/science/article/pii/S0305197807002402 [Accessed July 7, 2015].

Booker, A. et al., 2014. Chemical variability along the value chains of turmeric (Curcuma longa): A comparison of nuclear magnetic resonance spectroscopy and high performance thin layer chromatography. *Journal of Ethnopharmacology*, 152(2), pp.292–301. Available at: http://dx.doi.org/10.1016/j.jep.2013.12.042.

Buwa, L. V. & Van Staden, J., 2007. Effects of collection time on the antimicrobial activities of Harpephyllum caffrum bark. *South African Journal of Botany*.

Canter, P.H., Thomas, H. & Ernst, E., 2005. Bringing medicinal plants into cultivation: Opportunities and challenges for biotechnology. *Trends in Biotechnology*, 23(4), pp.180–185.

Chamorro, E. et al., 2008. Chemical composition of essential oil from Tagetes minuta L. leaves and flowers. *J.Argent.Chem.Soc.*, 96(1–2), pp.80–86. Available at: \\Robsrv-05\reference manager\Articles\12501.pdf.

Chao, J. et al., 2017. Improving the Concentrations of the Active Components in the Herbal Tea Ingredient, Uraria crinita: The Effect of Post-harvest Oven-drying Processing. *Scientific Reports*, 7(November 2016), p.38763. Available at:

http://www.nature.com/articles/srep38763.

Coates Palgrave, K., Coates Palgrave, P. & Coates Palgrave, M.C.N.-D., 1985. *Everyone's Guide to Trees of South Africa* 3rd editio., Thorold's Africana Books.

De Cahna, M.N. et al., 2015. Extract of Greyia radlkoferi and use thereof. Available at: www.google.com/patents/WO2013171720A1?cl=en.

De Cerqueira, M.D. et al., 2007. Seasonal variation and antimicrobial activity of Myrcia myrtifolia essential oils. *Journal of the Brazilian Chemical Society*, 18(5), pp.998–1003.

de la Cruz, P., 2004. Greyia radlkoferi., pp.1–3. Available at http://www.plantzafrica.com/plantefg/greyrad.htm.

de Matos Nunes, J. et al., 2014. Stress induction of valuable secondary metabolites in Hypericum polyanthemum acclimatized plants. *South African Journal of Botany*, 94, pp.182–189. Available at: http://dx.doi.org/10.1016/j.sajb.2014.06.014.

Dambolena, J.S. et al., 2010. Essential oils composition of Ocimum basilicum L. and Ocimum gratissimum L. from Kenya and their inhibitory effects on growth and fumonisin production by Fusarium verticillioides. *Innovative Food Science and Emerging Technologies*, 11(2), pp.410–414. Available at: http://dx.doi.org/10.1016/j.ifset.2009.08.005.

Dartora, N. et al., 2011. UPLC-PDA-MS evaluation of bioactive compounds from leaves of Ilex paraguariensis with different growth conditions, treatments and ageing. *Food Chemistry*, 129(4), pp.1453–1461. Available at: http://dx.doi.org/10.1016/j.foodchem.2011.05.112.

Demuner, A.J. et al., 2011. Seasonal variation in the chemical composition and antimicrobial activity of volatile oils of three species of leptospermum (Myrtaceae) grown in Brazil. *Molecules*, 16(2), pp.1181–1191.

Dhami, N. & Mishra, A.D., 2015. Phytochemical variation: How to resolve the quality controversies of herbal medicinal products? *Journal of Herbal Medicine*, 5(2), pp.118–127. Available at: http://linkinghub.elsevier.com/retrieve/pii/S2210803315000299.

Dogheim, S.M. et al., 2004. Pesticides and heavy metals levels in Egyptian leafy vegetables and some aromatic medicinal plants. *Food additives and contaminants*, 21(4), pp.323–330.

Duan, L.X. et al., 2012. Use of the metabolomics approach to characterize chinese medicinal material Huangqi. *Molecular Plant*, 5(2), pp.376–386. Available at: http://dx.doi.org/10.1093/mp/ssr093.

Dubey, N.K., Kumar, R. & Tripathi, P., 2004. Global promotion of herbal medicine: India's

opportunity. *Current Science*, 86(1), pp.37–41.

Druilhe P. *et al*. 1988. Activity of a combination of three Cinchona bark alkaloids against *Plasmodium falciparum* in vitro. Antimicrobial agents and chemotherapy 32 (2), 250-254.

Fan, G. et al., 2014. Metabolic discrimination of Swertia mussotii and Swertia chirayita known as "Zangyinchen" in traditional Tibetan medicine by 1H NMR-based metabolomics. *Journal of Pharmaceutical and Biomedical Analysis*, 98, pp.364–370. Available at: http://dx.doi.org/10.1016/j.jpba.2014.06.014.

Farag, M. a. et al., 2014. Metabolomics driven analysis of six Nigella species seeds via UPLC-qTOF-MS and GC-MS coupled to chemometrics. *Food Chemistry*, 151, pp.333–342. Available at: http://dx.doi.org/10.1016/j.foodchem.2013.11.032.

Farag, M. a., Porzel, A. & Wessjohann, L. a., 2012. Comparative metabolite profiling and fingerprinting of medicinal licorice roots using a multiplex approach of GC-MS, LC-MS and 1D NMR techniques. *Phytochemistry*, 76, pp.60–72. Available at: http://dx.doi.org/10.1016/j.phytochem.2011.12.010.

Gang, F. et al., 2010. Discussion on the Investigation Idea of Quality Control of Chinese Materia Medica Based on Metabolomics Technology. *World Science and Technology*, 12(6), pp.870–875. Available at: http://dx.doi.org/10.1016/S1876-3553(11)60033-1.

Govindaraghavan, S. & Sucher, N.J., 2015. Quality assessment of medicinal herbs and their extracts: Criteria and prerequisites for consistent safety and efficacy of herbal medicines. *Epilepsy & Behavior*. Available at: http://linkinghub.elsevier.com/retrieve/pii/S1525505015001092.

Hamilton, A.C., 2004. Medicinal plants, conservation and livelihoods. *Biodiversity and Conservation*, 13(8), pp.1477–1517.

Harbourne, N. et al., 2009. Effect of drying methods on the phenolic constituents of meadowsweet (Filipendula ulmaria) and willow (Salix alba). *LWT - Food Science and Technology*, 42(9), pp.1468–1473. Available at: http://www.sciencedirect.com/science/article/pii/S0023643809001297 [Accessed July 7, 2015].

Hartmann, H.. et al., 2011. *Plant Propagation. Principles and Practices.* Eighth edi., Sao Paulo, Prentic- Hall.

Hartmann, H.. et al., 1997. *Plant Propagation. Principles and Practices.* - sixth edi., Upper

Saddle River, New Jersey, Prentice Hall.

Hartmann, T. & Kester, D.E., 1983. *Plant Propagation—Principle and Practices* Fourth edi., Eaglewood Cliffs, New Jersey.

Heyman, H.M. et al., 2015. Identification of anti-HIV active dicaffeoylquinic- and tricaffeoylquinic acids in Helichrysum populifolium by NMR-based metabolomic guided fractionation. *Fitoterapia*, 103, pp.155–64. Available at: http://www.sciencedirect.com/science/article/pii/S0367326X15000891 [Accessed June 4, 2015].

Heyman, H.M. & Meyer, J.J.M., 2012. NMR-based metabolomics as a quality control tool for herbal products. *South African Journal of Botany*, 82, pp.21–32. Available at: http://www.sciencedirect.com/science/article/pii/S0254629912000543 [Accessed July 6, 2015].

Hosni, K. et al., 2011. Phenological variations of secondary metabolites from Hypericum triquetrifolium Turra. *Biochemical Systematics and Ecology*, 39(1), pp.43–50.

Isolabella, S. et al., 2010. Study of the bioactive compounds variation during yerba mate (Ilex paraguariensis) processing. *Food Chemistry*, 122(3), pp.695–699. Available at: http://www.sciencedirect.com/science/article/pii/S0308814610003134 [Accessed July 7, 2015].

Jiang, H. et al., 2006. Metabolic profiling and phylogenetic analysis of medicinal Zingiber species: Tools for authentication of ginger (Zingiber officinale Rosc.). *Phytochemistry*, 67(15), pp.1673–1685.

Kadu, C. a C. et al., 2012. Bioactive constituents in Prunus africana: Geographical variation throughout Africa and associations with environmental and genetic parameters. *Phytochemistry*, 83, pp.70–78. Available at: http://dx.doi.org/10.1016/j.phytochem.2012.06.001.

Kambizi, L., Adebola, P.O. & Afolayan, a. J., 2006. Effects of temperature, pre-chilling and light on seed germination of Withania somnifera; a high value medicinal plant. *South African Journal of Botany*, 72(1), pp.11–14.

Kim, H.K., Saifullah, et al., 2010. Metabolic classification of South American Ilex species by NMR-based metabolomics. *Phytochemistry*, 71(7), pp.773–84. Available at: http://www.sciencedirect.com/science/article/pii/S0031942210000427 [Accessed July 7,

2015].

Kim, H.K. et al., 2005. Metabolic fingerprinting of Ephedra species using 1H-NMR spectroscopy and principal component analysis. *Chemical & pharmaceutical bulletin*, 53(1), pp.105–109.

Kim, H.K., Choi, Y.H. & Verpoorte, R., 2010. NMR-based metabolomic analysis of plants. *Nature protocols*, 5(3), pp.536–549.

Kim, H.K. & Verpoorte, R., 2010. Sample preparation for plant metabolomics. *Phytochemical Analysis*, 21(1), pp.4–13.

Kim, N. et al., 2014. A metabolomic approach to determine the geographical origins of Anemarrhena asphodeloides by using UPLC-QTOF MS. *Journal of Pharmaceutical and Biomedical Analysis*, 92, pp.47–52. Available at: http://dx.doi.org/10.1016/j.jpba.2013.12.040.

Kim, N. et al., 2006. Application of novel HPLC method to the analysis of regional and seasonal variation of the active compounds in. *Food Chemistry*, 96(3), pp.496–502. Available at: http://www.sciencedirect.com/science/article/pii/S0308814605003018 [Accessed July 7, 2015].

Kim, N.K. et al., 2015. Seasonal Variations of Metabolome and Tyrosinase Inhibitory Activity of *Lespedeza maximowiczii* during Growth Periods. *Journal of Agricultural and Food Chemistry*, 63(38), pp.8631–8639. Available at: http://pubs.acs.org/doi/10.1021/acs.jafc.5b03566.

Kulkarni, M.G., Sparg, S.G. & Van Staden, J., 2007. Germination and post-germination response of Acacia seeds to smoke-water and butenolide, a smoke-derived compound. *Journal of Arid Environments*, 69(1), pp.177–187.

Kulkarni, M.G., Street, R. a. & Van Staden, J., 2007. Germination and seedling growth requirements for propagation of Dioscorea dregeana (Kunth) Dur. and Schinz - A tuberous medicinal plant. *South African Journal of Botany*, 73(1), pp.131–137.

Kwon, Y.K., Bong, Y.S., et al., 2014. An integrated analysis for determining the geographical origin of medicinal herbs using ICP-AES/ICP-MS and 1H NMR analysis. *Food Chemistry*, 161, pp.168–175. Available at: http://dx.doi.org/10.1016/j.foodchem.2014.03.124.

Kwon, Y.K., Ahn, M.S., et al., 2014. Discrimination of cultivation ages and cultivars of ginseng leaves using Fourier transform infrared spectroscopy combined with multivariate analysis.

Journal of Ginseng Research, 38(1), pp.52–58. Available at: http://dx.doi.org/10.1016/j.jgr.2013.11.006.

Lall, N., Mogapi, E., Canha, M.N. De, et al., 2016. Insights into tyrosinase inhibition by compounds isolated from Greyia radlkoferi Syzsyl using biological activity, molecular docking and gene expression analysis. *Bioorganic & Medicinal Chemistry*. Available at: http://dx.doi.org/10.1016/j.bmc.2016.09.054.

Lavola, A., 1998. Accumulation of flavonoids and related compounds in birch induced by UV-B irradiance. *Tree physiology*, 18(1), pp.53–58. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12651299.

Lebrun, A., Toussaint, A.. & Roggemans, J., 1998. Description of Syzygium paniculatum Gaertn. `Verlaine' and its propagation by stem cuttings. *Scientia Horticulturae*, 75(1–2), pp.103–111. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0304423898001228.

Lee, D.K. et al., 2013. Comparison of primary and secondary metabolites for suitability to discriminate the origins of Schisandra chinensis by GC/MS and LC/MS. *Food Chemistry*, 141(4), pp.3931–3937. Available at: http://dx.doi.org/10.1016/j.foodchem.2013.06.064.

Lee, S.Y. et al., 2016. Metabolite profiling of Neptunia oleracea and correlation with antioxidant and α-glucosidase inhibitory activities using 1H NMR-based metabolomics. *Phytochemistry Letters*, 16, pp.23–33.

Lemos, M.F. et al., 2015. Seasonality modifies rosemary's composition and biological activity. *Industrial Crops and Products*, 70, pp.41–47. Available at: http://dx.doi.org/10.1016/j.indcrop.2015.02.062.

Lubbe, A. et al., 2013. Seasonal accumulation of major alkaloids in organs of pharmaceutical crop Narcissus Carlton. *Phytochemistry*, 88, pp.43–53. Available at: http://dx.doi.org/10.1016/j.phytochem.2012.12.008.

Ma, C. et al., 2008. Metabolic fingerprinting investigation of Artemisia annua L . in different stages of development by gas chromatography and gas chromatography – mass spectrometry. , 1186, pp.412–419.

Mahrous, E. A. & Farag, M. A., 2015. Two dimensional NMR spectroscopic approaches for exploring plant metabolome: A review. *Journal of Advanced Research*, 6(1), pp.3–15. Available at: http://linkinghub.elsevier.com/retrieve/pii/S209012321400126X.

Makunga, N.P., Philander, L.E. & Smith, M., 2008. Current perspectives on an emerging formal

natural products sector in South Africa. *Journal of Ethnopharmacology*, 119(3), pp.365–375.

Manish Agarwal, Vartika Rai, S.K. and S.M., 2014. Effect of microbial load on therapeutically active constituent glycyrrhizin of. , 13(April), pp.319–324.

Máthé, Á. & Máthé, I., 2008. Quality assurance of cultivated and gathered medicinal plants. *Acta Horticulturae*, 765, pp.67–76.

Mbangcolo, M.M., 2008. *Aspects of Honeybush Tea (Cyclopia Species)*.

Mediani, A., Abas, F., Khatib, A., Maulidiani, H., Shaari, K., Choi, Y.H. & Lajis, N.H., 2012. 1H-NMR-based metabolomics approach to understanding the drying effects on the phytochemicals in Cosmos caudatus. *Food Research International*, 49(2), pp.763–770. Available at: http://www.sciencedirect.com/science/article/pii/S096399691200381X [Accessed April 16, 2015].

Mendonça-Filho, R.R., 2006. Bioactive Phytocompounds: New Approaches in the Phytosciences. *Modern Phytomedicine: Turning Medicinal Plants into Drugs*, pp.1–24.

Mesén, F., Newton, a. C. & Leakey, R.R.B., 1997. Vegetative propagation of Cordia alliodora (Ruiz & Pavon) Oken: the effects of IBA concentration, propagation medium and cutting origin. *Forest Ecology and Management*, 92(1–3), pp.45–54. Available at: http://www.sciencedirect.com/science/article/pii/S0378112796039606.

Mhamdi, B. et al., 2010. Effect of harvesting time on phenolic compounds and antiradical scavenging activity of Borago officinalis seed extracts. *Industrial Crops and Products*, 31(1), pp.e1–e4. Available at: http://www.sciencedirect.com/science/article/pii/S092666900900096X [Accessed July 7, 2015].

Murch, S.J. et al., 2003. Nickel contamination affects growth and secondary metabolite composition of St. John's wort (Hypericum perforatum L.). *Environmental and Experimental Botany*, 49(3), pp.251–257.

Ncube, B., Finnie, J.F. & Van Staden, J., 2012. Quality from the field: The impact of environmental factors as quality determinants in medicinal plants. *South African Journal of Botany*, 82, pp.11–20. Available at: http://dx.doi.org/10.1016/j.sajb.2012.05.009.

Ncube, B., Finnie, J.F. & Van Staden, J., 2011. Seasonal variation in antimicrobial and phytochemical properties of frequently used medicinal bulbous plants from South Africa.

South African Journal of Botany, 77(2), pp.387–396. Available at: http://dx.doi.org/10.1016/j.sajb.2010.10.004.

Nkomo, M.M. et al., 2014. Fusarium inhibition by wild populations of the medicinal plant Salvia africana-lutea L . linked to metabolomic profiling. , pp.1–9.

Ofori, D. a et al., 1996. Vegetative propagation of Militia excelsa by leafy stem cuttings : effects of auxin concentration , leaf area and rooting medium. , 84, pp.39–48.

Ogundele, K.O. & Animasaun, M.A., 2015. Influence of drying methods on the proximate and phytochemical composition of Moringa. , pp.1–7.

Olmez, Z., Gokturk, A. & Gulcu, S., 2006. Effects of cold stratification on germination rate and percentage of caper (Capparis ovata Desf.) seeds. *Journal of Environmental Biology*, 27(4), pp.667–670.

Ordoudi, S.A. et al., 2015. 1H NMR-based metabolomics of saffron reveals markers for its quality deterioration. *Food Research International*, 70, pp.1–6. Available at: http://www.sciencedirect.com/science/article/pii/S0963996915000368 [Accessed July 7, 2015].

Palgrave, K.C. & Palgrave, M.C., 2002. *Palgrave's Trees of Southern Africa* 3rd editio. E. J. Moll, ed., Cape Town: Struik Publishers.

Pavarini, D.P. et al., 2012. Exogenous influences on plant secondary metabolite levels. *Animal Feed Science and Technology*, 176(1–4), pp.5–16. Available at: <Go to ISI>://WOS:000308454000002.

Potisate, Y., Kerr, W.L. & Phoungchandang, S., 2015. Changes during storage of dried Moringa oleifera leaves prepared by heat pump-assisted dehumidified air drying. *International Journal of Food Science & Technology*, 50(5), pp.1224–1233. Available at: http://doi.wiley.com/10.1111/ijfs.12744.

Putri, SP. et al., 2013. Current metabolomics: Practical applications. *Journal of Bioscience and Bioengineering*, 115(6), pp.579–589. Available at: http://dx.doi.org/10.1016/j.jbiosc.2012.12.007.

Rasmussen, B. et al., 2006. Multivariate analysis of integrated and full-resolution 1H-NMR spectral data from complex pharmaceutical preparations: St. John's wort. *Planta medica*, 72(6), pp.556–563.

Rasoanaivo P. *et al*., 2011. Whole plant extracts versus single compounds for the treatment of

malaria: synergy and positive interactions. Malar J. 10 (1): S4

Reinten, E. & Coetzee, J.H., 2002. Commercialization of South African Indigenous Crops: Aspects of Research and Cultivation of Products. *Trends in New Crops and New Uses*, pp.76–80.

Maggini R., Kiferle C. & Pardossi A., 2014. Hydroponic production of medicinal plants. In D. alexandre M. Pereira, ed. *Medicinal Plants: Antioxidant Properties, Traditional Uses and Conservation Strategies*. Nova Science Publishers, pp. 91–116. Available at: https://www.novapublishers.com/catalog/product_info.php?products_id=46118.

Routray, W. & Orsat, V., 2014. Variation of phenolic profile and antioxidant activity of North American highbush blueberry leaves with variation of time of harvest and cultivar. *Industrial Crops and Products*, 62, pp.147–155. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0926669014005007.

Rusanov, K. et al., 2011. Traditional Rosa damascena flower harvesting practices evaluated through GC/MS metabolite profiling of flower volatiles. *Food Chemistry*, 129(4), pp.1851–1859. Available at: http://www.sciencedirect.com/science/article/pii/S0308814611008351 [Accessed July 7, 2015].

Sato, S. et al., 2004. Simultaneous determination of the main metabolites in rice leaves using capillary electrophoresis mass spectrometry and capillary electrophoresis diode array detection. *The Plant journal : for cell and molecular biology*, 40(1), pp.151–163.

Scognamiglio, M. et al., 2015. Chemical Composition and Seasonality of Aromatic Mediterranean Plant Species by NMR-Based Metabolomics. , 2015, pp.3–7.

Scognamiglio, M. et al., 2014. Seasonal phytochemical changes in Phillyrea angustifolia L.: Metabolomic analysis and phytotoxicity assessment. *Phytochemistry Letters*, 8, pp.163–170. Available at: http://www.sciencedirect.com/science/article/pii/S1874390013001511 [Accessed May 14, 2015].

Sellami, H.K. et al., 2013. Influence of growing conditions on metabolite profile of Ammi visnaga umbels with special reference to bioactive furanochromones and pyranocoumarins. *Phytochemistry*, 95, pp.197–206. Available at: http://dx.doi.org/10.1016/j.phytochem.2013.08.001.

Severino, L.S. et al., 2011. Propagation by stem cuttings and root system structure of Jatropha curcas. *Biomass and Bioenergy*, 35(7), pp.3160–3166.

Southwell, I. a & Bourke, C. a, 2001. Seasonal variation in hypericin content of Hypericum perforatum L. (St. John's Wort). *Phytochemistry*, 56(5), pp.437–441.

Taylor, J.L.S. & Van Staden, J., 2001. The effect of age, season and growth conditions on anti-inflammatory activity in Eucomis autumnalis (Mill.) Chitt. Plant extracts. *Plant Growth Regulation*.

Tchinda, N.D. et al., 2013. Improving propagation methods of Ricinodendron heudelotti Baill. from cuttings. *South African Journal of Botany*, 88, pp.3–9. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0254629913002573.

Teketay, D., 1996. The effect of different pre-sowing seed treatments , temperature and light on the germination of five Senna species from Ethiopia. , (Thulin 1989), pp.155–171.

Tugizimana, F., Piater, L. & Dubery, I., 2013. Plant metabolomics:A new frontier in phytochemical analysis. *South African Journal of Science*, 109(5), pp.1–11.

Verpoorte, R., Choi, Y.H. & Kim, H.K., 2007. NMR-based metabolomics at work in phytochemistry. *Phytochemistry Reviews*, 6(1), pp.3–14.

Van Der Kooy, F. et al., 2009. Quality control of herbal material and phytopharmaceuticals with MS and NMR based metabolic fingerprinting. *Planta Medica*, 75(7), pp.763–775.

Van der Kooy, F., Verpoorte, R. & Marion Meyer, J.J., 2008. Metabolomic quality control of claimed anti-malarial Artemisia afra herbal remedy and A. afra and A. annua plant extracts. *South African Journal of Botany*, 74(2), pp.186–189. Available at: http://www.sciencedirect.com/science/article/pii/S0254629907003742 [Accessed July 6, 2015].

Van Vuuren, S.F. et al., 2007. Seasonal and geographical variation of Heteropyxis natalensis essential oil and the effect thereof on the antimicrobial activity. *South African Journal of Botany*, 73(3), pp.441–448.

Van Wyk, B.E., 2011. The potential of South African plants in the development of new medicinal products. *South African Journal of Botany*, 77(4), pp.812–829.

Wang, Y. et al., 2004. Metabolomic strategy for the classification and quality control of phytomedicine: A case study of chamomile flower (Matricaria recutita L.). *Planta Medica*, 70(3), pp.250–255.

Wilson, P.J., 1993. Propagation Characteristics of Eucalyptus-Globulus Labill Ssp Globulus Stem Cuttings in Relation to Their Original Position in the Parent Shoot. *Journal of*

Horticultural Science, 68(5), pp.715–724. Available at: <Go to ISI>://A1993LX32100010.

Yang, D.-M., 2012. Antioxidant and Anti-Tyrosinase Activity from Olea Leaf Extract Depended on Seasonal Variations and Chromagraphy Treatment. *International Journal of Organic Chemistry*, 2(4), pp.391–397. Available at: http://www.scirp.org/journal/PaperDownload.aspx?DOI=10.4236/ijoc.2012.24054.

Zahmanov, G. et al., 2015. Metabolic differentiations of dwarf elder by NMR-based metabolomics. *Phytochemistry Letters*, 11, pp.404–409. Available at: http://www.sciencedirect.com/science/article/pii/S1874390014002432 [Accessed July 7, 2015].

Zhao, Y. et al., 2013. Effects of Postharvest Processing and Geographical Source on Phytochemical Variation of Corydalis Rhizoma. *Chinese Herbal Medicines*, 5(2), pp.151–157. Available at: http://www.sciencedirect.com/science/article/pii/S1674638413600234\nhttp://ac.els-cdn.com/S1674638413600234/1-s2.0-S1674638413600234-main.pdf?_tid=f28dfeea-ddc4-11e2-93c4-00000aacb362&acdnat=1372185053_946c71a1180a5063b4c312fecb030529.

Zobayed, S.M.A., Afreen, F. & Kozai, T., 2007. Phytochemical and physiological changes in the leaves of St. John's wort plants under a water stress condition. *Environmental and Experimental Botany*, 59(2), pp.109–116.

Zobayed, S.M. a, Afreen, F. & Kozai, T., 2005. Temperature stress can alter the photosynthetic efficiency and secondary metabolite concentrations in St. John's wort. *Plant Physiology and Biochemistry*, 43(10–11), pp.977–984.

CHAPTER 3

DEVELOPMENT OF VEGETATIVE AND SEED PROPAGATION PROTOCOLS FOR THE EFFICIENT CULTIVATION OF *GREYIA RADLKOFERI*

ABSTRACT

Greyia radlkoferi is one of the important and recently studied cosmeceutical trees or shrubs indigenous to South Africa. It offers a great potential as a source of extracts for the development of cosmeceutical products having the ability to treat hyperpigmentation. In order to develop the product, *G. radlkoferi* must be produced at a commercial scale. *Greyia radlkoferi* is known to regenerate naturally by seed. In an effort to introduce *G. radlkoferi* into cultivation, the effect of 5 temperature treatments ranging from 10°C-30°C on the germination of its seeds was investigated. The trial was carried out under laboratory-controlled conditions in incubators in continuous darkness. Germination of *G. radlkoferi* seeds was temperature dependent. Warm temperatures between 20°C and 25°C were more favourable for the germination of this species with optimum germination temperature being 25°C with 81% germination. In vegetative propagation by stem cuttings, the effect of cutting position, exogenous powdered rooting hormone (Seradix1 with 0.1% IBA, Seradix 2 with 0.3% IBA and 0.1% IBA, 0.3% IBA and 0.8% IBA liquid solution) and cutting position were studied in the glasshouse. Cutting position had a significant effect on rooting of *G. radlkoferi* cuttings with basal cuttings rooting better with 35% rooting as compared to 6% rooting attained for the apical cuttings. A clear trend in rooting response to application of rooting hormones was observed, with 0.1% Indole butyric acid (IBA) showing the highest rooting percentage. Considering the results of this study, seed propagation appears to be the most suitable technique for large-scale multiplication of *G. radlkoferi*. This data will contribute to the establishment of an efficient protocol for cultivation of *G. radlkoferi* for commercial purposes.

.

3.1 INTRODUCTION

Recent studies have shown that *G. radlkoferi* offers a great potential for the development of pharmaceuticals or cosmetic agents for use against dermatological disorders associated with hyperpigmentation (Lall *et al*. 2016). Before product development can commence, *G. radlkoferi* must be produced at a commercial scale through cultivation. Sustainable production of actives for commercialization requires a sustainable production of plant material in terms of volume and consistent supply. *Greyia radlkoferi* is known to be cultivated only for its ornamental display. It is mostly used as a decorative tree for inland gardens (Pooley 1993) and is popular as an ornamental plant (Coates Palgrave *et al*. 1985). There is a great difference between cultivating for ornamental use and cultivating for leaf production with the interest in bio-actives. Crop production for ornamental use focuses on the aesthetic value of the plant while production for bioactives is concerned with the bioactive product of interest. Cultivation offers the opportunity to optimize yield, achieve a uniform quality product and allows for control in every stage of the production system (Amujoyegbe *et al*. 2012). It might also allow for modification of concentrations of the biological active compounds through manipulation of growing environments.

Plant propagation is generally regarded as the first phase of the complete package of production of herbal medicine. The aim of propagation is to prove the reproducibility of plants for mass production. Two types of propagation methods, namely, vegetative (through asexual reproduction) and seed (through sexual reproduction) have been recognized as efficient means of rapid multiplication of plants (Hartmann *et al*. 2011). Selection of species specific and appropriate, easier, rapid propagation methods and ideal propagation conditions for mass propagation is important for commercial producers as it will increase yields leading to more availability of the plants to the market and thus increased profits. Propagation by seed and by cuttings is considered practical for use by developing farmers.

Among the various factors influencing the germination of a seed, temperature is the most prominent environmental factor regulating the timing of germination (Hartmann *et al*. 2011) of a plant. Temperature regulation of seed germination is attributed partly to dormancy release and

partly to climatic adaptation (Hartmann *et al*. 2011). Seed response to temperature depends on species, variety, growing region, quality of the seed and duration of time from harvest (Araya 2005). This means at any given time, space or set of conditions, seeds have a base temperature below which it will not germinate and an optimal temperature at which it germinates. Temperate region seeds require lower temperatures than tropical region seed. Seeds with high quality are able to germinate under a wider range of temperatures than low quality seeds (Copeland and McDonald, 1994).

Propagation by cutting is reported to be the most preferred method as it captures and conserve the genetic diversity of the plant; it is rapid, simple, economical and does not require much space (Hartmann *et al*. 1997). It provides high quality material in terms of homogeneity and accelerates production through quick establishment for improved quantity. Successful propagation of cuttings is determined by adventitious root formation (Hartmann *et al*. 2011). However, several factors including cutting type and rooting hormone affect the development of adventitious roots (Wilson 1993) and thus the success of the cuttings of a particular species.

It is generally known that hormones, particularly auxins promote the qualitative and quantitative development of adventitious roots of stem cuttings. Auxins achieve this function through their ability to promote the initiation of lateral root primordia and to enhance transport of carbohydrates to the cutting base (Hartmann *et al*. 1990). Indole butyric acid (IBA) is one of the most commonly used auxins to promote rooting of stem cuttings. However, the rooting response varies with different species and IBA concentrations ranging from no effect to a significant effect thereby suggesting a species-specific optimum IBA concentration. Concentrations of auxin substantially higher than those normally found in plant tissues may cause cell death (Hartmann *et al*. 1990).

Cutting position has been found to be closely related to the rooting ability of some species. The difference in rooting ability of stem cuttings obtained from different cutting position is attributed to either the cutting stem volume (length and diameter) which determines the storage capacity of carbohydrates produced pre- and post-severance (Leakey *et al*. 1994) or natural accumulation of endogenous auxin in the basal part of the stock plant (Wilson 1993).

The known mode of regeneration of *G. radlkoferi* in nature is by seeds. To our knowledge, there are no previous reports on the propagation of *G. radlkoferi.* Therefore, the aim of this study was to introduce *G. radlkoferi* into cultivation by determining the ideal propagation method for large-scale commercial production of this species.

The objectives of this study were,

i) To determine the effects of constant temperature and dark exposure on germination of *G. radlkoferi* seeds,

ii) To develop vegetative means of propagation for *G. radlkoferi* cuttings by defining optimum hormone treatments ideal for consistent high rooting percentage.

iii) To determine the effects of cutting position on rooting of *G. radlkoferi* stem cuttings.

iv) To determine the most suitable propagation method for propagation of *G. radlkoferi.*

3.2 MATERIALS AND METHODS

3.2.1 Propagation by seeds

Seeds were collected in August from flowers of trees planted at the Mothong African Heritage Trust garden in Mamelodi, Pretoria in October 2012 (GPS co-ordinates: $25^0$41′49.7″S $28^0$20′17.4″E). Seeds were collected when the flowers were dry and the seedpods were slightly open and were immediately used for the germination tests. Mature seeds of *G. radlkoferi* are dark brown in colour and are narrow, thin and almost cylindrical in shape. They are about 3-5mm in length or less and almost 1mm in width. However, the seedpods also contain of black, almost needle or linear shaped seeds, which were non-viable as they failed to germinate in all preliminary trials.

The study was carried out at the University of South Africa (UNISA) Science Campus (GPS co-ordinates: $26^0$9.042′S 27.904002′E). Germination behaviour of *G. radlkoferi* was tested in incubators (Memmert model, Lasec, South Africa) at constant temperatures of 10°C, 15°C, 20°C, 25°C and 30°C in continuous darkness. The selection of temperatures for germination of *G. radlkoferi* was based on preliminary studies. Incubators maintained the designated temperature to within ±1°C. To investigate the germination response of seeds to selected temperatures, three replicates, each in a separate plastic container (120mmx70mm) with a lid consisting of 25 freshly harvested seeds were prepared. The seeds were randomly dispersed on seed germination paper that was pre-moistened with distilled water. The plastic containers were then wrapped in a double layer of aluminium foil to ensure complete darkness, and incubated in germination chambers. The seed germination paper was moistened with distilled water as and when needed. The seeds were inspected daily over a period of 2 weeks and germinated seeds were counted. The seeds were considered to have completed germination upon radicle emergence through the seed coat (Hartmann *et al*. 2011). The data obtained was subjected to analysis of variance and Tukey's LSD multi comparison test at 5 % level was used to test the significance between the treatment means for germination percentage of seeds using GenStat® (Payne, 2014) statistical package.

3.2.2 Propagation by stem cuttings

The trials on vegetative reproduction of *G. radlkoferi* were carried out in the spring season in a glasshouse located at the Agricultural Research Council-Vegetable and Ornamental Plants (ARC-VOP) in Roodeplaat, Pretoria (GPS co-ordinates 25°59"S 28°35"E). The aim of this trial was to determine the effect of cutting type (apical or basal) on the rooting of *G. radlkoferi* treated with different rooting hormones using compost as a rooting media. Healthy young *G. radlkoferi* trees were purchased in September 2012 from Random harvest- a commercial supplier (nursery) based in Honeydew, Johannesburg and were kept in the shade-net at ARC-VOP. Cuttings were collected randomly from vigorously growing branches of mother stock trees which showed no sign of pests and diseases in October 2012. In an effort to prevent shoots from wilting, the cuttings were collected during early hours of the day (07h30-08h30) while they were still turgid and immediately put in a bucket of water to maintain high moisture levels.

To determine the cutting type; the cuttings prepared from the terminal sections of the shoot were regarded as apical cuttings and cuttings taken below the terminal section were regarded as basal cuttings. Ten centimeter (10cm) long basal and apical cuttings of between 3 and 5mm in diameter cut from the stock plants were treated with Seradix® 1 powder (0.1% IBA concentration) and Seradix® 2 powder (0.3% IBA concentration), and liquid solutions of 0.1% IBA, 0.3% IBA, 0.8% IBA. Before dipping in hormone treatments, cuttings were stripped of the bottom leaves, leaving only the top leaf intact. About 1 cm of the base of the apical and basal cuttings were dipped in IBA solutions for ten seconds based on the treatment. For Seradix® 1 powder and Seradix® 2 powder treatments, 1cm of the base of apical and basal cuttings were dipped in the powder and the excess powder was removed by slightly tapping the cutting prior to planting in growing medium. Immediately after the application of treatments, the cuttings were planted about 2cm deep in polyethylene planting bags (12cmx10cm) filled with pre-moistened composted bark as a growing medium. A stick was used to open a hole for planting in the growing media. This was done in order to prevent the applied treatments from brushing off upon insertion. After planting, the polyethylene bags were placed according to the statistical layout in a glasshouse. The cuttings were irrigated daily using a hosepipe.

The experimental design was a randomised complete block design with 2 cutting types, 6 treatments (Seradix® 1, Seradix® 2, 0.1% IBA, 0.3% IBA and 0.8% IBA and control) replicated in 3 blocks. Each treatment combination consisted of 10 cuttings and the total number of cuttings used for this trial was 360. The parameters that were measured were number of cuttings rooted, number of roots, root lengths, leaf sprouted and leaf number and were assessed after 30 days of growth. The data for roots was collected by carefully separating the cuttings from the bags and washing the root zone with water to remove the soil.

The data was analysed using GLM (generalized linear modelling) procedure of GenStat® statistical package (Payne 2014). Fisher's protected least significant difference was performed at 5 % level to compare treatment means. A probability level of 5% was considered significant for all tests.

3.3 RESULTS

3.3.1 Seed germination response to temperature

Temperature significantly affected germination time of *G. radlkoferi.* The seeds of *G. radlkoferi* started to germinate 6 days after sowing and germinated up to day 13 after sowing (Table 3.1). Seeds exposed to 20°C and 25°C were the first to germinate on day 6 after sowing, followed by 30°C on day 9 and 15°C on day 12. Seeds exposed to10°C failed to germinate over the whole period of the experiment (14 days/2 weeks). However, when these seeds were shifted from 10°C to 25°C after 14 days, germination commenced on day 21 (6 days after shifting) immediately recording 20% germination percentage (data not shown).

Table 3.1: Temperature effects on the days to beginning of germination and days to ending of germination of *G. radlkoferi.*

Temperature (°C)	Onset of germination (days)	End of germination (days)
10	-	-
15	12	13
20	6	11
25	6	11
30	9	13

The final germination percentage of *G. radlkoferi* seeds was affected significantly by temperature with most temperature treatments showing statistical significant differences at P≤0.05. Seed germination percentage increased with the increases in temperature from 15°C to an optimum point at 25°C and decreased thereafter at 30°C (Fig 3.1). The highest germination percentage was 81% at 25°C followed by seeds exposed to 20°C at 78%. However, the difference in germination percentage between the two temperature treatments was not significant (P> 0.05). No significant seed germination occurred at 15°C and 30°C while germination failed to occur at 10°C. Germination percentage of 47% was recorded at 30°C and the lowest germination percentage of 32% was recorded at 15°C.

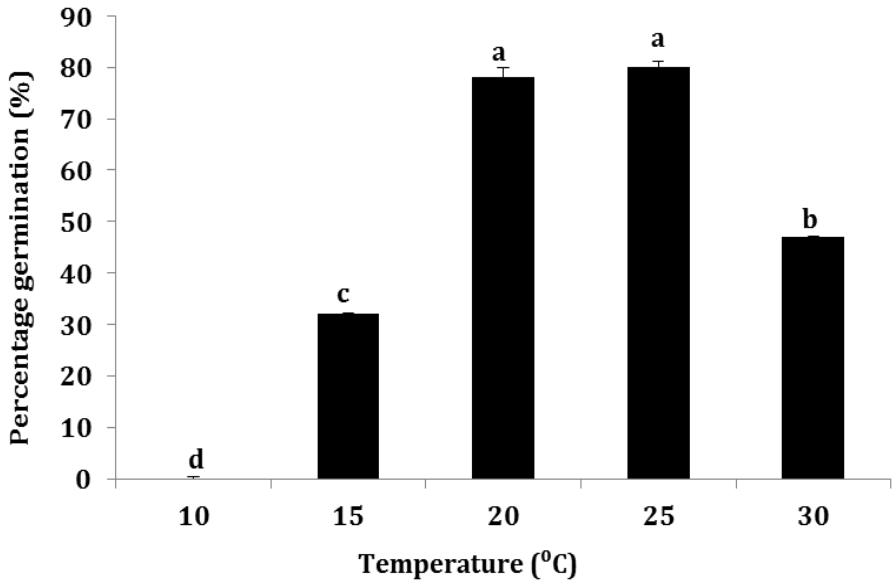

Figure 3.1: Seed germination of *G. radlkoferi* in response to various temperature treatments.

3.3.2 Propagation by stem cuttings

(a)The effect of cutting type

Highly significant differences (P<0.05) were obtained between basal and apical cuttings (Table 3.2). The thirty-five percent (35%) rooting percentage obtained in basal cuttings was five times higher than the 6% of rooting obtained in apical cuttings. Similarly, average root number of basal cuttings was 5 times higher than that of apical cuttings. Above forty percent (42.22%) of plants

sprouted leaves and obtained average leaf number of 27.62 in basal cuttings while only 5.56% of apical cuttings developed leaves with a 0.22 average leaf number. Stem diameters were measured before the onset of the experiment and during the analysis it was discovered that stem diameters of the basal cuttings were significantly larger than those of apical cuttings.

Table 3.2: Effect of cutting type on the percentage rooting, root number and leaf sprouting percentage and leaf number of *G. radlkoferi* stem cuttings.

Cutting type	Rooting percentage	Average root number	Percentage Leaf sprouting	Average leaf number	Stem diameter (mm)
Basal	35[a]	15.50[a]	42.22[a]	27.62[a]	0.83[a]
Apical	6.67[b]	2.88[b]	5.56[b]	0.22[b]	0.42[b]

Means with the same letter are not significantly different from each other at 5% level (F pr. <0. 05)

(b) Effect of hormone treatments

In terms of rooting percentage, root number, root length and percentage leaf sprouting, no significant differences were found between the 5 hormone treatments used including the control treatment. However, hormone treatment with 0.1% IBA recorded the highest values for all parameters measured (Table 3.3). Seradix 2 and 0.1% IBA produced significantly more leaves than all other hormone tratments. The rooting percentage, root number, root length and leaf number and percentage sprouting declined as the IBA concentration increased from 0.1% IBA to 0.8% IBA with 0.8% IBA recording values even lower than in the control treatment. The rooting percentage of cuttings treated with 0.1% IBA was 19% higher than that of the control cuttings.

(c) Interactive effects of cutting type and hormone treatments

No significant differences were found in interactive studies between the two cutting positions and the hormone treatments in terms of rooting percentage and the percentage of cuttings that sprouted (Figure 3.2 and Figure 3.3). However, basal cuttings in all treatments (with or without hormone) recorded the highest rooting percentage and the percentage of cuttings that sprouted compared to all the apical cuttings (with or without hormone). Considering basal cuttings only, the highest rooting percentage of 63% was obtained at 0.1% IBA (Figure 3.2) while the highest percentage of cuttings that sprouted leaves (53%) was obtained with Seradix 2 (Figure 3.3).

Table 3.3: Effects of hormone treatments on the rooting percentage, number of roots, number of leaves, sprouting percentage, and root length of *G. radlkoferi* cuttings.

Hormone treatments	Rooting percentage	Number of roots	Leaf sprouting percentage	Number of leaves	Root length
Seradix 1	23±0.07	19 ±7.01	30±0.06	8.24±2.62[b]	5.8
Seradix 2	23±0.06	16±6.56	30±0.06	21.51±4.25[a]	5.3
0.1% IBA	35±0.06	28±8.64	27±0.06	28.57±4.90[a]	9.4
0.3% IBA	15±0.05	11±5.30	16±0.05	8.24±2.63[b]	4.1
0.8% IBA	11±0.05	7±4.29	15±0.05	5.55±2.15[b]	2.5
Control	16±0.6	12±5.62	25±0.06	11.42±3.09[b]	3.0

Means without letters and means with the same letter are not significantly different from each other at 5% level (F pr. <0. 05)

The rooting percentage of cuttings treated with 0.1% IBA was 33% higher than that of the control cuttings. Similar to the results tabulated in Table 3.3, a decline in rooting and leaf sprouting percentage with the increase in IBA concentration was also observed in the interaction study in basal and apical cuttings.

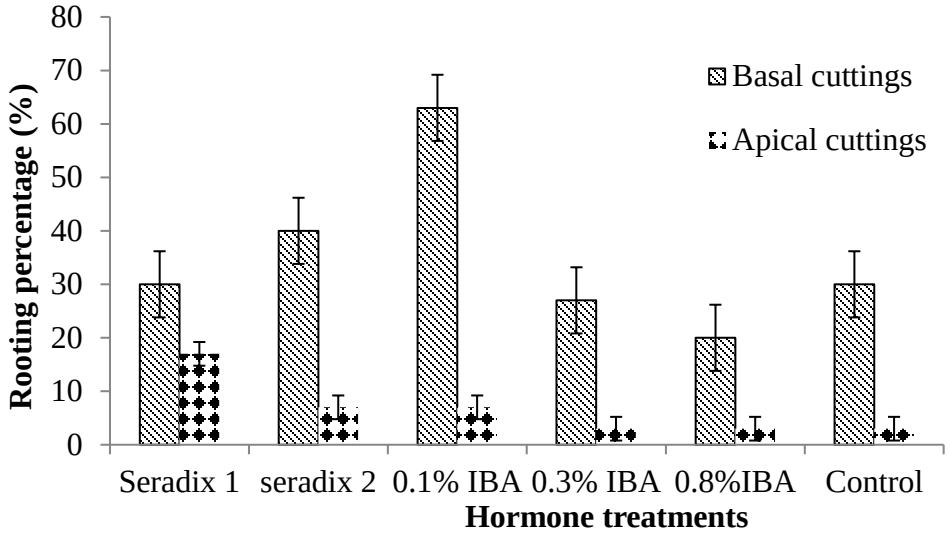

Figure 3.2: Interactive effects of hormone treatments and cutting type on the rooting percentage of *G. radlkoferi* stem cuttings.

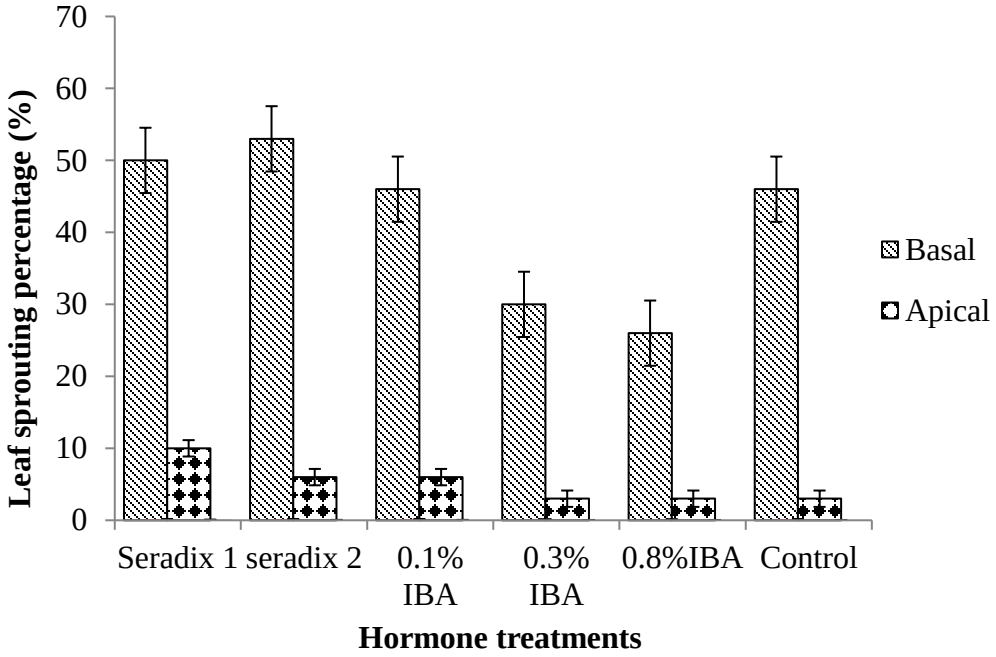

Figure 3.3: Interactive effects of hormone treatments and cutting type on percentage leaf sprouting of *G. radlkoferi* stem cuttings.

3.4 DISCUSSION

Temperature is the most important environmental condition that regulates the timing of germination (Hartmann *et al*. 2011) of species. It can promote or inhibit the germination process partly due to dormancy release and partly due to climatic adaptations. In this study, seed germination results indicate that germination percentage and time to germination were clearly affected by exposure to a range of constant temperatures (from 10°C to 30°C) in the dark after 14 days of germination. Germination of the seeds responded differently to the five temperature treatments. A large percentage (81%) of *G. radlkoferi* seeds germinated at 25°C. These results fall within the range between 15°C and 30°C of optimum temperatures for germination as reported for most plant species (Copeland and McDonald, 1994).

Poor germination at very low and high temperatures reported in legume species such as *V. unguiculata* (Balkaya 2004), and in Brassica species (Tokumasu Satoru *et al*. 1985) is assumed to indicate that little or no germination will take place during winter or mid-summer seasons in the field. This could be seen as a protection mechanism against excessive seedling mortality (Chanyenga *et al*. 2012). In the same line of thought, field emergence of *G. radlkoferi* can also be predicted by associating the temperature requirements for germination with seasonal soil temperature. During germination, *G. radlkoferi* shows intolerance to cold (<15°C) and hot (>25°C) temperatures which are common characteristics of winter and mid-summer seasons respectively. This indicates that winter and summer seasons are not suitable for germinating *G. radlkoferi* as little to no germination may be expected to occur. *Greyia radlkoferi* however, requires warm temperatures (20°C -25°C) to germinate, indicating that seeds may germinate well in the early spring season. *Greyia radlkoferi* seeds which had initially failed to germinate at 10°C in the course of the trial attained 20% germination just 6 days after they were transferred to 25°C and thus confirming the appropriate temperature needs for this plants. These results are in agreement with those of Kulkarni *et al*. (2007) where *Dioscorea dregeana* seeds which had failed to germinate upon exposure to 10°C, achieved a 30% germination percent after they were shifted from 10°C to 25°C. Furthermore, the temperature requirements obtained for *G. radlkoferi* germination in this study is typical temperature-requirements for germination of tropical and/or subtropical plants (Hartmann *et al*. 2011).

The onset of germination of *G. radlkoferi* seeds began on the 6th day following exposure to 20°C and 25°C temperature and proceeded at a rapid speed reaching end of germination within five days (day 11) with high germination percentages (81%). These results indicate that *G. radlkoferi* seeds have the ability to germinate fast when the germination conditions are favourable.

The exogenous application of hormones did not significantly influence the rooting of *G. radlkoferi* at $P \leq 0.05$, however, a noticeable difference in percentage rooting between the treatments was observed with optimum rooting percentage obtained in 0.1% IBA. A striking characteristic of the current results was a decline in rooting percentage in concentrations higher than 0.1% (i.e. 0.3% IBA and 0.8% IBA) as evidenced in hormone treatment analysis and in the interaction between hormones and cutting type analysis. This decline in rooting percentage with increase in IBA concentration is a common characteristic of studies in which IBA is used as a rooting hormone. Araya (2005) noted a decline in rooting promotion when IBA concentration higher than 0.1% was used. Furthermore, rooting rate, number of leaves, roots and root-length were reduced in IBA concentrations greater than 100 μg/l in *Ricinodendron heudelotti* Baill (Tchinda *et al*. 2013) while a decline in rooting percentage with IBA concentrations greater than 0.2% was shown in *Milicia excels* (Ofori *et al*. 1996). In such studies, the decline in rooting percentage in response to IBA concentrations higher than the optimum is associated with inhibition in rhizogenesis caused by exogenous application of high levels of the hormone. According to Hartmann *et al*. (1990) concentrations of auxin substantially higher than those normally found in plant tissues may cause cell death. Therefore, the use of exogenous auxins at concentrations appropriate for a particular species is essential. However, such inference cannot be drawn from the results of current study since the difference between hormone treatments was not significant.

Cutting type had a great influence on the rooting, mean root number, leaf sprouting and mean leaf number of *G. radlkoferi* with all of these parameters higher in basal cuttings than in cuttings taken from the apex. The current findings on high rooting ability of basal cuttings are in agreement with studies undertaken on *Syzygium paniculutum* (Lebrun *et al*. 1998), *Dalbergia melanoxylon* (Amri *et al*. 2010) and *Ricinodendron heudelotti (*Tchinda *et al*. 2013). Hartmann &

Kester (1983) states that the best rooting of cuttings is usually found from the basal portions of shoots and there is a gradient in rooting response from top to base.

The high rooting ability in basal cuttings over apical cuttings can be attributed to the cutting stem volume (influenced by basipetal gradients in stem length and diameter) which determines the storage capacity of carbohydrates produced pre- and post-severance (Leakey *et al*. 1994). This could be true for *G. radlkoferi* considering that the total mean stem diameter of basal cuttings was significantly higher than that of apical cuttings. Based on stem diameter data it can be assumed that basal cuttings of *G. radlkoferi* had enough carbohydrate reserves to provide for not just rooting, but also leaf development at post-severance. Alternatively, the high rooting ability of basal cuttings over apical cuttings can be attributed or associated with natural accumulation of endogenous auxin in the basal part of the stock plant (Wilson 1993). Depending on the endogenous level of growth-regulating substances at time of severance, exogenous application of auxin may be promotive, ineffective or even inhibitory for the rooting of cuttings (Hartmann *et al*. 1990). In the current study a large percentage of basal cuttings (with or without hormone) developed roots, compared to apical cuttings (with or without hormone), with the highest rooting percentage of 63% obtained in 0.1% IBA. However, the interaction between cutting type and hormone was not statistically significant suggesting that little or no rooting enhancement is as a consequence of auxins. On the contrary, 63% rooting achieved by the basal cuttings treated with 0.1% IBA cannot be completely ignored. According to Hartmann *et al*. (2011) if the rooting ability of a new cultivar is over 50%, propagation of such cultivar by cuttings is economically feasible.

3.5 CONCLUSIONS

The germination percentage and the time to the onset of germination of *G. radlkoferi* was affected by exposure to constant temperatures. *Greyia radlkoferi s*eeds germinated optimally under continuous dark exposure at temperatures of 25°C. Temperatures below 15°C and temperatures above 25°C were unfavourable for germination of *G. radlkoferi* seeds. Based on this investigation, seeds of this species would be expected to germinate well when planted during spring after the occurrence of cold winter temperatures and before the occurrence of hot mid-summer temperatures.

The temperatures that were determined as favourable for germination of *G. radlkoferi* seeds do not only assist in predicting appropriate seasons for growing *G. radlkoferi*, but new locations for cultivating this plant. However, germination is a function of multiple environmental factors. In this study, the experiments were conducted under controlled environmental conditions of constant temperatures, whereas under natural conditions temperature is subject to diurnal fluctuations. Due to some study limitations, the effect of other environmental factors affecting germination could not be included. Future studies should focus on the effects of other responsible factors and perhaps their interactions, to refine the results obtained in this study. Seedling survival studies would also be suitable to determine the success of seed propagation of this plant.

Cutting type was the major influencing factor in vegetative production of *G. radlkoferi* cuttings. It affected the rooting percentage, average root number and leaf development. A large percentage of basal cuttings developed a high number of roots and leaves compared to apical cuttings and rooting was achieved within 30 days. Thus compared to apical cuttings, basal cuttings are a better planting material for vegetative reproduction of *G. radlkoferi*.

Although the use of exogenous hormones did not have a significant effect in rooting of *G. radlkoferi* cuttings, increased rooting and leaf development was achieved upon application of hormones, with the highest percentage of 63% rooting obtained in basal cuttings. Further work is needed on the other factors contributing to successful rooting of cuttings such as growing media, age of the stock plant and perhaps the use of a non-mist propagation system. The conclusions drawn from exogenous hormone observations are based only on the effect of one hormone. There is no knowledge of how other exogenous hormones such as IAA would affect the rooting percentage of *G. radlkoferi.*

Considering the results of the two types of propagation techniques used in the current study, the limited vegetative material per tree and the factors studied, seed propagation appears to be the suitable technique for large-scale multiplication of *G. radlkoferi*. The information provided in this study can be used as a practical contribution for establishment of a commercial nursery for cultivation of *G. radlkoferi.*

3.6 REFERENCES

Amri, E. et al., 2010. Vegetative propagation of African Blackwood (Dalbergia melanoxylon Guill. & Perr.): effects of age of donor plant, IBA treatment and cutting position on rooting ability of stem cuttings. *New Forests*, 39(2), pp.183–194. Available at: http://link.springer.com/10.1007/s11056-009-9163-6.

Amujoyegbe, B.J., Agbedahunsi, J.M. & Amujoyegbe, O.O., 2012. Cultivation of medicinal plants in developing nations : means of conservation and poverty alleviation. *International Journal of Medicinal and Aromatic Plants*, 2(2), pp.345–353.

Araya, H. T., 2005. Seed germination and vegetative propagation of bush tea (*Athrixia Phylicoides*) *by* Hintsa Tesfamicael Araya. *Submitted in partial fulfilment of the requirements for the degree MSc (Agric) Horticulture Department of Plant Production and Soil Science In t*. University of Pretoria.

Balkaya, L., 2004. Modelling the effect of temperature on the germination speed in some legume crops. *Journal of Agronomy*, 3(3), pp.179–183.

Chanyenga T F, Geldenhuys Coert J. & Weldesemayat Sileshi, G., 2012. Germination response and viability of an endangered tropical conifer Widdringtonia whytei seeds to temperature and light. *South African Journal of Botany*, 81, pp.25–28.

Coates Palgrave, K., Coates Palgrave, P. & Coates Palgrave, M.C.N.-D., 1985. *Everyone's Guide to Trees of South Africa* 3rd editio., Thorold's Africana Books.

Copeland, L.O. & Mcdonald, M.B., 1994. Seed Science and Technology. Third edition., Chapman and Hall, 29 West 35th Street, New York, USA.

Hartmann, H.. et al., 2011. *Plant Propagation. Principles and Practices.* Eighth edi., Sao Paulo, Prentic- Hall.

Hartmann, H.. et al., 1997. *Plant Propagation. Principles and Practices.* - sixth edi., Upper Saddle River, New Jersey, Prentice Hall.

Hartmann, H., Kester, D. & Davies, F., 1990. *Plant Propagation: Principles and Practices* 5th editio., Englewood Cliffs, NJ Prentice-Hall.

Kulkarni, M.G., Street, R. a. & Van Staden, J., 2007. Germination and seedling growth requirements for propagation of Dioscorea dregeana (Kunth) Dur. and Schinz - A tuberous medicinal plant. *South African Journal of Botany*, 73(1), pp.131–137.

Lall, N. et al., 2016. Insights into tyrosinase inhibition by compounds isolated from Greyia

radlkoferi Syzsyl using biological activity, molecular docking and gene expression analysis. *Bioorganic & Medicinal Chemistry*.

Leakey, R.R.B., Newton, A.C. and Dick, J.McP., 1994. Capture of genetic variation by vegetative propagation: processes determining success. In: R.R.B. Leakey and A.C. Newton (Editors), Tropical Trees: the Potential for Domestication and the Rebuilding of Forest Resources. HMSO, London, pp. 72-83.

Lebrun, A., Toussaint, A.. & Roggemans, J., 1998. Description of Syzygium paniculatum Gaertn. `Verlaine' and its propagation by stem cuttings. *Scientia Horticulturae*, 75(1–2), pp.103–111. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0304423898001228.

Ofori, D. a et al., 1996. Vegetative propagation of Militia excelsa by leafy stem cuttings : effects of auxin concentration , leaf area and rooting medium. *Forest Ecology and Management 84(1):39-48*, 84, pp.39–48.

Payne, R.W., 2014. Introduction to GenStat® for Windows^{TM} (17th Edition), VSN International, Hemel Hempstead, Hertfordshire, UK. Available at: Website: http://www.genstat.co.uk/.

Pooley E., 1993. *The Complete Field Guide to Trees of Natal, Zululand and Transkei* 1st Editio., Natal Flora Publications Trust.

Tchinda, N.D. et al., 2013. Improving propagation methods of Ricinodendron heudelotti Baill. from cuttings. *South African Journal of Botany*, 88, pp.3–9. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0254629913002573.

Tokumasu Satoru, Kanada, I. & Kato, M., 1985. Germination Behaviour of Seeds as Affected by Different Temperatures in Some Species of Brassica. *Engei Gakkai zasshi*, 54(3), pp.364–370.

Wilson, P.J., 1993. Propagation Characteristics of Eucalyptus-Globulus Labill Ssp Globulus Stem Cuttings in Relation to Their Original Position in the Parent Shoot. *Journal of Horticultural Science*, 68(5), pp.715–724. Available at: <Go to ISI>://A1993LX32100010.

CHAPTER 4

PRUNING AND HARVESTING EFFECTS ON LEAF YIELD OF CULTIVATED *GREYIA RADLKOFERI* TREES.

ABSTRACT

For product development, leaf production of *G. radlkoferi* must be available in quantities required and sustainable to the industry. Information on the agronomic management of *G. radlkoferi* trees especially those that would enhance leaf production is lacking. The purpose of this study was to determine the effect of pruning and harvesting frequencies on fresh and dry weights of *G. radlkoferi* leaves. Four pruning treatments ('pruned but not tipped', 'tipped but not pruned', 'not pruned nor tipped' as well as 'pruned and tipped') and three harvesting periods (monthly, bimonthly and once–off) were applied. Results revealed that harvesting bimonthly is the best harvesting practice which can be adopted for *G. radlkoferi* with fresh weight yields of 238 g per tree or 2.38 tons/hectare dry weight yield of 83 g per tree or 0.830 tons/hectare. Bimonthly harvests showed higher leaf fresh weights and therefore increased leaf production compared to trees that were harvested monthly and once-off. Furthermore, this study suggests that a suitable pruning practice for *G. radlkoferi* would be either to 'prune only' or 'cut back' the main stem rather than a combination of the two treatments.

4.1 INTRODUCTION

There is a worldwide increase in interest in the production of herbal products derived from wild indigenous plants. This increase is incited by the large plant biodiversity available for exploitation as well as consumer needs for safer and cheaper alternative products. As a result scientific research in South Africa has increasingly progressed to exploring the possibility of developing and commercializing products obtained from indigenous plants (Van Wyk 2008). However, the attention given to the use of plant resources obtained from indigenous plants requires an increase in medicinal plant research that investigates production techniques of such plants having promising potential as cultivated crops. *Greyia radlkoferi*, an indigenous plant in South Africa has been identified for commercialization for the production of cosmeceutical products that can treat hyperpigmentation. Leaves are the outmost important part of this plant as its ethanolic extracts and its isolated compounds highly inhibited the enzyme tyrosinase and further reduced melanin in melanocyte studies.

Young *G. radlkoferi* trees have a tendency to grow straight and tall with leaves clustered towards the stem tips (Wentzel n.d.), possessing little to no branching (Figure 4.1). This type of development results in limited vegetative growth and thus limits availability of leaf material per plant. Sustainable production of active ingredients for commercialization requires a more reliable supply and volume of plant material. The herbal market requires standardized plant material not just qualitatively, but also in quantitative terms, meaning increased biomass production (Maggini *et al*. 2014). Success in maintaining abundance and adequate leaf material supply will require continuous focus on developing best production practices that will ensure sustainable levels of production. Therefore, the shoot development structure of *G. radlkoferi* requires that it should be in some way trimmed to promote branching, to increase yields, and to facilitate harvesting. Failure to cut the main trunk might result in trees with no branches and leaves will mainly be growing on the primary stems. This could be achieved through silvicultural practices such as pruning.

Pruning involves the selective removal of a part of a plant; either the shoot (branches, bud) or the root system (Preece & Read 2005). Plants are pruned for a number of reasons depending on the purpose for which they are grown or used. Pruning can be done to improve plant health, to

correct structural defects, to achieve a desired shape, to save space, to improve appearance and function (Preece & Read 2005). According to these authors plants are often pruned to stimulate branching through disruption of apical dominance and thus resulting in a bushy plant. This can be achieved through application of shoot pruning which essentially is the artificial removing of leaf bearing branches of the plant. The operation is aimed at keeping the size and vegetative vigor of the plant in a condition most conducive for maximum vegetative growth and cropping (Tea Research Institute of Sri Lanka 1986).

In order to achieve the desired outcome in terms of growth and appearance, various basic types of pruning are used i.e. heading cuts and thinning cuts. In heading cuts the tip of the main branch is cut with the intension to encourage the growth of side stems and thus making denser, compact foliage trees. In thinning cuts, the branches are removed entirely, leaving no buds to grow. Their energy is diverted into remaining branches, which grow more vigorously.

According to our knowledge, information on how cultivated *G. Radlkoferi* trees could be managed is lacking. Traditional agricultural practices that could influence leaf yield production such as pruning and harvesting frequency on *G. radlkoferi* have never been documented. Understanding the fundamental response of this tree to pruning as well as harvesting periods may help to increase leaf biomass production and improve supply. Therefore, the objectives of the current study were to determine the effects of harvesting frequency and pruning on leaf fresh and dry matter yield of cultivated *G. radlkoferi.*

Figure 4.1: Photograph of a small *G. radlkoferi* tree from Mothong experimental site in Mamelodi

4.2 MATERIALS AND METHODS

4.2.1 Study Site and Climate

The study was conducted in Mothong African Heritage Trust garden site, in Mamelodi, Pretoria (Figure 4.2). Mamelodi township is at an elevation of 1307m above sea level (Climate-data.org n.d.). It experiences mild, generally warm temperate climates with the average annual temperature of 17.5^{0}C. January is the hottest month of the year with average temperatures of 22.1^{0}C while June is the coldest month with average temperatures of 10.8^{0}C. The rainfall is significant in Mamelodi with precipitation even during the driest month. Precipitation is the lowest in July with an average of 6 mm and reaches its peak in January with an average of 134 mm. The rainfall averages 694mm per year.

Figure 4.2: Aerial view of Mothong African Heritage Trust garden site

4.2.2 Source of plant material

Greyia radlkoferi trees of around 1 m in height were purchased from Random harvest; a commercial supplier (nursery) based in Honeydew, Johannesburg and were kept in the shade-net at the Agricultural Research Council-Vegetable and Ornamental Plants (ARC-VOP) in Roodeplaat, Pretoria.

4.2.3 Planting and maintenance of trees

The plantation was established during the planting season in September 2013. The trees were transported from ARC-VOP to Mothong African Heritage Trust gardens where they were planted in an experimental area of 800m^2 in pits of 50 cm deep with open diameters of 50 cm each. The pits were filled with composted bark and soil in 1:1 ratio. The trial was conducted with three replicates in a randomized complete block design with 12 treatments consisting of 4 trees each and a total of 48 trees in each block. Individual trees within a treatment were planted at 1 m apart and the treatments were spaced at 2 m apart from each other while the blocks or replicates were placed at 3 m spacing from each other. The trees were irrigated immediately after planting and further irrigation was done every two weeks. Spot weeding by hand-picking was only done directly around the trees and no pesticide or herbicides were applied. The trees were left for establishment until October 2013 before pruning was done. The main stem of the trees was cut back by 30 cm from the top and they were referred to as tipped treatments. The plantation consisted of 4 treatments consisting of trees that were ‘pruned but not tipped’, ‘tipped but not pruned’, ‘not pruned nor tipped’ and those that were ‘pruned and tipped’. Three harvesting periods which were selected for yield comparison were monthly, bimonthly and once-off.

Monthly and bimonthly harvest was done on the 15th day of the month from January to May 2014 while once-off harvest was only done at the end of the harvesting period, that is, May 2014.

4.2.4 Determination of leaf yield and statistical analysis

Harvesting of leaf material began 3 months following the cutting back of the main stem. Harvesting was done using pruning shears and every second leaf was picked from each tree within the experimental plots at each harvesting period. This approach to harvesting was undertaken so that trees were not completely stripped of all their leaves. Harvested leaves were grouped according to treatments and immediately taken to the laboratory where they were weighed to determine fresh leaf yield using a weighing balance. After recording fresh leaf weight values, the leaves were allowed to dry for 2 weeks in a well-ventilated room away from the sun after which leaf dry weight values were determined using a weighing balance. Yield was expressed by the leaf biomass produced per plant.

The data obtained was subjected to analysis of variance. Tukey's LSD multi comparison test at 5 % level was used to test the significance between the treatment means using GenStat® (Payne, 2014) statistical package.

4.3 RESULTS

4.3.1 Pruning effects

Pruning experiments ('prune or no prune' and 'tip or no tip') did not seem to have influenced fresh and dry weight yields of *G. radlkoferi* as there was no particular trend observed in the treatments (Figure 4.3). However, when data analysis excluded harvest frequencies, a clear difference in leaf yield was observed among treatments (Figure 4.4).

Plants that were 'tipped but not pruned' recorded higher fresh leaf weights of 180g per plant followed by those that were 'not tipped but pruned' with 173g per plant. The lowest fresh leaf weights of 151g per plant were observed in plants that were 'tipped and pruned'. On the other hand, higher dry leaf weight yields of 68g per plant were recorded in plants that were neither tipped nor pruned. Similar to fresh weight yields, the second highest dry leaf yield was observed in plants that were 'not tipped but pruned' and the lowest leaf dry weights in plants that were 'tipped and pruned'. The 'no tip but pruned' plants recorded 62g per plant dry leaf yield while 'tipped and pruned' treatment recorded 54g per plant dry leaf weight. However, the differences observed among pruning treatments were not significant.

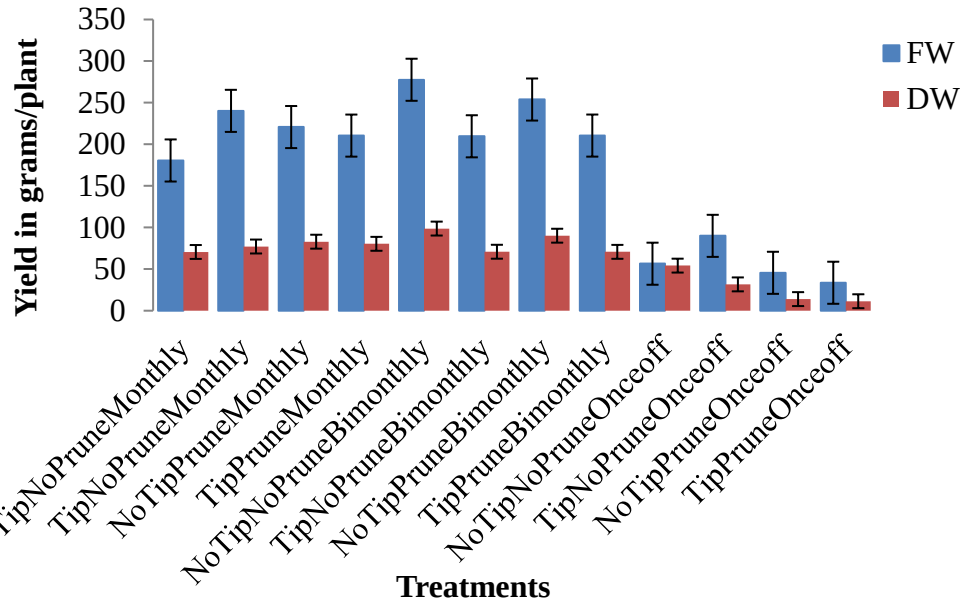

Figure 4.3: Pruning and harvesting treatment effects on the yield of *G. radlkoferi* leaves. FW= fresh weight and DR = dry weight.

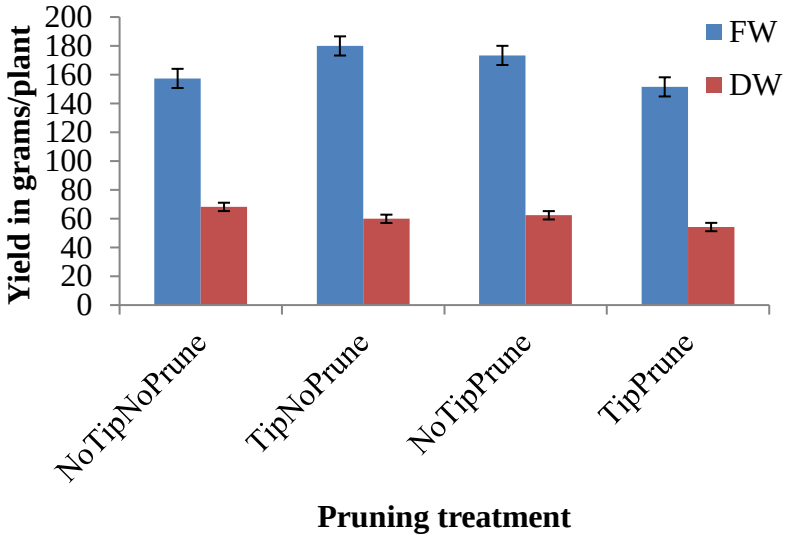

Figure 4.4: Effect of four pruning treatments on total average fresh and dry weight leaf yield of *G. radlkoferi* leaves. FW= fresh weight and DR = dry weight.

4.3.2 Harvesting frequency effects

Figure 4.5 shows the variation in average leaf fresh and dry weight yield of *G. radlkoferi* according to frequencies of harvests, and this graph clearly shows that harvesting frequency affected the fresh and dry leaf yield of *G. radlkoferi*. Maximum leaf yields in terms of fresh and dry weights were acquired in plants whose leaves were harvested at bimonthly intervals (Figure 4.3) recording fresh and dry yields of 238g and 83g per plant respectively compared to monthly and once-off harvests. At monthly harvests, fresh yield of 213g per plant and dry yields of 78g per plant were recorded while the lowest fresh yields of 50g per plant and dry yields of 28g per plant were recorded in the once-off harvest.

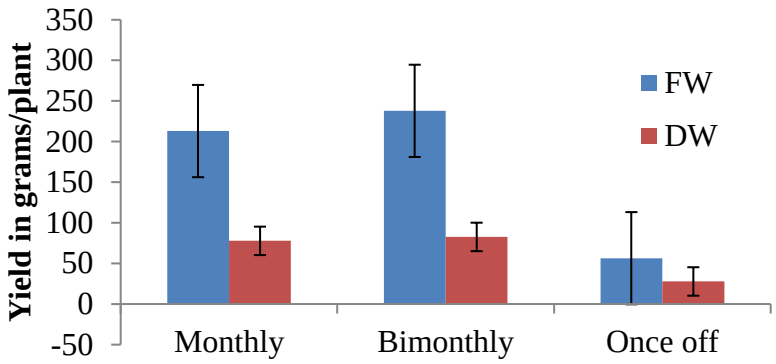

Figure 4.5: Effect of harvesting frequency (monthly, bimonthly and once-off) on the leaf yield of *G. radlkoferi* leaves. FW = Fresh weight and DW = Dry weight.

4.4 DISCUSSION

Harvesting frequency affected the fresh and dry weight yield of *G. radlkoferi* leaves. Low fresh and dry weight yield recorded for the monthly harvests suggests that this treatment was too frequent to allow vigorous growth of *G. radlkoferi* leaves. Harvesting once in the growing season resulted in a poor total leaf yield per plant. The yield obtained would not be sufficient for continuous supply to the market and for sustainable development of products throughout the year. Increasing the harvesting interval from once every month to once in two months improved the leaf yield (fresh and dry weights). Bimonthly harvests proved to be the best management

technique for leaf harvesting of *G. radlkoferi* trees, because it gave the highest average fresh and dry matter yields of 238g and 83g per tree respectively. This indicates that the replenishment of biomass is high when leaves are harvested every two months. In agreement with these results, was the results obtained in harvesting frequency studies undertaken on *Centella asiatica* (Rahajanirina *et al*. 2016). *Centella asiatica* harvested monthly and on annual basis yielded poor biomass yields while high biomass yields were obtained when harvesting frequency was spaced to two, three or four months apart.

The results of the current study showed that leaf yields (fresh and dry weights) were affected by pruning treatments. *Greyia radlkoferi* seemed to prefer one form of pruning practice i.e. either pruning or tipping rather than the combination of the two treatments as higher fresh yields were obtained in plots whose plants were 'not pruned but tipped' and those whose trees were 'pruned but not tipped'. Therefore, 'tipping' and 'pruning' treatments were regarded as pruning practices best suitable for management of *G. radlkoferi* trees for increased leaf production.

4.5 CONCLUSIONS

The results of the present study show that quantity of leaf yield of *G. radlkoferi* depends on harvesting frequency. Harvesting of leaves at bimonthly intervals showed a favourable increase in leaf yield with fresh weight yields of 238 g per tree or 2.38 tons/per hectare as well as dry weight yield of 83 g per tree or 0.830 tons/hectare. This is based on a spacing of 1x1 m (10 000 trees/hectare) and different spacings might be investigated in future to determine the effect on the yield. Amongst the harvesting periods studied, bimonthly harvests can be regarded as the best management technique for leaf harvesting of *G. radlkoferi* trees.

Considering the pruning studies, a suitable pruning practice or approach for increased leaf production of *G. radlkoferi* in this particular study would be either to 'tip' (cut back the main stem by 30cm) only or to 'prune' (cut the main trunk) only.

4.6 REFERENCES

Climate-data.org. (n.d.). Climate_ Mamelodi - Climate graph, Temperature graph, Climate table - Climate-Data. Retrieved March 2, 2016, from http://en.climate-data.org/location/27164/

Payne, R.W. (Ed.) Introduction to GenStat® for Windows™ (17th Edition), VSN International, Hemel Hempstead,Hertfordshire, UK. © 2014 VSN International. Website: http://www.genstat.co.uk/

Preece, J. E., & Read, P. E. (2005). *The Biology of Horticulture- An Introductory Textbook* (2nd Editio). Wiley.

Rahajanirina, V., Faramalala, M. H., Roger, E., Michel, J., Pock, L., Danthu, P., & Zebrowski, C. (2016). Effects of harvest frequency on leaf biomass and triterpenoid content of Centella asiatica (L .) Urb from Madagascar, *2*(1), 1–8.

Tea Research Institute of Sri Lanka. (1986). *Handbook on Tea*. (A. K. P. Sivapalan, S. Kulasegaram, Ed.). Tea Research Institute of Sri Lanka.

Van Wyk, B. E. (2008). A broad review of commercially important southern African medicinal plants. *Journal of Ethnopharmacology*, *119*(3), 342–355. http://doi.org/10.1016/j.jep.2008.05.029

Wentzel, J. (n.d.). Photos of South Afircan Plants - Image_ Greyia radlkoferi.

CHAPTER 5

METABOLOMIC PROFILE AND ANTI-TYROSINASE ACTIVITY OF *GREYIA RADLKOFERI* LEAF EXTRACTS IN RESPONSE TO SEASONAL CHANGES

ABSTRACT

Considering the high anti-tyrosinase activity previously reported for *G. radlkoferi* leaf extracts, this study was conducted to investigate the influence of seasons on the tyrosinase activity of this plant. Tyrosinase enzyme inhibition was investigated against monophenolase (tyrosine) with kojic acid as positive control. Furthermore, the metabolic profiles of *G. radlkoferi* in different seasons were also investigated using NMR spectroscopy and multivariate statistical analysis with an aim to evaluate and monitor their changes as influenced by seasons. LC-MS (LC-QqQ-MS), MAGMa and CSIFingerID were further used to confirm the identity of the annotated metabolites. In comparisons to other seasons, bioactivity studies showed a high tyrosinase inhibitory activity in leaves harvested in winter with IC_{50}=30.3 µg/ml. In metabolomics studies, the orthogonal partial least square discriminant analysis (OPLS-DA) of the ^{1}H-NMR spectra, provided a clear class separation according to the harvest season. This study found the metabolic profiles of spring and winter extracts to be clearly different from each other with the winter metabolome positively associated to sucrose, alanine, acetamide and gallocatechin-(4 alpha->8)-epigallocatechin. Catechins, a sub-group of flavonoids commonly found in green tea are known for their ability to inhibit the enzyme tyrosinase. Thus the increased anti-tyrosinase activity in winter harvested leaves could be attributed to the availability of this catechin compound. Metabolomics approach using NMR and LC-MS (LC-QqQ-MS) were found to be useful in characterizing *G. radlkoferi* leaf extracts according to harvesting seasons and in revealing the secondary metabolites. Therefore, this study concludes that in order to obtain high bioactivity products, the best suitable time for harvesting leaf samples is in late autumn-early winter.

5.1 INTRODUCTION

In recent years, focus on traditional plant research either for medicinal, cosmeceutical and nutraceutical use has increased and several studies have provided evidence to show the immense potential use of these plants in traditional systems. Natural products of interest are usually extracts and/or essential oils and their active biological compounds.

Several factors and one of them being seasonal changes (Ncube *et al*. 2012; Dhami & Mishra 2015) influence the production of phytochemical compounds in plants. Some metabolites may accumulate at a particular period as a response to seasonal changes. These fluctuations in the production of phytochemical compounds cause notable changes in the chemical profile of any plant material thus compromising the quality of the bioactive compound. Biological activity, which is dependent on the chemical composition, is similarly subjected to this variation. Evidence of such changes in chemical composition and in biological activity of plant extracts are presented in several studies undertaken on this subject matter (Southwell & Bourke 2001; De Cerqueira *et al*. 2007; Van Vuuren *et al*. 2007; Marzouki *et al*. 2009; Hussain *et al*. 2010; Paolini *et al*. 2010; Bapela *et al*. 2008; Br & Sheth 2011; Demuner *et al*. 2011;Ncube *et al*. 2011; Lubbe *et al.* 2013; Scognamiglio *et al*. 2014; Tomar *et al*. 2015; Lemos *et al*. 2015; Scognamiglio *et al*. 2015; Soni *et al*. 2015; Kim *et al.* 2015). The variation in chemical composition and bioactivity in response to seasons is attributed to the climate changes such as temperature, soil humidity, rainfall as well as different stages of plant metabolism (Lemos *et al*. 2015).

The chemical composition, that is, the concentration and availability; and bioactivity are some of the important factors deciding the application of a biological or commercial product (Dhami & Mishra 2015). Variation in these characteristics within plant material may cause routine complications for the validation of its medicinal efficacy. Absence or loss of activity in a medicinal plant product may result in the medicinal plant being perceived as having no medicinal value, whilst factors affecting variation in metabolite production have not been considered. The herbal market requires standardized plant material with not only high and constant concentrations of the bioactive compound, but less variation in biological activity (Maggini *et al*. 2014). In order to maintain high quality standards, monitoring of the variation on the composition, either in quality or quantity and the biological activity in response to factors

affecting secondary metabolite production, should be of significant importance during commercial production of herbal plant material.

Biotechnology techniques such as metabolomics coupled with multivariate data analysis provides a platform to evaluate challenges brought about by these environmental changes such as seasons on the secondary metabolite production. Plant metabolomics is able to achieve this through its ability to monitor and detect changes in plant metabolite levels in response to environmental changes. The success of the metabolomics technique in detecting changes in chemical composition of important medicinal plants in response to seasons, has been shown in a few studies (Gazim *et al*. 2010; Falasca *et al*. 2014; Scognamiglio *et al*. 2014; Kim *et al*. 2015; Scognamiglio *et al*. 2015).

According to our knowledge, there is no available data on the influence of seasons, on the chemical composition and the bioactivity of *G. radlkoferi* extracts. Therefore, the objectives of this chapter were to evaluate the effects of seasonal changes on the chemical composition as well as on the anti-tyrosinase activity of *G. radlkoferi* ethanolic leaf extracts.

5.2 MATERIALS AND METHODS

5.2.1 South African seasons

South Africa has a weather pattern with four distinct seasons. Winter is the coldest and driest season and covers the period May to July. Most vegetation shed leaves during this dry winter period. Spring follows winter and it is the warm, windy and dry season, which is characterized by flowering in most of the plant species. Spring occurs from mid-August to late-October. Summer succeeds spring and is the longest of the seasons stretching from November to February. Summer is the rainy season (except for the Cape floristic region with winter rainfall) and is characterized by high temperatures. All the vegetation is green in summer. Autumn marks the transition from summer into winter and usually falls between March and April. Plant leaves begin senescing during this season.

5.2.2 Metabolomics analysis

The untargeted metabolomics analysis methods described below are adapted from Maree and Viljoen (2012) and Mediani *et al*. (2012).

5.2.2.1 Plant material collection

For the purpose of this study, *G. radlkoferi* leaf material was collected in September 2014 to June 2015 from two sites: (i) cultivation site based at Mothong, in Mamelodi township of Pretoria, Gauteng and at UNISA Science campus gardens in Florida, a suburb situated in Johannesburg, Gauteng. Plant material was collected over the four seasons of the year in spring (15 September 2014), summer (15 December 2014), autumn (15 March 2015) and in winter (15 June 2015). Leaves were thoroughly washed with water to remove dirt particles before they were dried in a well-ventilated room, protected from direct sunlight for two weeks at room temperature. The dried leaf material was then ground to a fine powder using a hand grinder.

5.2.2.2 Extraction of metabolites

Extracts were prepared using a direct extraction method for metabolomics. Powdered leaf material of 50 mg per sample was weighed in 2ml Eppendorf tubes for extraction and analysis. Added to the sample was 0.75 ml of deuterated methanol (CH_3OH-d4) and 0.75 ml of potassium dihydrogen phosphate (KH_2PO_4), buffered in deuterium water (D_2O) (pH 6.0) containing 0.1% (w/w) TSP (Trimethylsilylpropionic acid sodium salt). In order to mix the reagents, they were vortexed at room temperature for 1 min. The mixture was then ultrasonicated for 20 minutes to break down the cell wall after which it was centrifuged for 20 minutes to separate the supernatant from the pellet. The supernatant from each tube was then transferred to a 5mm NMR tube for analysis.

5.2.2.3 Data acquisition by NMR analysis

NMR spectral data was obtained using 600 MHz NMR spectrometer (Varian Inc, California, USA), with 32 scans recorded.

5.2.2.4 Data mining and pre-processing

MestReNova software (9.0.1, Mestrelab Research Spain) was used for the pre-processing of the spectral data. Manual phase adjustment and baseline correction were conducted in all sample spectra as well as calibration with an internal standard TSP to 0.0 ppm. The chemical shift of δ4.70-4.90 and δ 3.23-3.36, representing water and methanol respectively (Mediani *et al.* 2012), were excluded from further analysis. MestReNova was further used for bucketing of NMR spectra. The spectral intensities were therefore reduced to integrated regions, also referred to as buckets or bins, of equal width (0.04 ppm each) corresponding to the region of 0.04-10.00ppm. The ASCII generated files were then imported to Microsoft Excel 2010 after which they were imported to Simca-software (13.0, Umetrics, Umeå, Sweden) for secondary variable labelling.

5.2.2.5 Multivariate data analysis

Simca-software was used for Multivariate data analysis (MDA) by means of Principal component analysis (PCA) and orthogonal partial least square discriminatory analysis (OPLS-DA) using Parreto scaling method. 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 factors 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 (Maree and Viljoen, 2012), in order to see the differences or similarities between the samples by the factors measured.

5.2.3 Anti-tyrosinase Assay

The anti-tyrosinase assay methods was adapted from Yang *et al*. (2012).

5.2.3.1 Reagents preparation

Sigma Aldrich (South Africa) supplied reagents used for this part of the study. Preparations of the buffer, substrate, extracts and enzyme are detailed in Appendix 8.1. Briefly, plant extracts and the positive control (kojic acid) were dissolved in dimethyl sulphoxide (DMSO) to a final

concentration of 20 mg/ml. Potassium phosphate buffer (KH_2PO_4) (pH 6.5) was then used to further dilute the plant extracts in a 24-well plate to eight concentrations, ranging from 600 to 37.5 µg/ml (Figure 5.1). L-tyrosine (2 mM) was used as a substrate.

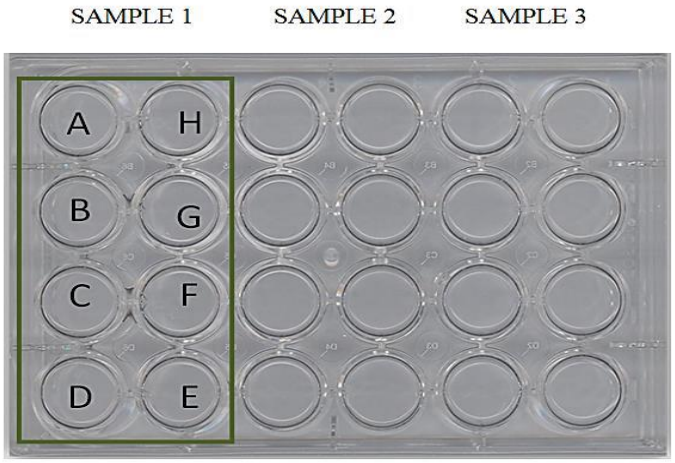

Figure 5.1: Representation of plant extract dilutions from the highest concentration (A= 600 µg/ml) to the lowest concentration (H= 37.5 µg/ml) using a phosphate buffer (pH 6.5).

5.2.3.2 Extraction for anti-tyrosinase assay

Ten grams of air-dried leaf material harvested at different seasons were each extracted with 100 ml of 70% absolute ethanol. The mixtures were left for 48 h on a shaker at room temperature and then filtered through a Whatman filter paper (15cm). The solvent was then evaporated under the ventilated fume cupboard for a week to yield dry extracts.

5.2.3.3 Colorimetric tyrosinase inhibition assay

The tyrosinase inhibition assay was conducted as described by Mapunya *et al.* (2011). For each sample (2 mg each), 70 µl of the dilutions (Figure 5.1) were added to the wells of a 96-well plate. All samples (plant extracts and kojic acid-positive control) were added in triplicate, and 30 µl of the prepared enzyme (333 units/ml) was then added to each well of a 96-well plate as illustrated in Figure 5.2. The plate was incubated for 5 minutes at room temperature. After the incubation, 110 µl of the substrate, L-tyrosine was added to the wells and the plate was further incubated at room temperature for 30 minutes, which resulted in a colour change.

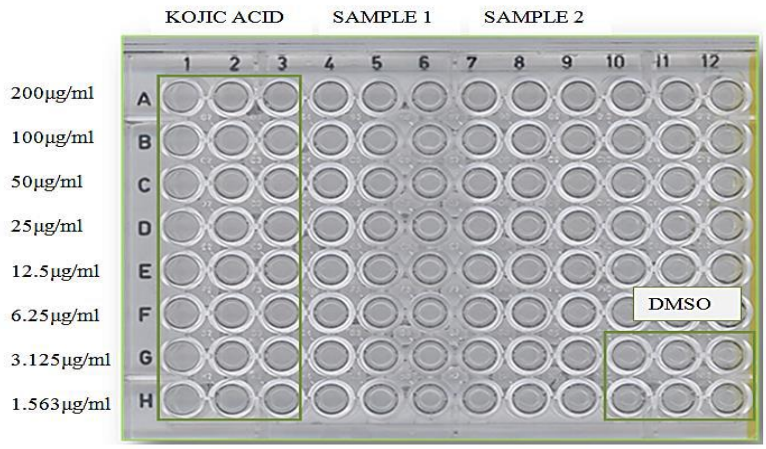

Figure 5.2: A 96-well plate representation of the tyrosinase inhibition assay. Positive control (Kojic acid), DMSO (negative control) and plant extracts were tested in triplicate. Kojic acid and plant extracts were tested from 200 µg/ml to 1.563 µg/ml and DMSO was tested only at 200 µg/ml.

5.2.3.4 Statistical analysis

The BIOTEK power XS multi-well plate reader (A.D.P, Weltevreden Park, South Africa) was used to read optical densities in each well during the 30 minutes of incubation. Using optical densities, IC_{50} values were calculated for each sample using GraphPad prism software. This was done by comparing the inhibitory effects of samples relative to the effect of the DMSO control. The IC_{50} values represent the concentrations at which a sample inhibited 50% of the tyrosinase enzyme. The coefficients of determination (R^2) were also determined by GraphPad prism for statistical significance.

5.2.4 Liquid chromatography-mass spectroscopy (LC-MS)

Triple quad based LC-MS (LC-QqQ-MS) was used in order to confirm the compounds annotated from NMR-metabolomics and chemometric studies.

5.2.4.1 Sample preparation

The leaf material was ground to a fine powder and 5 mg dry weight material was extracted with 1.5 ml (75% MeOH:25% water). Samples were sonicated in an ultrasonic bath for 5 min and

centrifuged for 15 min with a table centrifuge set at 15 000 rpm. To remove cell debris, the supernatant was filtered through 0.2 micron syringe filters (Sartorius Minisart RC 4) with 1 ml plastic discardible pipette. Extractions were performed in triplicate for statistical processing.

5.2.4.2 LC-MS data acquisition and sample analysis

HPLC was carried out using a Waters Alliance 2795 HT system with a column oven. For chromatographic separation a Discovery HS F5 Supelguard column (2.0 x4 mm, 3 µm particle and an analytical column Discovery HS F5 (2.1 x150 mm, particle size 3µm) were used (Sigma-Aldrich). Five microliters of each sample was injected into the system for LC-PDA-MS analysis. Degassed solutions of formic acid:ultrapure water ($1:10^3$, v/v; eluent A) and formic acid:acetonitrile ($1:10^3$, v/v; eluent B) were pumped at 0.19 mL min^{-1}into the HPLC system. The gradient applied started at 5% B and increased linearly to 35% B in 45 min. Then, for 15 min the column was washed and equilibrated before the next injection. The column temperature was kept at 40°C and the samples at 20°C. The room temperature was maintained at 20°C.

For MS detection, ESI conditions were set as follow: capillary voltage: 2.5 kV, sample cone voltage: 17 V, MCP detector voltage: 1600 V, source temperature: 120 C, desolvation temperature: 350 C, cone gas flow: 50 L/h, desolvation gas flow: 450 L/h, m/z range: 100–1000, scan time: 0.1 s, interscan delay: 0.02 s, mode: centroid, lockmass: leucine enkephalin (556.3 lg/mL), lockmass flow rate: 0.4 mL/min, mass accuracy window: 0.5 Da. The molecular formula assignments software that was used is MassLynx XS™ (Waters Corporation, Milford, MA, USA). Mass accuracy of all the m/z values in all the acquired spectra were automatically corrected during acquisition based on calibration curves, lockmass and dynamic range extended. The MS detector was set to collect both negative (ESI-) and positive (ESI+) ions.

5.2.4.3 Annotation and identification of compounds

5.2.4.3.1 NMR data

The metabolite signals of NMR spectra were assigned based on comparisons with the chemical shifts of compounds in NMR databases such as Chenomx NMR software suite (Version 8.3) and

the Human Metabolome Database (www.hmdb.ca/). Some of the compounds were annotated based on previously published literature.

5.2.4.3.2 LC-MS data

An automated chemical structure annotation and identification of *G. radlkoferi* leaf extracts was carried out using MAGMa and CSIFingerID respectively. MAGMa obtainable from http://www.emetabolomics.org/magma was used to propose the most likely structure of compounds that were in *G. radlkoferi* leaf extracts, based on the fragmentation pattern (see Appendix 8.3). MAGMa is a computational method used to support the data analysis by annotating all fragmented compounds in LC−MSn data sets with candidate molecules taken from large chemical databases such as PubChem or the Human Metabolite Database (Ridder *et al*. 2013). The method is based on an algorithm for candidate substructure annotation of multistage accurate mass spectral trees recently developed by Rider *et al*. (2012). Alternative candidates are ranked on the basis of the calculated matching score. The fragmentation trees obtained from MAGMa were then transferred to CSIFingerID (http://www.csi-fingerid.org/). CSIFingerID is a new approach used in untargeted LC-MS data to identify newly detected compounds in situations where there is lack of a comprehensive reference database (Davies, 2015). According to Davis (2015), CSIFingerID calculates fragmentation trees from known reference spectra, fragmentation tree similarities as well as PubChem (CACTVS) and Klekota–Roth fingerprints. Each molecular property is predicted by an individual "support vector machine" (SVM) which together make up the nerve centre for the approach. The SVMs yield probabilities for the presence or absence of a particular molecular property in any given compound based on the MS/MS spectral data. With the system trained, it becomes possible to move to the actual analysis of the new data. The system takes one or more MS/MS spectra, the unknown analyte to be identified and calculates the similarities shown in the unknown data set against the MS/MS spectra and fragmentation trees in the training set. The SVMs then predict the presence or absence of all molecular properties for the unknown compound providing a probability fingerprint. This fingerprint can then be used as a search criterion against the much bigger molecular structure databases such as PubChem. Each potential solution chemical structure has its calculated fingerprint scored against the unknown to provide a hit-list to the user. The highest

ranking score represents the most probable compound and was then compared to the results obtained in MAGMa to derive at compound identification.

5.3 RESULTS

5.3.1 Seasonal variation effect on anti-tyrosinase activity

As chemical composition of extracts potentially affects the biological activity, the study evaluates whether or not seasonal variation affects the anti-tyrosinase activity of *G. radlkoferi* extracts. Seasonal variation effected notable changes on the anti-tyrosinase activity of ethanol leaf extracts of *G. radlkoferi* (Figure 5.3). The IC_{50} (50% tyrosinase inhibition concentration) values were high in spring and summer compared to autumn and winter. The highest tyrosinase inhibitory activity was recorded in winter collected plants with an IC_{50} value of 30.3±1.8 µg/ml and the lowest in spring with an IC_{50} value of 114.9±34.5 µg/ml.

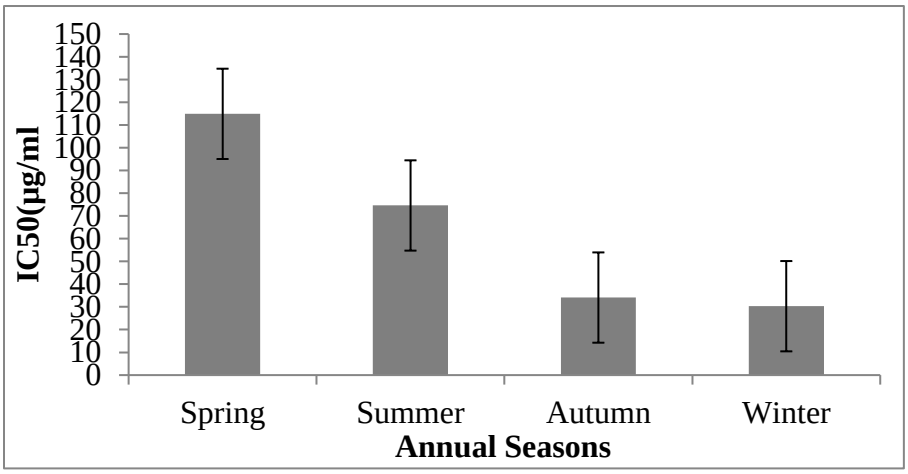

Figure 5.3: Tyrosinase inhibitory activity by ethanol extracts of *G. radlkoferi* leaves harvested at different seasons. The higher the IC_{50} value, the lower the activity and vice versa.

The colorimetric assay of the tests can be observed on the 96-well plates presented in Figure 5.4 Brownish-orange wells visible in the seasonal samples as well as in the kojic acid samples represent the formation of melanin while clear wells represent the inhibition of the enzyme by the extracts and the kojic acid.

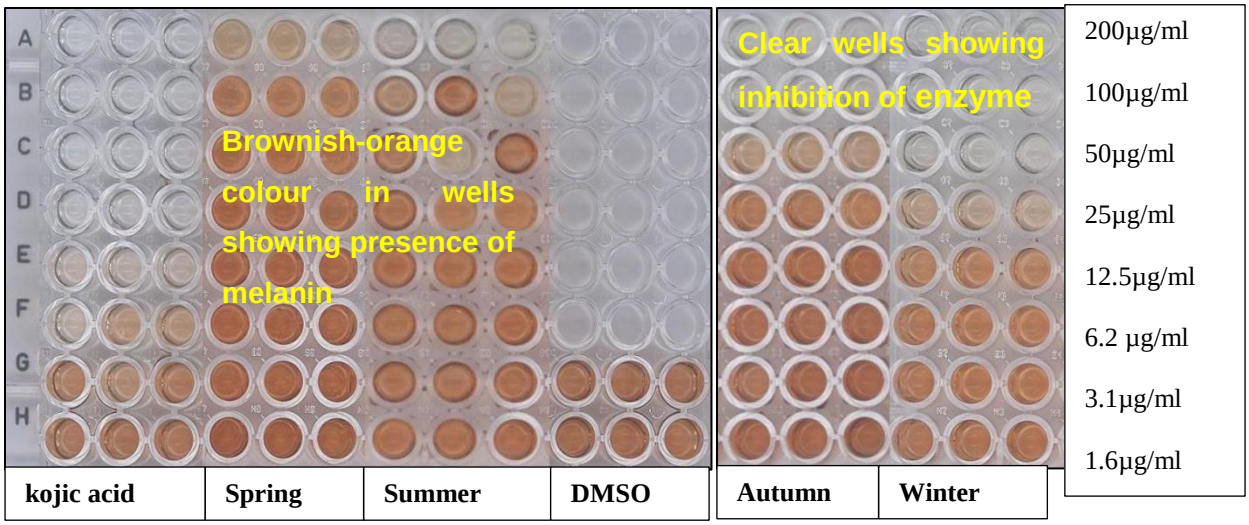

Figure 5.4: Colorimetric anti-tyrosinase plate for seasonal screening of ethanol leaf extracts of *G. radlkoferi* growing in UNISA campus gardens.

5.3.2 Seasonal variation effects on metabolomic profile of the extract

Thirty methanol extracts were prepared from *G. radlkoferi* leaves collected at different seasons (summer, autumn, winter and spring). Thereafter ^{1}H-NMR-based metabolomics approach was used to determine seasonal effects on metabolite profiles of this plant. Representative ^{1}H-NMR spectra showing profiles of spring and winter samples are shown in Appendix 8.2.1-8.2.2. Multivariate data analysis using PCA and OPLS-DA models was used to determine whether the metabolic fingerprint of the extracts were sufficiently different to distinguish between seasons. Each point in the PCA and/or OPLS-DA score scatter plot represents an individual sample. Samples with similar origins will cluster together in different areas in the ellipse. Data points representing Mothong and UNISA campus gardens collected leaves were assigned classes according to the four seasons of the year.

Upon exposure of the data to MVA models, significant outliers were identified based on the visual observations of the PCA score plots as well as the application of Distance to Model X (DMod[X]) and were excluded from the data sets. The final PCA did not show any variation between the data points (Figure 5.5A). There was no separation observed between spring, summer, autumn and winter collected samples. The PCA model showed a degree of clustering with a goodness of fit (R^2) of 0.97 and the predictability (Q^2) of 0.83. However, when the data

sets were exposed to OPLS-DA, the score plot showed different behaviors for extracts collected from different seasons in both PC1 and PC2 (Figure 5.5B). Samples collected in spring and summer were clustered together indicating that these samples present more similarities with each other than the winter and autumn. On the other hand, samples collected in winter and autumn were clearly separated from each other and from the other seasons with each forming its own cluster. In general, spring and summer collected samples showed a positive loading along PC1 while autumn collected samples showed a negative loading. Along PC2 spring, summer and autumn collected samples showed a negative loading while winter collected samples showed a positive loading. This observation indicate that samples collected in each of these two seasons varies significantly to each other and to the rest of the other seasons. The OPLS-DA model showed a degree of clustering, with the goodness-of-fit and predictability as indicated by R^2X and Q^2Y values of 0.962 and 0.48 respectively.

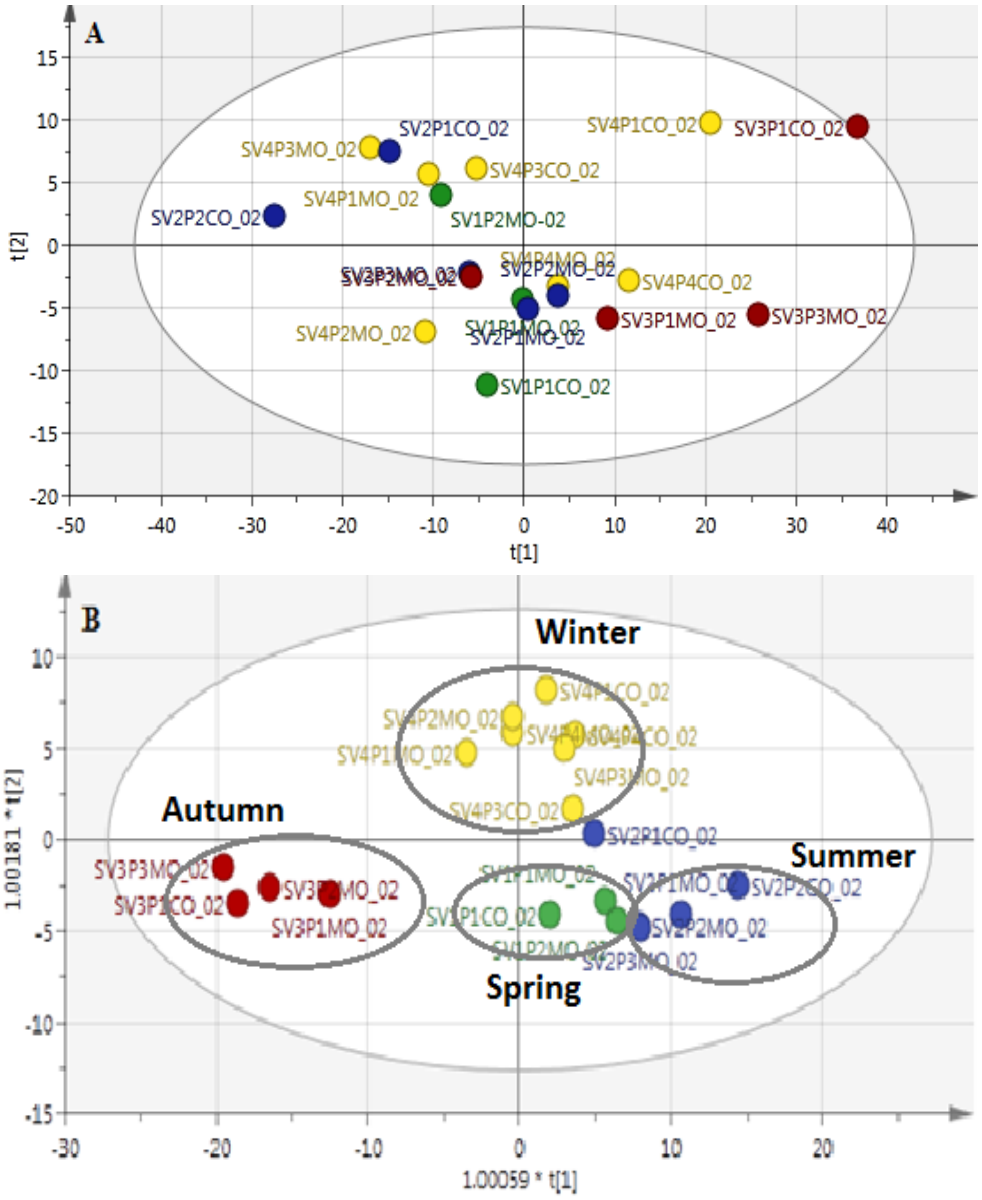

Figure 5.5: Score scatter plot of PCA (A) and OPLS-DA (B) performed on *G. radlokoferi* leaf extracts A) collected at difference seasons. SV1 representing spring (green), SV2 representing summer (blue) SV3 representing autumn (red) and SV4 representing winter (yellow).

A pairwise scatter plot of spring against winter collected samples was generated (Figure 5.6A). The selection of these two seasons for comparisons was informed by the tyrosinase activity results which showed the highest anti-tyrosinse activity in winter and the lowest in spring (refer to Figure 5.3). The OPLS-DA score plot showed a clear separation between the samples collected in different seasons with the winter collected samples clustered on the far right to the spring collected samples. The samples showed a degree of clustering, with the goodness-of-fit and predictability as indicated by R^2X and Q^2Y values of 0.975 and 0.842 respectively.

In order to reveal metabolites, which distinguished spring from winter, collected samples, a contribution plot was generated from the OPLS-DA analysis only including winter and spring samples (Figure 5.6B). The contribution plot showed ^{1}H-NMR spectral regions (in ppm) that contributed to the observed separation between winter and spring extracts. The buckets or bars seen in figure 5.6B represent the specific ^{1}H-NMR spectral regions of *G. radlkoferi* responsible for the difference between winter and spring seasons. The negative bars represent ^{1}H-NMR spectral regions positively associated with winter, but negatively associated with spring while the positive bars represent ^{1}H-NMR spectral regions positively associated with spring, but negatively associated with winter. When the chemical shifts of interest were correlated to classes of secondary metabolites found within the plant kingdom, the most prominent bars associated with winter were tentatively associated with the sugar region (3 – 5 ppm). Primary and secondary metabolites that were responsible for differentiating winter to the spring season were annotated from the contribution plot as acetamide, alanine, gallocatechin, epigallocatechin, and sucrose, and these are outlined in Table 5.1. However, analysis of the LC-MS data using MAGMa and CSIFingerID (http://www.csi-fingerid.org/) led to the identification of gallocatechin-(4 alpha->8)-epigallocatechin (mass =610.13226) as the catechin present in *G. radlkoferi* leaf extracts. The structure of gallocatechin-(4 alpha->8)-epigallocatechin is represented in Appendix 8.4. It was noted that the structure of gallocatechin-(4 alpha->8)-epigallocatechin is comprised of gallocatechin and epigallocatechin components. The presence of gallocatechin-(4 alpha->8)-epigallocatechin compound was highly associated with extracts of winter when compared to the extracts of samples collected in spring.

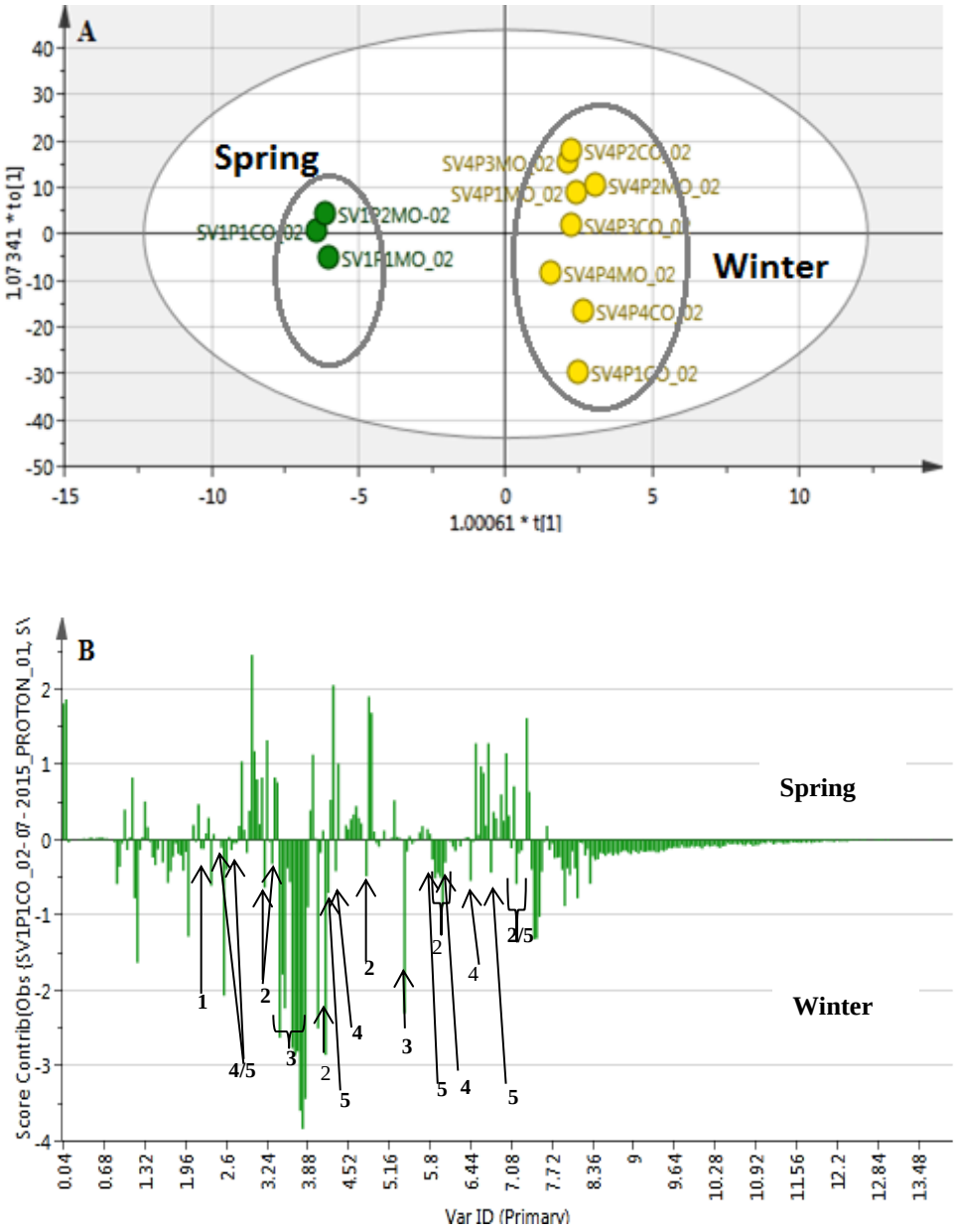

Figure 5.6: OPLS-DA scatter plot (A) and contribution plot (B) of spring and winter collected samples. 1=acetamide, 2= sucrose, 3= alanine, 4= gallocatechin, 5=epigallocatechin.

Table 5.1: ^{1}H-NMR spectral regions annotated compounds that contributed to the separation of winter and spring harvested leaves of *G. radlkoferi*.

Treatment	Compounds	^{1}H-NMR Chemical shifts (ppm)	Referenced chemical shifts (ppm)	References	Chenomx (ppm)	Assigned numbers
Winter	Acetamide	2.0	2.005	https://www.drugbank.ca/spectra/nmr_one_d/2530 (drugbank accessed 23.01.2017)		1
	Sucrose	3.4 3.5 3.7 3.86 3.78 4 4.16 5.42	3.43 3.53 3.76 3.84 3.80 4 4.19 5.4	(Le Gall *et al*. 2004)	3.4 3.5 3.6 3.7 3.8 3.9 4 4.2 5.4	2
	Alanine	1.5	1.5	(Le Gall *et al*. 2004)	1.5	3
	*Gallocatechin	2.58 2.87 3.92 - 5.84 6.00 6.48	2.51 2.87 3.97 4.51 5.87 6.01 6.45	(Davis *et al*. 1996; Yuan *et al.* 2014)		4

*Epigallocatechin	2.74 2.84 4.21 - 5.89 6.00 6.59	2.72 2.84 4.19 4.82 5.91 6.01 6.57	(Davis *et al*. 1996)	5

5.4 DISCUSSION

Tyrosinase activity of *G. radlkoferi* was significantly affected by seasonal changes with the highest activity in winter. These results suggest that harvesting *G. radlkoferi* leaves in winter will prove useful as the inhibition properties of tyrosine, an enzyme responsible for melanin reduction were the highest during this season.

In metabolomics studies, the PCA model failed to show separation for different seasons as the data sets were scattered all over the ellipse. The lack of clustering pattern in the PCA score plot could be due to variabilty brought about by the effects of other factors such as site of collection or growing conditions superceeding the effect of seasons. The effect of these factors can be controlled by increasing the biological replications which may however be time-consuming and costly. However, when the same data was analysed using the OPLS-DA model, owing to the lack of discrimination in the PCA analysis, distinct class groupings were formed according to seasons. These results indicate that the metabolic profiles of *G. radlkoferi* leaf extracts were different enough to distinguish between seasons suggesting that leaf metabolites went through changes from one season to the other. However, spring and summer samples were grouped together in the ellipse indicating that the metabolites of those seasons were more similar.

Gallocatechin and epigallocatechin were annotated as secondary metabolites that were strongly associated with extracts of winter harvested leaves. However, confirmation of the presence of these compounds in leaf extracts of *G. radlkoferi* using LC-MS, MAGMa and CSIfingerID led to the identification of gallocatechin-(4 alpha->8)-epigallocatechin. Structural similarities between gallocatechin, epigallocatechin and gallocatechin-(4 alpha->8)-epigallocatechin were noted. The structure of gallocatechin-(4 alpha->8)-epigallocatechin is comprised of gallocatechin and epigallocatechin units and hence it was not easily annotated from ^{1}H-NMR spectra. Gallocatechin and epigallocatechin belong to catechins, a subgroup of flavonoids commonly found in green tea (Davis *et al*. 1996; No *et al*. 1999; Parvez *et al.* 2007). Catechins have been widely reported as being effective tyrosinase inhibitors (No *et al*. 1999; Cheng *et al*. 2007; Sato & Toriyama 2009; Abdillahi *et al*. 2011) and therefore might be effective in treating hyperpigmentation disorders. However, literature relating to gallocatechin and anti-tyrosinase activity was not found. Epigallocatechin on the other hand has been reported in several studies

for its ability to inhibit tyrosinase activity. A United States invention titled 'Reducing Tyrosinase Activity', lists epigallocatechin as one of the effective tyrosinase inhibitor with 17.5% tyrosinase inhibition activity at 0.15 mg/ml (Hara & Honda 1986). Other catechins listed as tyrosinase inhibitors in the invention include epicatechin with 11.9%, epicatechin gallate with 95.1% and epigallocatechin gallate with 79.5%. A subsequent study by No and co-authors reported a 40% of tyrosinase enzyme inhibition by (-)-epigallocatechin obtained from green tea extracts when L-tyrosine was used as a substrate (No *et al.* 2009). Other catechins that had been investigated by the latter author for their anti-tyrosinase inhibition include (-)-epicatechin 3-0-gallate with 64%, (-)-gallocatechin 3-0-gallate with 73%, (-)-epigallocatechin 3-0-gallate with 59% and epicatechin and catechin with 2% tyrosinase inhibitory activities (No *et al*. 1999). Gallocatechin-(4 alpha->8)-epigallocatechin possess two sites for quionone formation (believed to be mechanism of tyrosinae inhibition) (Parvez *et al*. 2007). This information together with the facts that this compound is comprised of epigallocatechin, a well-known tyrosinase inhibitor, suggest that gallocatechin-(4 alpha->8)-epigallocatechin is probably able to inhibit tyrosinase activities similarly to known catechins with proven anti-tyrosinase abilities.

Phenolic compounds play an important role by replacing other compounds in cellular metabolism during the winter season (Chen *et al*. 2012). Phenolic compound contents are affected by environmental factors such as temperature, UV light, rainfall, and soil conditions (Lemos *et al*. 2015). The synthesis of flavonoids in particular is significantly affected by low temperatures (Janmohammadi 2012). Cold acclimation of plants leads to remarkable increase in phenylalanine ammonia-lyase (PAL) activity resulting in the accumulation of flavonoids depending upon the range of low temperature to which the plants are subjected (Stefanowska *et al*. 2002). However, the effect of these conditions on phenolic compounds varies with different species. The evidence is presented in an article reviewing various publications on effect of seasonal variation on secondary metabolites of various medicinal plants (Soni *et al*. 2015). In *Mentha longifolia* the increased accumulation of flavonoids was observed in winter while in *Pseudobombax marginatum* the increase was observed in summer and in *Mellitus melissophyllum* the increase was observed in spring seasons. Our results suggest that the accumulation of phenolic compounds in the leaves of *G. radlkoferi* harvested in winter might be an adaptation to low-temperature stress.

The current study showed that primary metabolites such as sugars and amino acids were affected by seasons. The levels of sucrose were increased in *G. radlkoferi* leaves harvested in June, which is the beginning of the winter season in South Africa. South African winter is associated with coldest temperatures and driest conditions in Gauteng. Low temperature subjects growing plants to cold stress and induces carbohydrate changes in many plant species (Chen *et al*. 2012). Sugar metabolism plays an important role in the survival of cold stress. Sucrose has been reported as one of the soluble sugars, which accumulate in plants as a reserve carbon source in preparation for cold conditions (Chen *et al*. 2012). Therefore, the sucrose observed in winter harvested leaves could be regarded as one of the measures taken by *G. radlkoferi* to cope with seasonal changes.

The activity of medicinal plants depends upon the chemical constituents present in the plant (Soni *et al*. 2015). However, different seasons of the year could directly or indirectly affect the availability of some precursors that the plant needs for the biosynthesis of the active ingredients. Kim and co-authors (2015) established a correlation between the changes in bioactivities and the changes in metabolites of *Lespedeza maximowiczii* according to seasonal variations using Pearson's correlation coefficients (r). These authors discovered 9 and 7 secondary metabolites that were positively correlated with tyrosinase inhibition activity in leaves and stems respectively. However, out of these 16 metabolites, only four which were common between these plant organs were present in the larger quantities in both winter leaves and stems of *Lespedeza maximowiczii.* The four compounds which were common between these plant organs were regarded as the compounds responsible for increased tryosinase inhibition activity. Based on this view we can then conclude that the improvement of anti-tyrosinase activity as well as determination of a possible anti-tyrosinase inhibitor, in winter harvested *G. radlkoferi* leaves suggests a possible correlation between the bioactivity and the preserved chemical compounds.

The robustness of a model generated is explained by means of Q^2 and R^2 in the case of the PCA model and Q^2Y and R^2Y in the case of an OPLS-DA model. $R^2(R^2Y)$ indicates the amount of data explained by the model and gives a general overview of the fitness of the model whereas $Q^2(Q^2Y)$ is a measure of the predictability of the model (Eriksson *et al*. 2006). The PCA model generated in the current study sufficiently explained the data (R^2=0.97) and the model was good

at predicting the data with Q^2=0.83. Generally, the Q^2/Q^2Y value greater than 0.5 is regarded as good and the Q^2/ Q^2Y value greater than 0.9 is regarded as excellent (Eriksson *et al*. 2006). The generated OPLS-DA model for all the four seasons explained the data well with (R^2Y)=0.96, however its OPLS-DA model was not a good model as it did not predict the data well (Q^2Y=0.48). Furthermore, there was a huge difference between R^2Y and Q^2Y values. According to Eriksson *et al*. (2006) the difference between R^2Y and Q^2Y larger than 0.2-0.3 indicate the presence of many irrelevant model terms or a few outlying data points. On the other hand, the OPLS-DA model generated for the pairwise score plot of spring samples against winter sample was indeed a good model. It explained and predicted data very well with R^2Y =0.959 and Q^2Y=0.901.

5.5 CONCLUSIONS

NMR-based metabolomics approach in combination LC-MS (LC-QqQ-MS) was able to confirm that the production of primary and secondary metabolites in the leaves of *G. radlkoferi* changes in response to seasonal variations. The tyrosinase inhibitory activity assay further substantiated the results of the spectroscopy studies by showing differences in the inhibitory activity across the seasons. Winter was determined as the season with highest tyrosinase enzyme inhibition activity with IC_{50}=30.3 µg/ml. Gallocatechin-(4 alpha->8)-epigallocatechin was identified as the secondary metabolite that was highly associated with winter and thus probably responsible for the increased antityrosinase activity in *G. radlkoferi* leaf extracts. Testing gallocatechin-(4 alpha->8)-epigallocatechin for anti-tyrosinase activity are future research activities which will probably confirm the marked increase in tyrosinase activity in winter and possible synergistic effects with previously identified compounds. Therefore this study concludes that the best harvesting time for optimum bioactivity of *G. radlkoferi* is in late autumn-early winter.

5.6 REFERENCES

Abdillahi, H.S., Finnie, J.F. & Van Staden, J., 2011. Anti-inflammatory, antioxidant, anti-tyrosinase and phenolic contents of four Podocarpus species used in traditional medicine in South Africa. *Journal of Ethnopharmacology*, 136(3), pp.496–503. Available at: http://dx.doi.org/10.1016/j.jep.2010.07.019.

Bapela, M.J., Lall, N. & Meyer, J.J.M., 2008. Seasonal variation of naphthoquinones in Euclea natalensis subspecies natalensis. *South African Journal of Botany*, 74(2), pp.218–224. Available at: http://www.sciencedirect.com/science/article/pii/S0254629907004115 [Accessed July 7, 2015].

Br, A.R. & Sheth, F., 2011. Range of Seasonal Phytochemical Variations in Calotropis procera. *Int. J. Med. Arom. Plants*, 1(2), pp.180–183.

De Cerqueira, M.D. et al., 2007. Seasonal variation and antimicrobial activity of Myrcia myrtifolia essential oils. *Journal of the Brazilian Chemical Society*, 18(5), pp.998–1003.

Chen, T. et al., 2012. Seasonal changes in non-structural carbohydrates and sucrose metabolism enzymes in two Sabina species. *Acta Physiologiae Plantarum*, 34(1), pp.173–180.

Cheng, K.-T. et al., 2007. New constituent from Podocarpus macrophyllus var. macrophyllus shows anti-tyrosinase effect and regulates tyrosinase-related proteins and mRNA in human epidermal melanocytes. *Chemical & pharmaceutical bulletin*, 55(5), pp.757–761.

Davies AN (2015) A new approach to identifying unknown trace level analytes by tandem mass spectrometry without reference spectroscopic database support: CSI:FingerID. SpectroscopyEurope 27 (5), pp. 13-15

Davis, A.L. et al., 1996. 1H and13C NMR Assignments of Some Green Tea Polyphenols. *Magnetic Resonance in Chemistry*, 34(11), pp.887–890. Available at: http://doi.wiley.com/10.1002/(SICI)1097-458X(199611)34:11<887::AID-OMR995>3.0.CO;2-U.

Demuner, A.J. et al., 2011. Seasonal variation in the chemical composition and antimicrobial activity of volatile oils of three species of leptospermum (Myrtaceae) grown in Brazil. *Molecules*, 16(2), pp.1181–1191.

Dhami, N. & Mishra, A.D., 2015. Phytochemical variation: How to resolve the quality controversies of herbal medicinal products? *Journal of Herbal Medicine*, 5(2), pp.118–127.

Available at: http://linkinghub.elsevier.com/retrieve/pii/S2210803315000299.

Eriksson, L. et al., 2006. Multi- and megavariate data analysis: Advanced applications and method extensions, 1st edn, Umetrics AB, Umea.

Falasca, A. et al., 2014. Seasonal changes in the metabolic fingerprint of Juniperus communis L. berry extracts by 1H NMR-based metabolomics. *Metabolomics*, 10(1), pp.165–174.

Gazim, Z.C. et al., 2010. Seasonal variation, chemical composition, and analgesic and antimicrobial activities of the essential oil from leaves of Tetradenia riparia (Hochst.) Cdd in suthern Brazil. *Molecules*, 15(8), pp.5509–5524.

Hara, Y. & Honda, M., 1986. United States Patent. , pp.54–55.

Hussain, A.I. et al., 2010. Seasonal variation in content, chemical composition and antimicrobial and cytotoxic activities of essential oils from four mentha species. *Journal of the Science of Food and Agriculture*, 90(11), pp.1827–1836.

Janmohammadi, M., 2012. Metabolomic analysis of low temperature responses in plants. Current Opinion in Agriculture 2012 1(1), pp.1–6.

Kim, N.K. et al., 2015. Seasonal variations of metabolome and tyrosinase inhibitory activity of *Lespedeza maximowiczii* during growth periods. *Journal of Agricultural and Food Chemistry*, 63(38), pp.8631–8639. Available at: http://pubs.acs.org/doi/10.1021/acs.jafc.5b03566.

Le Gall G., Colquhoun IJ., Defernez M. 2004. Metabolite profiling using ^{1}H NMR spectroscopy for quality assessment of green tea, *Camellia Sinensis* (L.). J. Agric. Food Chem., 52 (4), pp. 692-700.

Lemos, M.F. et al., 2015. Seasonality modifies rosemary's composition and biological activity. *Industrial Crops and Products*, 70, pp.41–47. Available at: http://dx.doi.org/10.1016/j.indcrop.2015.02.062.

Lubbe, A. et al., 2013. Seasonal accumulation of major alkaloids in organs of pharmaceutical crop Narcissus Carlton. *Phytochemistry*, 88, pp.43–53. Available at: http://www.sciencedirect.com/science/article/pii/S0031942212005304 [Accessed July 7, 2015].

Mapunya, M. B.; Hussein, A. A.; Rodriguez, B.; Lall, N. 2011.Tyrosinase activity of *Greyia flanaganii* (Bolus) constituents. *Phytomedicine* 18, 1006-1012.

Maree, J.E. & Viljoen, A.M., 2012. Phytochemical distinction between Pelargonium sidoides

and Pelargonium reniforme - A quality control perspective. *South African Journal of Botany*, 82, pp.83–91. Available at: http://dx.doi.org/10.1016/j.sajb.2012.07.007.

Marzouki, H. et al., 2009. Seasonal and Geographical Variation of Laurus nobilis L . Essential Oil from Tunisia. *The Open Natural Products Journal*, pp.86–91.

Mediani, A. et al., 2012. 1H-NMR-based metabolomics approach to understanding the drying effects on the phytochemicals in Cosmos caudatus. *Food Research International*, 49(2), pp.763–770.

Ncube, B., Finnie, J.F. & Van Staden, J., 2012. Quality from the field: The impact of environmental factors as quality determinants in medicinal plants. *South African Journal of Botany*, 82, pp.11–20. Available at: http://dx.doi.org/10.1016/j.sajb.2012.05.009.

Ncube, B., Finnie, J.F. & Van Staden, J., 2011. Seasonal variation in antimicrobial and phytochemical properties of frequently used medicinal bulbous plants from South Africa. *South African Journal of Botany*, 77(2), pp.387–396. Available at: http://dx.doi.org/10.1016/j.sajb.2010.10.004.

No, J.K. et al., 1999. Inhibition of tyrosinase by green tea components. *Life Sciences*, 65(21), p.PL241-PL246. Available at: http://www.sciencedirect.com/science/article/pii/S0024320599004920.

Paolini, J. et al., 2010. Chemical composition, intraspecies variation and seasonal variation in essential oils of Calendula arvensis L. *Biochemical Systematics and Ecology*, 38(5), pp.865–874.

Parvez, S. et al., 2007. Naturally Occurring Tyrosinase Inhibitors: Mechanism and Applications in Skin Health, Cosmetics and Agriculture Industries. *Phytotherapy Research*, 21, pp.805–816.

Ridder L et al., 2013. Automatic chemical structure annotation of an LC-LMS(n) based metabolic profile from green tea. *Anal Chem.* 85 (12), pp.6033-6040.

Maggini R., Kirfele C. & Pardossi A., 2014. Hydroponic production of medicinal plants. In D. alexandre M. Pereira, ed. *Medicinal Plants: Antioxidant Properties, Traditional Uses and Conservation Strategies*. Nova Science Publishers, pp. 91–116. Available at: https://www.novapublishers.com/catalog/product_info.php?products_id=46118.

Sato, K. & Toriyama, M., 2009. Depigmenting effect of catechins. *Molecules*, 14(11), pp.4425–4432.

Scognamiglio, M. et al., 2015. Chemical Composition and Seasonality of Aromatic Mediterranean Plant Species by NMR-Based Metabolomics. *Journal of Analytical Methods in Chemistry*, 2015, pp.3–7.

Scognamiglio, M. et al., 2014. Seasonal phytochemical changes in Phillyrea angustifolia L.: Metabolomic analysis and phytotoxicity assessment. *Phytochemistry Letters*, 8, pp.163–170. Available at: http://www.sciencedirect.com/science/article/pii/S1874390013001511 [Accessed May 14, 2015].

Soni, U., Brar, S. & Gauttam, V., 2015. Effect of seasonal variation on secondary metaboltes of medicinal plants. *International Jurnal of Pharmaceutical Sciences and Research*, 6(9), pp.3654–3662.

Southwell, I. a & Bourke, C. a, 2001. Seasonal variation in hypericin content of Hypericum perforatum L. (St. John's Wort). *Phytochemistry*, 56(5), pp.437–441.

Stefanowska, M., Kuraś, M. & Kacperska, A., 2002. Low temperature-induced modifications in cell ultrastructure and localization of phenolics in winter oilseed rape (Brassica napus L. var. oleifera L.) leaves. *Annals of Botany*, 90(5), pp.637–645.

Tomar, N.S., Sharma, M. & Agarwal, R.M., 2015. Phytochemical analysis of Jatropha curcas L . during different seasons and developmental stages and seedling growth of wheat (Triticum aestivum L) as affected by extracts / leachates ... Phytochemical analysis of Jatropha curcas L . during different sea. , 21(July), pp.83–92.

Van Vuuren, S.F. et al., 2007. Seasonal and geographical variation of Heteropyxis natalensis essential oil and the effect thereof on the antimicrobial activity. *South African Journal of Botany*, 73(3), pp.441–448.

Yang, D.-M., 2012. Antioxidant and Anti-Tyrosinase Activity from Olea Leaf Extract Depended on Seasonal Variations and Chromagraphy Treatment. *International Journal of Organic Chemistry*, 2(4), pp.391–397. Available at: http://www.scirp.org/journal/PaperDownload.aspx?DOI=10.4236/ijoc.2012.24054.

Yuan, Y. et al., 2014. Simultaneous determination of caffeine, gallic acid, theanine, (−)-epigallocatechin and (−)-epigallocatechin-3-gallate in green tea using quantitative 1H-NMR spectroscopy. *Analytical Methods*, 6(3), pp.907–914. Available at: http://pubs.rsc.org/en/content/articlehtml/2014/ay/c3ay41369a.

CHAPTER 6

METABOLITE PROFILING AND ANTI-TYROSINASE ACTIVITY OF PHYTOCHEMICAL CONSTITUENTS OF *GREYIA RADLKOFERI* LEAF EXTRACTS SUBJECTED TO DIFFERENT DRYING METHODS

ABSTRACT

Drying is the most common method for post-harvest preservation of medicinal plants. However, changes in structure and bioactivity of certain metabolites occur in the process and these could compromise the medicinal properties of the plant. Therefore, this study was conducted to evaluate the effects of drying on the metabolite profile as well as the anti-tyrosinase activities of *G. radlkoferi* leaf extracts. During processing, leaf material was exposed to three commonly used drying methods, namely, sun, oven and air-drying. Tyrosinase enzyme inhibition was investigated against monophenolase (tyrosine) with kojic acid as positive control. Then ^{1}H-NMR based metabolomics approach coupled with multivariate data analysis was applied to distinguish variations among extracts of leaf material processed with the three drying techniques and possible annotate metabolites responsible for the differences. Following this, LC-QqQ-MS, MAGMa and CSIFingerID were further used to confirm the identity of the annotated metabolites. Extracts prepared from air-dried material showed a high tyrosinase inhibition with an IC_{50} value of 17.80 µg/ml compared to other drying methods. Amongst other annotated compounds, air-dried material was dominated by gallocatechin-(4 alpha->8)-epigallocatechin. Catechins are flavonoids commonly found in green tea and are known for their ability to inhibit the enzyme tyrosinase. Thus the increased anti-tyrosinase activity in extracts of air-dried leaves can probably be attributed to the availability of gallocatechin-(4 alpha->8)-epigallocatechin. In addition to its high tyrosinase inhibition activity, air-dried samples preserved important metabolites compared to the other drying methods. Therefore, air drying could be recommended as the most suitable method for processing *G. radlkoferi* leaf material.

6.1 INTRODUCTION

At post-harvest, preparation of extracts for either herbal products, essential oil or cosmeceutical product development, begins with the drying of the raw plant material. Drying involves the removal of excess moisture from the plant with an aim to inhibit enzymatic degradation and to limit microbial growth and thus preserving the shelf life of the plant material (Rocha *et al*. 2011; Mediani *et al*. 2012; Harbourne *et al*. 2009). Drying allows for the quick conservation of the medicinal qualities of the plant material in an uncomplicated manner (Muller & Heindl 2006). However, the drying process in medicinal plants is known to alter the metabolic contents of the plants. These metabolites are actually chemical constituents found in the extract of a medicinal plant and are of utmost importance as they are responsible for the plant's efficacy in treating illnesses and diseases, as well as its cytotoxicity (Fennell *et al*. 2004).

Several methods are used to dry medicinal plant material. These include open air (shaded from direct sunlight), direct sunlight, in drying ovens, in solar driers, indirect fire, baking, lyophilization (freeze drying), microwave and infrared devices. Air drying and oven drying are reported to be the most preferred drying methods for herb processing due to their low cost and easy application (Mediani *et al.* 2012) which can be undertaken by small scale farmers. On the other hand, oven drying is reported to have an extensive negative impact on bioactivity of plant extracts, because of the heat effect on the phytochemical content. Therefore, it becomes desirable to establish the variability in raw material processed using different methods of drying.

The quality of herbal products is highly related to the attributes of the raw materials. Therefore, it becomes important to investigate and choose the processing method that mostly preserves the secondary metabolites of the plant material of interest. Such investigations can be achieved with metabolomics approach. As mentioned in the previous chapter, metabolomics can be used to evaluate and monitor metabolite variation within an organism under various conditions.

To our knowledge, there is no available data on the influence of the drying method on the metabolomic profile of *G. radlkoferi*. Therefore, the aim of the current study is two-fold; (i) to investigate phytochemical variation in air, sun and oven-dried *G. radlkoferi* leaf material as well as (ii) to evaluate the anti-tyrosinase activity of *G. radlkoferi* leaf extracts. The information

obtained from the current study will be useful as a guide to select the best methods of drying to be applied for the sake of efficiency, conservation of metabolite constituents and its anti-tyrosinase activity.

6.2 MATERIALS AND METHODS

6.2.1 Plant material collection

Greyia radlkoferi leaf material was collected from the cultivation site at Mothong African Heritage Trust gardens, Mamelodi township in Pretoria, Gauteng. Fresh and undamaged, non-infested leaves of *G. radlkoferi* were collected in April 2015. Upon harvest, leaves were washed thoroughly with water to remove dirt particles.

6.2.2 Plant material preparation

Leaf material was separated into three batches and it was subjected to different drying methods; namely oven, air and sun drying methods. For oven, drying the freshly harvested leaf material was dried in a laboratory oven operating under forced-air ventilation at 55°C for 12h. The selected temperature was based on recommendations of Muller and Heindl (2006). These authors recommended low drying temperatures of between 30°C and 50°C as they are thought to protect sensitive active ingredients. The air-dried samples were exposed to a well-ventilated room, protected from direct sunlight at ambient temperatures of between 21°C and 25°C for 2 weeks. For sun, drying the freshly harvested leaf material was spread on trays before they were exposed to direct sunlight for 5 days (Adeyemi *et al.* 2014). The leaves were turned occasionally to ensure even drying. The dried leaves from the three drying methods were ground separately with a hand grinder and stored in a -80^{0}C freezer until needed for further analysis.

6.2.3 Metabolomics analysis

The method used for metabolomics analysis used in this chapter is similar to the one used in chapter 5, section 5.2.3.2 to section 5.2.3.5

6.2.4 Anti-tyrosinase assay

The method followed for tyrosinase assay used in the current chapter is the same as the method used in chapter 5, section 5.2.3.1 to section 5.2.3.4

6.2.5 Liquid chromatograph-mass spectroscopy (LC-MS)

The method for LC-QqQ-MS is similar to the one used in chapter 5, section 5.2.4.1 to 5.2.4.3

6.3 RESULTS

6.3.1 Effect of drying methods on the anti-tyrosinase activity

The results of the anti-tyrosinase activity showed that *G. radlkoferi* leaves processed using different drying methods, successfully inhibited the enzyme tyrosinase. Similar to kojic acid (Figure 6.1 A), the percentage of tyrosinase inhibition by leaf extracts of *G. radlkoferi* increased with the increase in extract concentration (Figure 6.1). *Greyia radlkoferi* leaf extracts of sun, oven and air-dried samples were able to inhibit tyrosinase enzyme even at the lowest concentrations.

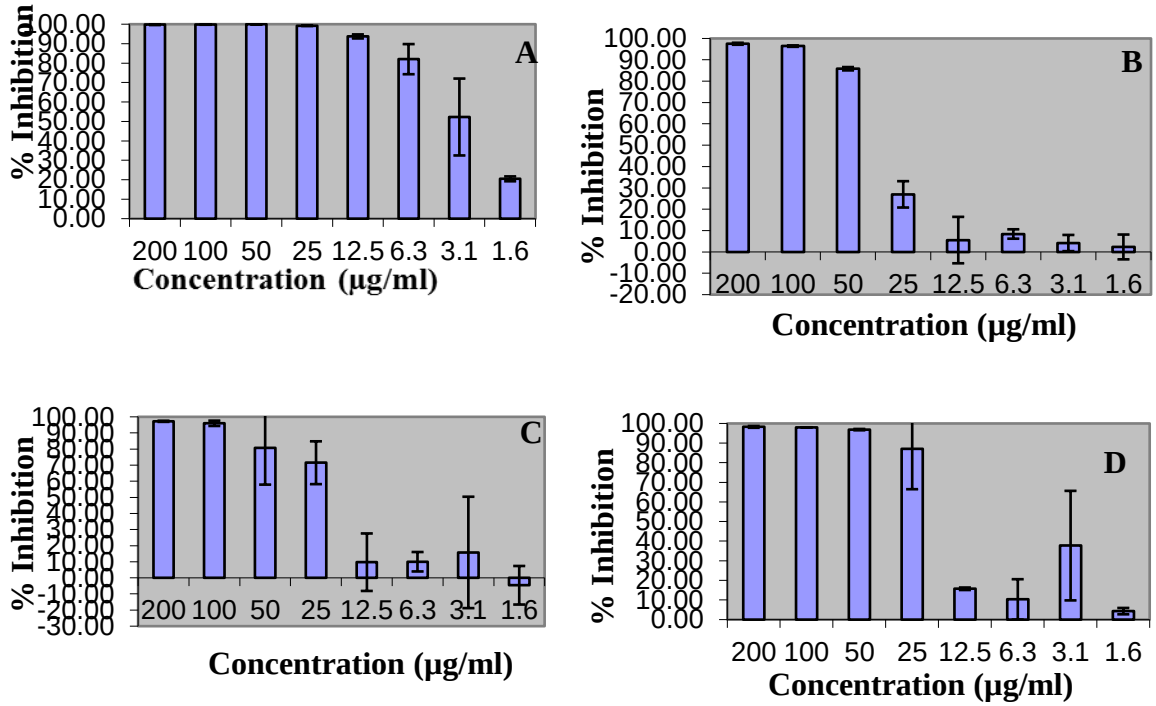

Figure 6.1: Percentage tyrosinase activity of kojic acid (A) and *Greyia radlkoferi* ethanol leaf extracts exposed to different drying methods (B=Oven-dried, C=Sun-dried, D=Air-dried).

The 50% tyrosinase inhibition concentration (IC_{50}) of *G. radlkoferi* ethanolic extracts processed with different drying methods is presented in Table 6.1. In general, leaf extracts of *G. radlkoferi* showed low tyrosinase inhibition in all treatments as compared to kojic acid (control). However, comparing the three drying methods, the highest inhibitory activity against tyrosinase enzyme was observed in the extracts of the air-dried samples with an IC_{50} value of 17.80 µg/ml. Extracts of sun-dried samples exhibited an IC_{50} inhibition activity of 19.82 µg/ml while the extracts of oven-dried samples exhibited the lowest IC_{50} inhibition activity of 32.45 µg/ml.

Table 6.1: The fifty percent inhibitory (IC_{50}) concentration of *Greyia radlkoferi* ethanol leaf extracts exposed to different drying treatments.

Drying Treatments	**IC_{50} values (µg/ml)**	**R^2values**
Oven drying	32.45	0.9862
Sun drying	19.82	0.8401
Air drying	17.80	0.8524
kojic acid	3.843	0.9419

A high IC_{50} value represents a low inhibitory activity and a low IC_{50} value represents a high inhibitory activity.

6.3.2 The effect of drying methods on chemical constituents

In order to reveal variations in metabolites present in *G. radlkoferi* leaves after they were processed using three different drying methods, ^{1}H-NMR-based metabolomics analysis was used to detect the holistic metabolite profiles of *G. radlkoferi* methanol leaf extracts. Representative ^{1}H-NMR spectra showing profiles of sun-dried, air-dried and oven-dried leaves are shown in Figure. 6.2. These ^{1}H-NMR spectra revealed varying peak signals indicating differences in the chemical composition of *G. radlkoferi* leaf extracts of sun-dried, air-dried and oven-dried leaves. When comparing the three spectra, air-dried samples showed the highest and concentrated intensity peak signals in the aliphatic region (0.5-3.0 ppm) and the aromatic region (5.5-9.0 ppm) while sun-dried samples showed the highest intensity peak signals in the carbohydrate region (3.00-5.5. ppm). Based on the results obtained from anti-tyrosinase activity studies, the current study compared the spectra of air-dried samples to those of oven and sun-dried samples (Figure 6.3).

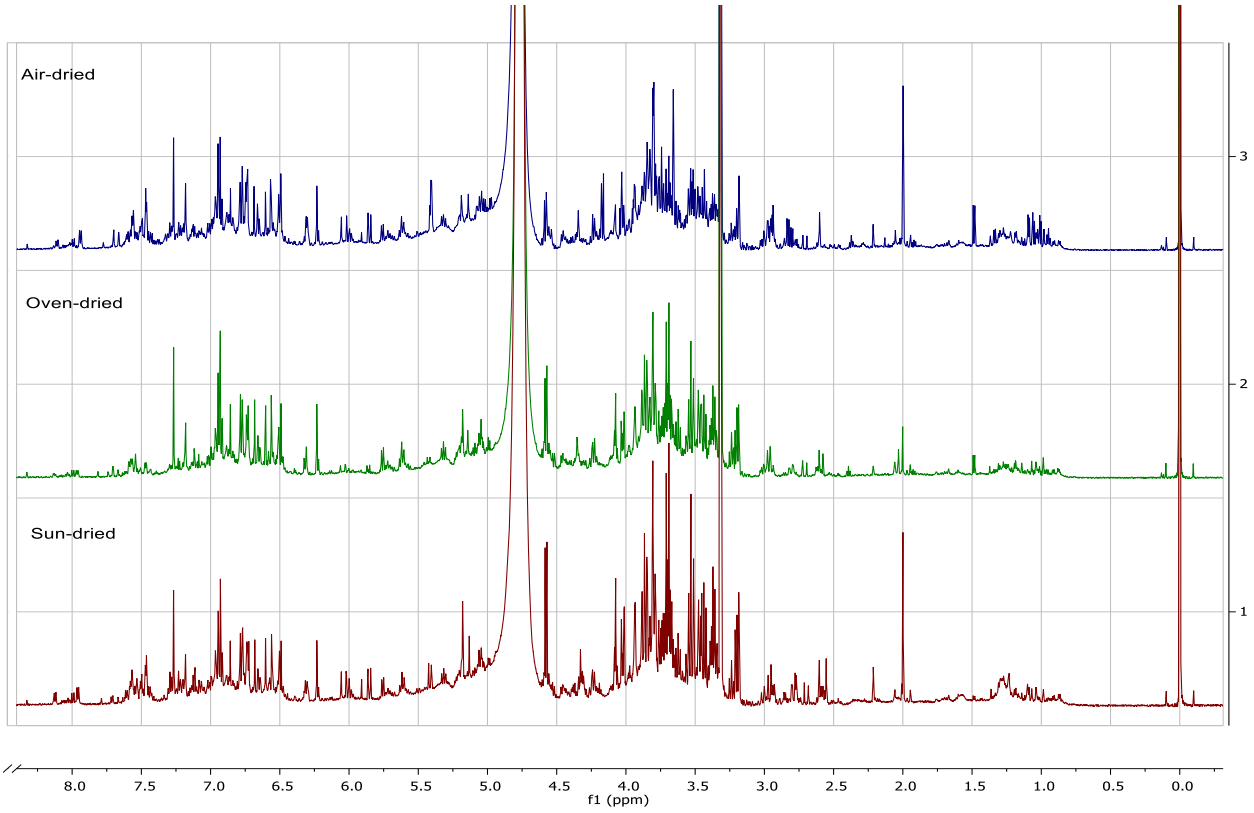

Figure 6.2: The 600 MHz ^{1}H-NMR spectra of *G. radlkoferi* leaf extratcs processed by different drying methods. Blue = air-dried, green = oven-dried and red = sun-dried material.

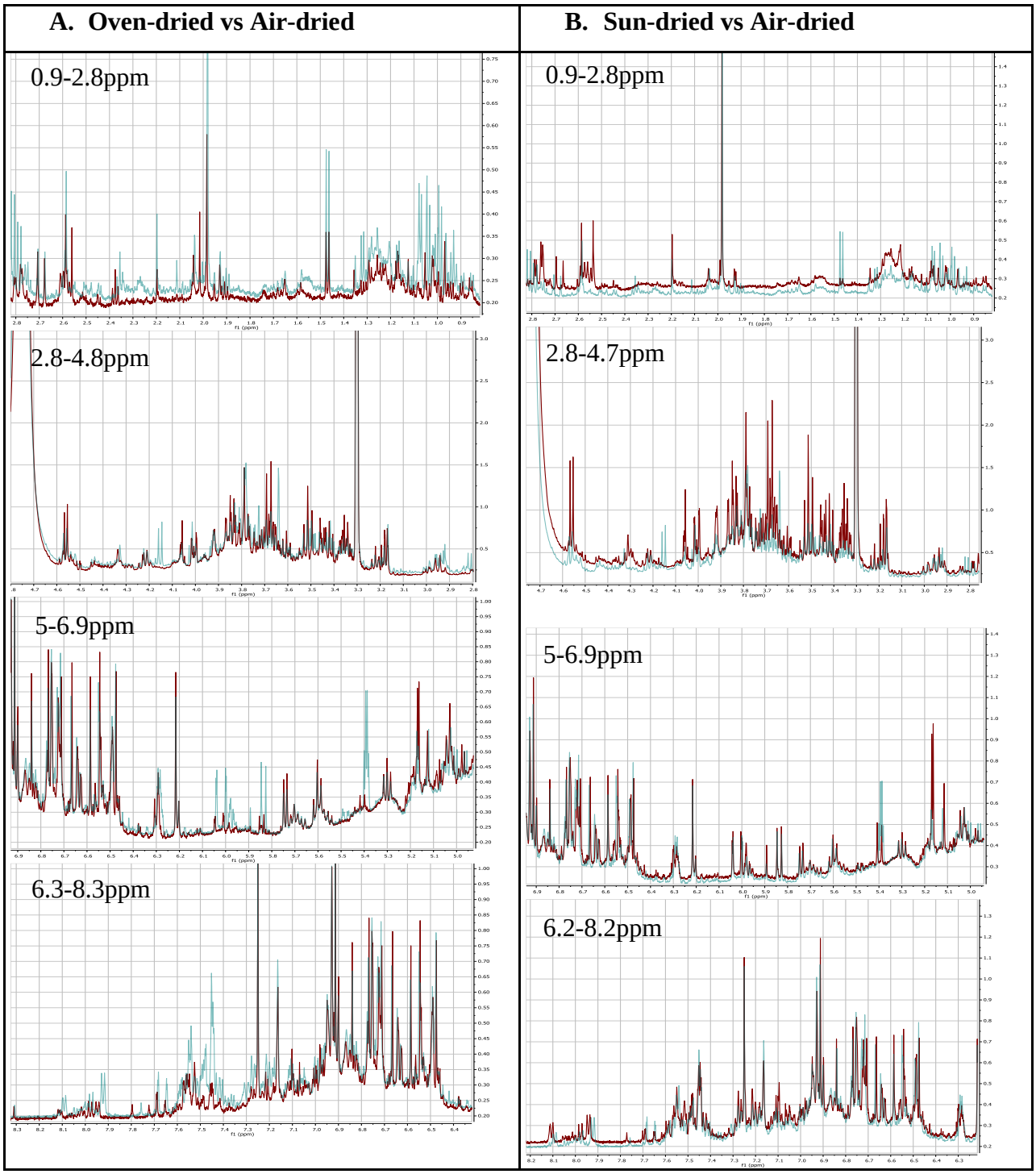

Figure 6.3: Snapshots comparing ^{1}H-NMR spectra of (A) air-dried versus oven-dried and (B) air-dried versus sun-dried samples of *G. radlkoferi* from 0.9 ppm to 8.3ppm. Blue colour represents spectra for air-dried samples (A&B) and red represent spectra for oven (A) and sun dried (B).

In the snapshots of superimposed spectra of air-dried versus oven-dried samples, air-dried samples generally showed higher peak intensities throughout the spectral regions [aliphatic region (0.5-3.0ppm) and aromatic region (5.5-9.0 ppm)] in comparison to oven-dried samples. Even though peak intensities of air-dried samples seemed to be higher than those of sun-dried samples, the differences were not so obvious in the snapshots.

Multivariate data analysis conducted with PCA, OPLS-DA and loading plots were used to reduce, analyze and conceptualize the information contained in *G. radlkoferi* extracts, which were processed using different drying methods. The samples were classified according to sun-dried, shade-dried and oven-dried groups. A principle component analysis (PCA) was generated by exposing the samples to several principal components (PCs) which were then presented graphically using a score plot. PCA score plot showed a few significant outliers and these were excluded from the data sets. The final plot of the PCA scores of the first two components (PC1 and PC2) showed separation in sun-dried and air-dried samples (Figure 6.4A). Although not clearly delinieted, more of the air dried samples showed a negative loading along PC2 while more of the sun-dried samples showed a positive loading along PC2 indicating that these samples were distinct from each other. Oven-dried samples however did not show separation from either the sun or the air-dried samples. The PCA showed a goodness-of-fit and predictability as indicated by R^2 and Q^2 values of 0.908 and 0.785 respectively. Further analysis of the data set using OPLS-DA, revealed clear separation and distinct clustering of samples processed with the three drying methods according to the method of drying used. (Figure 6.4B). Air-dried samples of *G. radlkoferi* clearly distinguished its self from the sun-dried and oven-dried samples along PC1 and PC2. Air-dried samples showed a negative loading along PC1 and PC2 while sun-dried samples and oven-dried samples showed a positive loading along PC1 and PC2 respectively. The OPLS-DA score plot showed good model quality with a goodness-of-fit and predictability as indicated by R^2X and Q^2Y values of 0.966 and 0.95 respectively. (Figure 3B)

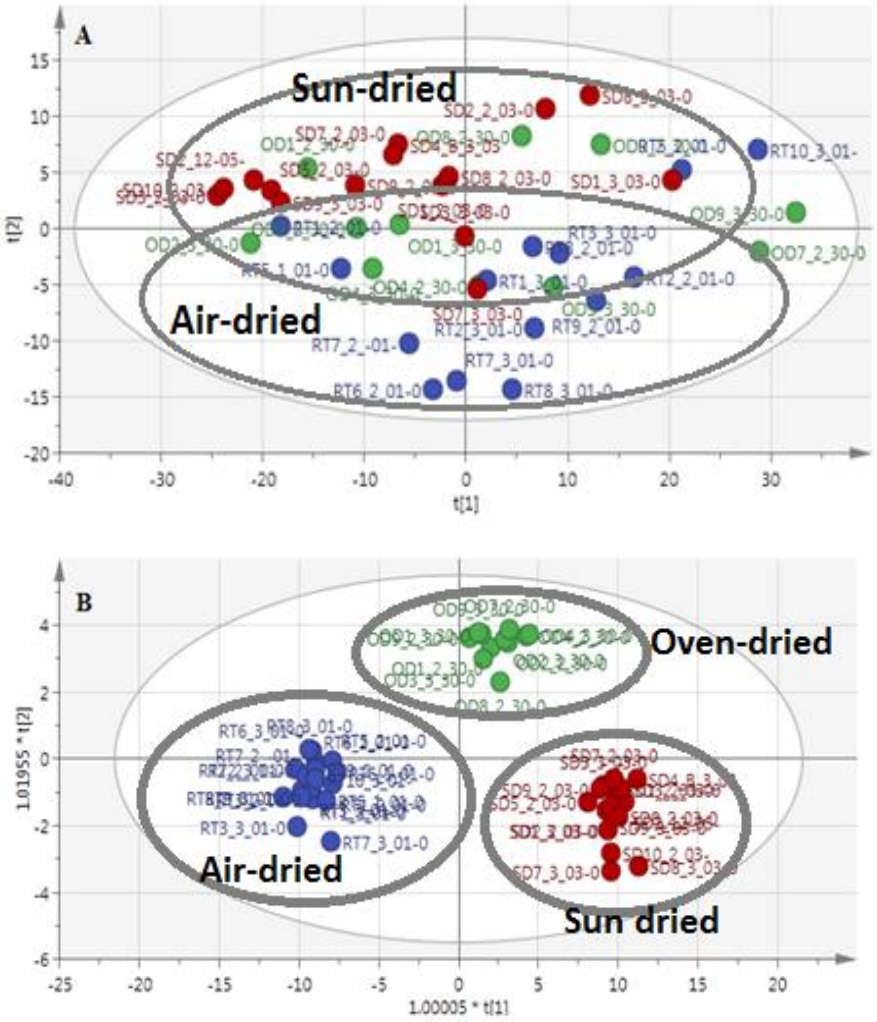

Figure 6.4: Score scatter plot of PCA (A) and OPLS-DA (B) performed on *G. radlokoferi* leaf extracts exposed to different drying methods. Oven drying (OD) represented by the green color, sun drying (SD) represented by the red color and air drying (RTD) represented by the blue color.

The pair-wise comparisons score plots of the three drying methods were performed to demonstrate the degree of clustering between air-dried samples against the sun-dried and the oven-dried samples (Figure 6.5A and Figure 6.6A). Corresponding loading plots were generated

in order to illustrate the most relevant variables that affect the sample differentiation between the groups (Figure 6.5B and Figure 6.6B).

Figure 6.5A below shows the OPLS-DA score plot of oven and air-dried samples and its corresponding loading plot. A clear separation of samples into distinct groupings was also noted for sun-dried and oven-dried plants with R^2X of 0.96 and Q^2X of 0.99 respectively. The loading plot in Figure 6.5B shows that bars corresponding to aliphatic, carbohydrates and aromatic chemical shifts were dominantly present in the air-dried samples compared to the oven-dried samples. However, these bars originate from different chemical shifts and thus represent different compounds.

The OPLS-DA score plot and corresponding loading plots of air-dried and sun-dried samples are presented in Figure 6.6. A distinct separation of classes according to the drying methods was observed with a goodness of fit and predictability as presented by R^2X of 0.92 and Q^2X of 0.99 respectively (Figure 6.6A). Similar to loading plot of air-dried and oven-dried samples, loading plot of air-dried and sun-dried samples (Figure 6.6B) shows that bars corresponding to aliphatic, carbohydrate and aromatic chemical shift regions were dominantly present in the air-dried samples compared to the sun-dried samples. However, these bars originate from different chemical shifts and thus represent different compounds.

Primary and secondary metabolites responsible for differentiating one drying method to the other were observed by noting the regions of the chemical shifts in the loading plot that differs from one treatment to the other. Compounds differentiating air to oven-dried samples were annotated as alanine, sucrose, acetamide, epigallocatechin and gallocatechin (Table 6.2) while those differentiating air to sun-dried samples include threonine, valine, alanine, leucine, isoleucine, sucrose, epigallocatechin and gallocatechin. Sun-dried samples was positively associated with β-glucose while oven-dried samples was positively associated with of β-glucose and asparagine.

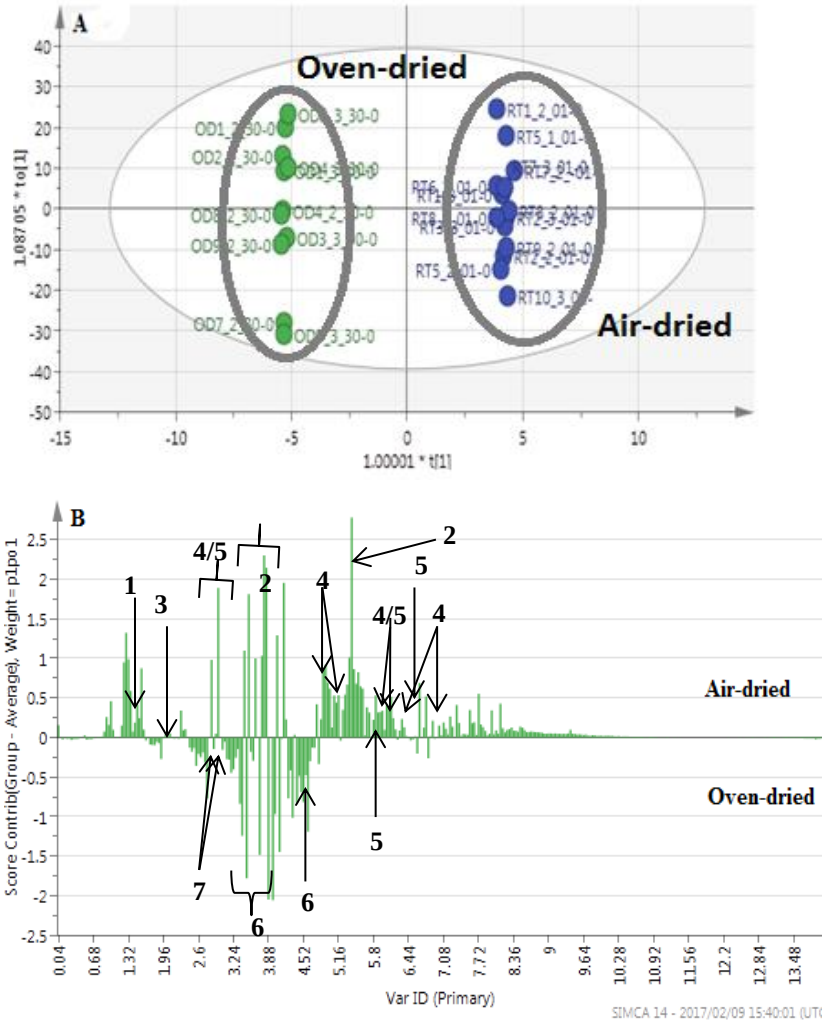

Figure 6.5: Score scatter plot (A) and score loading plot (B) from OPLS-DA on air-dried and oven-dried *Greyia radlokoferi* leaf extracts. 1=alanine; 2=sucrose; 3=acetamide; 4= epigallocatechin; 5=gallocatechin; 6=glucose; 7= asparagine.

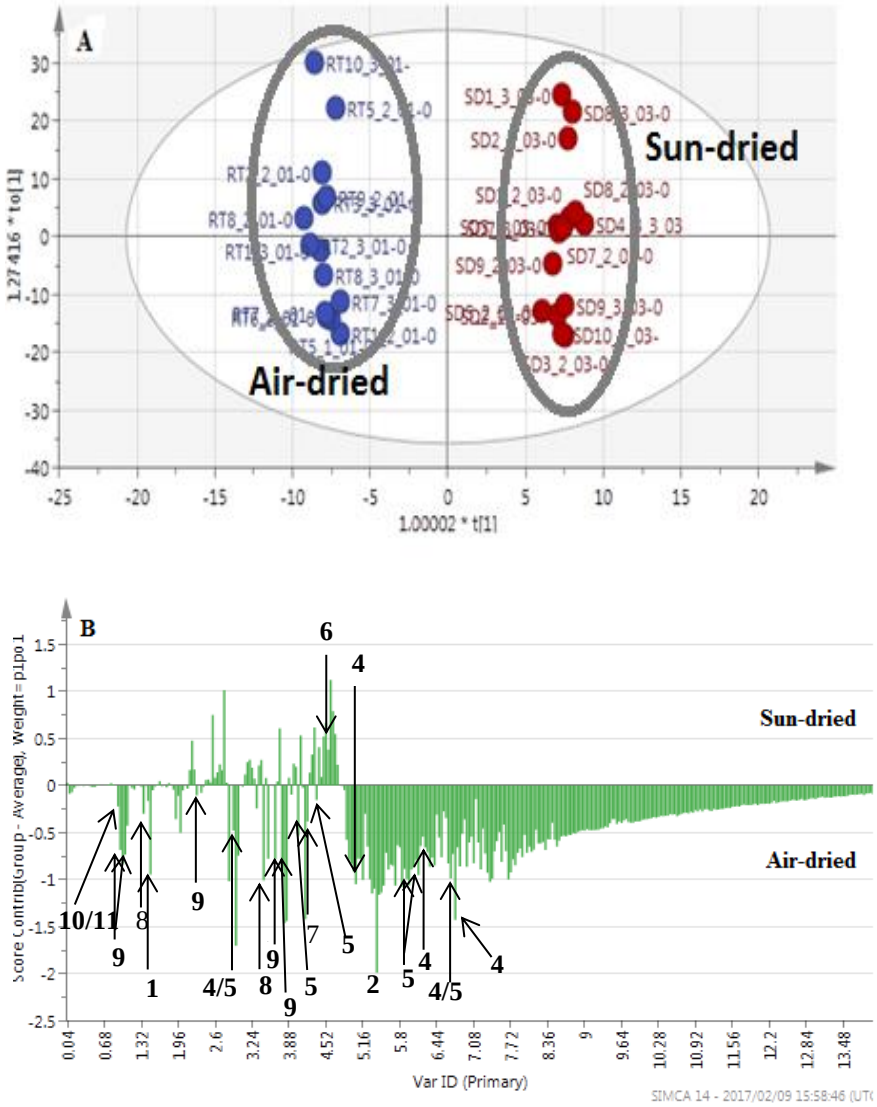

Figure 6.6: Score scatter plot (A) and score loading plot (B) from OPLS-DA on sun-dried and air-dried *Greyia radlokoferi* leaf extracts. 1=alanine; 2=sucrose; 4=epicagallocatechin; 5=gallocatechin; 6=glucose; 8=threonine; 9=valine; 10=leucine; 11=isoleucine.

Table 6.2: Specific NMR regions and annotated compounds that contributed to the separation of sun-dried, air-dried and oven-dried leaf extracts of *G. radlkoferi*.

Treatment	Compound	^{1}H-NMR Chemical shifts (ppm)	Chenomx (ppm)	Human Metabolome Database	Literature (Davis *et al*. 1996)	Assigned numbers
Air/oven-dried	Alanine	1.47	1.5	2.005		1
		Undetectable	3.68			
	Sucrose	3.42	3.4	3.46		2
		3.51	3.5	3.55		
		3.67	3.6	3.67		
		3.74	3.7	3.75		
		3.84	3.8	3.87-3.89		
			3.9	4.04		
		4.02	4.0			
		4.15	4.2	4.21		
		5.39	5.4	5.40		
	Acetamide/ acetate	1.98	2.0			3
	*Epigallocatechin	2.73			2.72	4
		2.84			2.84	
		4.15			4.19	
		4.95			4.82	
		5.89			5.91	
		6.00			6.01	
		6.54			6.57	
	*Gallocatechin	2.58			2.52	5
		2.87			2.87	
		3.96			3.97	
		4.52			4.51	
		5.83			5.8	
		6.0			6.01	
		6.44			6.45	
Oven/Air-dried	β-Glucose	3.2-3.24		3.21-3.23		6
		3.32-3.37		3.37-3.41		
		3.49		3.49		
		3.64-3.74		3.68-3.77		
		3.76-3.82		3.81-3.84		

		3.85 3.87 4.56 4.8		3.87 3.89-3.90 4.60-4.64 4.85	
	Asparagine	2.81 2.95		2.81 2.95	7
Air/Sun-dried	Threonine	1.32 3.48 4.22		1.3165 3.574 4.24	8
	Valine	0.96 1.02 2.260 3.64		0.976 1.029 2.261 3.601	9
	Alanine	1.47	1.5	1.46	1
	Leucine	0.93 undetectable 3.73		0.948 1.700 3.722	10
	Isoleucine	0.93 1.00 1.32 Undetectable Undetectable 3.64		0.926 0.997 1.248 1.45 1.968 3.661	11
	Sucrose	3.42 3.51 3.67 3.74 3.76-3.84 4.02 4.15 5.39	3.4 3.5 3.6 3.7 3.8 3.9 4.0 4.2 5.4		2
	*Epigallocatechin *Gallocatechin	see Air/oven drying above			5 6
Sun/Ove	β-Glucose	3.2-3.24		3.21-3.23	7

n-dried	3.32-3.37	3.37-3.41
	3.49	3.49
	3.64-3.74	3.68-3.77
	3.76-3.82	3.81-3.84
	3.85	3.87
	3.87	3.89-3.90
	4.56	4.60-4.64
	Undetectable	4.85

However, analysis of the LC-MS data using MAGMa in combination with CSIFingerID (http://www.csi-fingerid.org/) led to the identification of gallocatechin-(4 alpha->8)-epigallocatechin (mass =610.13226) instead of gallocatechin and epigallocatechin as previously annotated from ^{1}H-NMR spectra. The presence of gallocatechin-(4 alpha->8)-epigallocatechin compound was highly associated with extracts of air-dried leaves when compared to oven and sun-dried leaves.

6.4 DISCUSSION

Processing of the plant material through drying serves as a conservation means for raw medicinal plant material as well as the initial stage for preparation of extracts for herbal products. There are different methods of drying medicinal plants; however, the choice of the correct drying methods is necessary for good quality products. During the drying process, some changes in the structure and bioactivity of certain metabolites might occur (Abdul-Hamid *et al*. 2015). Thus, in the present study the biological activities of the air-dried, sun-dried and oven-dried *G. radlkoferi* leaf samples were investigated. Accordingly metabolomics analysis was applied to investigate the variations in metabolites of the air-dried, sun-dried and oven-dried *G. radlkoferi* leaf samples.

The present study revealed that the different drying methods studied greatly affected the metabolite profile and the bioactivity of the *G. radlkoferi* leaf extracts. Air drying (protected from direct sunlight) exhibited the highest anti-tyrosinase activity in comparison to the other drying methods, indicating that this could be the most favourable method for drying *G. radlkoferi* leaves for medicinal plant preparation. The IC_{50} value of 17.80 µg/ml reported for air-dried *G. radlkoferi* leaf extracts in this study is very similar to the values reported in the previous studies. Lall and co-authors previously reported anti-tyrosinase inhibition with an IC_{50} value of 17.96 µg/ml by air-dried *G. radlkoferi* leaf extracts (Lall *et al*. 2016).

Varying peak signals observed in the representative ^{1}H-NMR spectra and the superimposed ^{1}H-NMR spectra revealed that different drying methods affect the metabolite profiles of *G. radlkoferi*. In this study, certain groups of metabolites dominated certain chemical shift regions of the spectra depending on the method of drying. This study expected sun-drying and/or oven drying methods to increase the availability of flavonoids in the extract as literature reports these to be highly induced as a defence mechanism in response to stress associated with

heat/temperature (Wahid *et al*. 2007). However, this was not the case with *G. radlkoferi* leaf extracts processed with different drying methods. A depletion of chemical constituents in the oven-dried and sun-dried groups, especially in the aliphatic and aromatic region was evident in the loading plots. Oven-dried and sun-dried leaf extracts were mostly dominated by the sugars rather than the other metabolite groups. The possible explanation for this observation might be that metabolites of *G. radlkoferi* leaves are either sensitive to the thermal temperature used in the current study (55^{o}C) and/or to the combination of heat and drought stress effects. The physical colour changes of dried leaves upon exposure to heat-drying determines the breakdown of phenolic compounds. Julkunen-Tiitto & Sorsa (2001) attributed the changes in color (from green to brown) of heat-dried leaves to advanced decomposition of the phenolics. *Greyia radlkoferi* leaves turned brown upon exposure to sun and oven-drying while air-dried leaves remained green (data not shown).

Further analysis of data using multivariate analysis confirmed the existence of differences in the profiles of air-dried, sun-dried and oven-dried samples. OPLS-DA score plots demonstrated that the metabolic characteristics of these samples were different. Air-dried, sun-dried and oven-dried groups were different enough to distinguish between the different drying methods and thus they were separated in clusters according to the drying method used. Similarly, pairwise OPLS-DA plots clearly differentiated air-dried and oven-dried groups as well as air-dried and sun-dried samples. The corresponding loading plots further revealed that metabolites of *G. radlkoferi* went through some changes during the different drying processes. The air-dried group was set apart from the sun-dried and oven-dried groups by the presence of aliphatic and the aromatic compounds which were, however absent or in lower concentration in the oven-dried and sun-dried groups. The chemical shifts corresponding to those bars led to the annotation of amino acid (asparagine), an amide derived from acetic acid (acetamide), a sugar (sucrose), epigallocatechin and gallocatechin as metabolites associated with air-dried material and thus differentiate the air-dried to the oven-dried group. Amino acids such as valine, alanine, leucine and isoleucine differentiated the air-dried group from the sun-dried group. Glucose and asparagine were highly associated with the oven-dried samples while sun-dried samples were highly associated with glucose and thus differentiated these drying methods to the air-dried group.

Similar to the previous chapter, LC-MS was used to identify gallocatechin-(4 alpha->8)-epigallocatechin as the metabolite present in leaf extracts of *G. radlkoferi*. In general, the spectras of the catechins are almost similar differing with only a few peak regions. Furthermore, gallocatechin-(4 alpha->8)-epigallocatechin is comprised of epigallocatechin and gallocatechin components and hence it was initially difficult to annotate this compound with NMR, and thus the use of LC-MS for confirmation of the compounds. Several plant metabolites are associated with the anti-tyrosinase activity of plant extracts including the phenolic compounds. These phenols particularly flavonoids are able to inhibit the tyrosinase enzyme due to their ability to chelate copper in the active site. Air-dried leaves were highly associated with a flavonoid of the catechin group, gallocatechin-(4 alpha->8)-epigallocatechin and accordingly probably is resonsible for the improved tyrosinase inhibition effect of *G. radlkoferi*. Even though gallocatechin-(4 alpha->8)-epigallocatechin has never been tested for anti-tyrosinase activity, catechins in general are well known tyrosinase inhibitors (No *et al*. 1999; Cheng *et al*. 2007; Sato & Toriyama 2009; Abdillahi *et al*. 2011). Therefore, the high tyrosinase inhibitory ability of air-dried leaves could be attributed the presence of this catechin. Detailed information regarding gallocatechin-(4 alpha->8)-epigallocatechin is presented in the discussion in chapter 5.

Temperature has been identified as one of the critical factors affecting catechin stability. Catechins in green tea are reported to be susceptible to degradation by many environmental factors which subsequenlty limit their dietetic and pharmaceutical applications (Li *et al*. 2011). Furthermore, a study on drying of apricots demonstrated the sensitivity of catechins to temperature (Madrau *et al*. 2009). These authors demonstrated a decrease in dry matter of epicatechins with the increase in drying temperatures ranging from 55-75°C. This also supports the reduced intensity peaks in gallocatechin-(4 alpha->8)-epigallocatechin in the oven and sun-dried *G. radlkoferi* leaf extracts and related decrease in activity.

The PCA model generated in the current study sufficiently explained the data (R^2=0.908) and the model was good at predicting the data with Q^2=0.785. High goodness of fit values and high predictability values were obtained for pairwise score plots of oven against air dried and that of sun against air dried material with R^2Y=0.96 and Q^2Y=0.95 as well as R^2Y=0.92 and Q^2Y=0.96

respectively. All the models generated in the current study explained and predicted the data very well, thus it was considered as an excellent model.

6.5 CONCLUSIONS

Metabolomics approach using ^{1}H-NMR and LC-MS combined with biological activity analysis was used to explore the effects that different postharvest processing methods have on *G. radlkoferi* leaf extratcs. The findings of the current study demonstrated that the application of air-dried, sun-dried and oven-dried methods caused significant changes in the metabolomic profile of leaves *G. radlkoferi.* These changes were noted in both primary and secondary metabolites. Metabolomics approach using ^{1}H-NMR and LC-MS (LC-QqQ-MS) successfully determined acetamide, sucrose and gallocatechin-(4 alpha->8)-epigallocatechin as the chemical constituents contributing to the variation of leaf extract metabolites and was positively associated with air-dried compared to the oven-dried samples. Amino acids such as valine, alanine, leucine, isoleucine; glucose and gallocatechin-(4 alpha->8)-epigallocatechin contributed to the variation and was positively associated with the air-dried compared to the sun-dried samples. Glucose and asparagine were highly associated with the oven-dried leaf extracts while sun-dried extract was dominated by glucose only and thus differentiated these two drying methods from the air-dried group. Air drying preserved most metabolites during processing while sun and oven drying clearly depleted some metabolites especially amino acids and some aromatic compounds. Temperature above 55^{o}C were clearly not suitable for drying of *G. radlokoferi* leaf extracts. Air drying *G. radlokoferi* leaves also improved the anti-tyrosinase activity (IC_{50} =17.80 µg/ml) of leaf extracts and gallocatechin-(4 alpha->8)-epigallocatechin is considered as responsible for this increased anti-tyrosinase activity. Therefore this study concludes that for a better preservation of natural quality and medicinal benefits, *G. radlkoferi* leaves should be processed by air drying away from direct sunlight and at room temperature.

6.6 REFERENCES

Abdillahi, H.S., Finnie, J.F. & Van Staden, J., 2011. Anti-inflammatory, antioxidant, anti-tyrosinase and phenolic contents of four Podocarpus species used in traditional medicine in South Africa. *Journal of Ethnopharmacology*, 136(3), pp.496–503.

Abdul-Hamid, N.A. et al., 2015. Influence of Different Drying Treatments and Extraction Solvents on the Metabolite Profile and Nitric Oxide Inhibitory Activity of Ajwa Dates. *Journal of Food Science*, 80(11), pp.H2603–H2611.

Adeyemi, SB., Ogundele, KO., Animasaun, A.M., 2014. Influence of drying methods on the proximate and phytochemical composition of Moringa oleifera Lam. *Global journal of medicinal plant research*, 2(1), pp.1–5.

Cheng, K.-T. et al., 2007. New constituent from Podocarpus macrophyllus var. macrophyllus shows anti-tyrosinase effect and regulates tyrosinase-related proteins and mRNA in human epidermal melanocytes. *Chemical & pharmaceutical bulletin*, 55(5), pp.757–761.

Fennell, C.W. et al., 2004. Assessing African medicinal plants for efficacy and safety: Agricultural and storage practices. *Journal of Ethnopharmacology*, 95(2-3), pp.113–121.

Harbourne, N. et al., 2009. Effect of drying methods on the phenolic constituents of meadowsweet (Filipendula ulmaria) and willow (Salix alba). *LWT - Food Science and Technology*, 42(9), pp.1468–1473.

Julkunen-Tiitto, R. & Sorsa, S., 2001. Testing the Effects of Drying Methods on Willow Flavonoids, Tannins, and Salicylates. *Journal of Chemical Ecology*, 27(4), pp.779–789.

Lall, N. et al., 2016. Insights into tyrosinase inhibition by compounds isolated from Greyia radlkoferi Syzsyl using biological activity, molecular docking and gene expression analysis. *Bioorganic & Medicinal Chemistry*.

Li, N., Taylor, L.S. & Mauer, L.J., 2011. Degradation kinetics of catechins in green tea powder: Effects of temperature and relative humidity. *Journal of Agricultural and Food Chemistry*, 59(11), pp.6082–6090.

Madrau, M.A. et al., 2009. Effect of drying temperature on polyphenolic content and antioxidant activity of apricots. *European Food Research and Technology*, 228(3), pp.441–448.

Mediani, A. et al., 2012. 1H-NMR-based metabolomics approach to understanding the drying effects on the phytochemicals in Cosmos caudatus. *Food Research International*, 49(2),

pp.763–770. Available at: http://www.sciencedirect.com/science/article/pii/S096399691200381X [Accessed September 25, 2015].

Muller, J. & Heindl, A., 2006. Drying of medicinal plants. In R. J. Bogers, L. E. Craker, & D. Lange, eds. *Medicinal and Aromatic Plants*. Netherlands: Springer, p. 252.

No, J.K. et al., 1999. Inhibition of tyrosinase by green tea components. *Life Sciences*, 65(21), pp.PL241–PL246.

Rocha R. P., Melo E.C. & Radünz L.L., 2011. Influence of drying process on the quality of medicinal plants: A review. *Journal of Medicinal Plants Research*, 5(33), pp.7076–7084.

Sato, K. & Toriyama, M., 2009. Depigmenting effect of catechins. *Molecules*, 14(11), pp.4425–4432.

Wahid, A. et al., 2007. Heat tolerance in plants: An overview. *Environmental and Experimental Botany*, 61(3), pp.199–223.

CHAPTER 7

GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

Medicinal plant cultivation could prove to be a solution in terms of improvement of quality and quantity demands from the market for herbal formulations and preparations. Cultivation ensures the availability of the plant material of interest in quantities required by the industry. Furthermore, it provides a platform to monitor the production of bioactive compound and thus detect conditions favouring optimum production of these compounds. To ensure appropriate methods for establishment of *G. radlkoferi* into cultivation and continuous supply of leaf material to the market, this study investigated different propagation methods, pruning methods and frequency of leaf harvesting. To investigate the conditions favouring optimum production of secondary metabolites, changes in the metabolomic profiles of *G. radlkoferi* were monitored in response to seasonal variation (pre-harvest) and to different drying methods (post-harvest). The study further investigated the bioactivity of the leaf extracts of *G. radlkoferi* at different seasons and drying methods against tyrosinase enzyme with an aim to establish if a relationship exists between bioactivity and the chemical profiles.

The investigation into propagation of *G. radlkoferi* using two propagation methods, namely seed and stem cutting propagation led to the conclusion that seed propagation is the most suitable technique for establishment and large-scale multiplication of *G. radlkoferi* plants. Impressive results of 81% germination were obtained in seeds germinated at 25°C. Considering cutting propagation trials, poor rooting percentages were obtained in stem cuttings when factors affecting rooting such as cutting type and rooting hormones were considered. Basal cuttings were determined as cutting type that produced the best rooting percentage, even though the rooting percentage was very poor (35%) without application of hormones. It is well known by now that the propagation of a new cultivar using cuttings is considered economically feasible if the rooting ability exceeds 50% and thus cutting propagation is not considered successful for establishment of *G. radlkoferi*. Application of rooting hormones (0.1% IBA) however increased the rooting percentage to 63%, in basal cuttings even though it was not statistical significant and therefore a scientifically-based conclusion could not be drawn. Perhaps studies considering other

factors contributing to successful rooting of cuttings in addition to the ones investigated in the current study may improve rooting of *G. radlkoferi* to acceptable levels. Additional factors to consider include different hormones, growing media, age of the stock plant and the use of a non-mist propagation system.

This study also revealed that the germination percentage and the onset of germination of *G. radlkoferi* were affected by exposure to constant temperatures. Very low temperatures associated with winter (< 15°C) and very high temperatures associated with summer (>25°C) were not suitable for germination of *G. radlkoferi s*eeds. Seeds of this species germinated optimally between 20°C and 25°C suggesting spring, as a suitable season to germinate *G. radlkoferi.* Temperature requirements of *G. radlkoferi* could further assist in predicting new locations for cultivating this plant. Having reached these conclusions, this study acknowledges that seed germination is a function of multiple environmental factors. Our experiments were conducted under controlled environmental conditions at constant temperatures, whereas under natural conditions temperature is subject to diurnal fluctuations as well as dark and light fluctuations. The environmental factors such as diurnal temperatures and light exposures were not considered in this study due to equipment limitations. Future studies should focus on the effects of additional factors and perhaps their interaction to refine the results obtained in this study, although a high germination percentage was obtained with the current experimental conditions. Propagation studies provided an understanding of the establishment of this plant in new and controlled environments.

Once the medicinal plant of interest has been established by means of propagation, it becomes necessary to investigate means of optimizing leaf production as well as suitable harvesting frequencies for continuous and improvement of supply quantities to the market. Leaves of *G. radlkoferi* were harvested monthly, bimonthly and once-off (end of growth season) from trees that were exposed to various pruning methods after which leaf weight and dry matter were determined. The quantity of leaf yield of *G. radlkoferi* was found to be dependent on harvesting frequency. Harvesting of leaves at bimonthly intervals showed a favourable increase in leaf yield quantities indicating that there is an allowance for growth of new leaves from one harvesting period to the next. Monthly harvest allows a limited time for re-growth of leaves while

harvesting at the end of the growth season means supply is limited to one harvest in a year. Bimonthly harvests were thus regarded as the best management technique for leaf harvesting of *G. radlkoferi* trees. Fresh weight yield of 238 g per tree or 2.38 tons/hectare were obtained while dry weight yield of 83g per tree or 0.830 tons/hectare was obtained. A suitable pruning practice or approach for increased leaf production could not be determined, as there was no significant difference in fresh and dry leaf yield of *G. radlkoferi* among the four applied pruning methods.

The potential use of *G. radlkoferi* for cosmeceutical purpose is associated with the presence of certain metabolites responsible for the bioactivity of the plant. These metabolites are prone to pre-harvest and post-harvest changes. Changes in metabolites affect the biological activity of the plant extracts and thus cause negative consequences to the final herbal product. For example, absence, loss or low activity in medicinal plant material and/or its products may be perceived as having limited or no medicinal value at all, while factors affecting variation in metabolite production have not been considered. Therefore, the understanding of how pre- and post-harvest factors affect the composition of various secondary metabolites in plants is important in the commercial production of raw medicinal plant material and its products for quality improvement.

To investigate metabolite variations as affected by seasons and drying methods, this study used a standard direct extraction protocol for NMR with a two phase deuterated solvent system, which was methanol, and water. Since a non-targeted metabolomics approach was followed, as many compounds as possible, from the widest polarity range were targeted for extraction. The polarity and pH of the solvents used are known to affect the range of metabolites that can be extracted. This study was aimed at extracting compounds from *G. radlkoferi* both of polar and non-polar nature in a single extraction. Furthermore, combining deuterated methanol with potassium dihydrogen phosphate buffer (KH_2PO_4) (to avoid possible fluctuations in chemical shifts of signals in the NMR spectra) in deuterated water with a pH of 6 is known to be able to extract an extensive range of metabolites which might include phenolics, terpenoids, fatty acid, organic acids, amino acids and carbohydrates in a single extraction. The metabolomics extraction method used, therefore, ensured that all the metabolites extracted by ethanol (for the anti-tyrosinase assays) will be obtained, as well as additional metabolites that are extractable by the wider polarity range of the solvent used, to successfully apply the non-targeted approach.

Based on the outcomes of this study, untargeted metabolomics approach using NMR has proven to be an ideal tool that can applied for quality control of raw medicinal plant material in the field and at postharvest. ^{1}H-NMR spectra demonstrated changes in the production of chemical constituents in the leaves of *G. radlkoferi* in response to seasonal changes and to different drying methods. These metabolite changes were not only limited to secondary metabolites (which are often of medicinal value) but to primary metabolites as well. ^{1}H-NMR spectra profiles provided a complete picture of the plant metabolome for each season (winter, spring, summer and autumn) and for each drying method (air, sun and oven drying). The metabolite changes presented in this study may aid in promoting the understanding of the functioning of the whole metabolome of *G. radlkoferi* under various conditions. Understanding of the functioning of the whole plant metabolome may be useful in molecular studies that seek to manipulate the plant metabolome for increased chemical constituents.

Reduction of ^{1}H-NMR data using multivariate data analysis (OPLS-DA) further demonstrated variations in different seasons and different drying methods. The sample groups were separated into clusters based on different seasons and drying methods. The clustering observed indicates that the OPLS-DA model was a valid model for the current study as it demonstrated high goodness of fit (R^2 and R^2Y) as well as good predictability values (Q^2 and Q^2Y).

The NMR regions representing chemical shifts of metabolites possibly responsible for the differences in OPLS-DA score plots of different seasons (winter and spring) and different drying methods (air, sun and oven drying) were revealed through loading plots. Correlating these chemical shifts to classes of secondary metabolites found within the plant kingdom by means of Human metabolome database, Chenomx and literature search led to successful annotation of metabolites differentiating the extracts of different seasons and the extracts of leaves dried with different drying methods. Chemical constituents distinguishing winter and spring samples are tabulated in Chapter 5 (Table 5.1) and those distinguishing air, sun and oven-dried samples are tabulated in Chapter 6 (Table 6.2). Metabolites which became of high interest were flavonoids of the catechin group, gallocatechin and epigallocatechin which were highly associated with extracts of winter harvested leaves and those of air-dried leaves. Upon the

annotation of these constituents, the current study further made use of other methods to confirm the identity of the annotated compounds. LC-MS (LC-QqQ-MS), MAGMa and CSIfingerID successfully identified only one flavonoid of the catechin group, gallocatechin-(4 alpha->8)-epigallocatechin in the extracts of *G. radlkoferi* leaves. This compound was highly associated with extracts of winter harvested leaves in the seasonal study and extracts of air-dried leaves in the drying study and structurally is comprised of gallocatechin and epigallocatechin components. Generally, catechins are well-known for their tyrosinase inhibition properties and thus a potential in treating hyperpigmentation disorders. Epigallocatechin [one of the components of gallocatechin-(4 alpha->8)-epigallocatechin] in particular, has proven tyrosinase inhibition ability and its potential in treating hyperpigmentation disorders in several studies and in a patented invention. Furthermore, extracts highly associated with gallocatechin-(4 alpha->8)-epigallocatechin exhibited the highest tyrosinase inhibition activity in the current study. In previous studies linking metabolomics and bioactivity, many authors have successfully established co-relations between bioactivity and the presence of a particular compound in the highly active extracts. Based on these views, the current study can therefore suggest that gallocatechin-(4 alpha->8)-epigallocatechin is responsible for the high tyrosinase inhibition activity exhibited by extracts of winter harvested and extracts of air-dried leaves. Gallocatechin-(4 alpha->8)-epigallocatechin could be working in synergy with other compounds with increased tyrosinase inhibition properties as previously isolated by Lall and co-authors in 2016 from *G. radlkoferi* leaf extracts. The outcomes of the current studies suggest that for optimum quality of extracts and metabolites of its medicinal value, *G. radlkoferi* leaves must be harvested in winter and the prefered drying method is air drying. The highest leaf yield was however noticaeble with the bimonthly harvesting towards late summer and recommendations are therefore to delay harvesting to this period (late summer season) to allow enough time for optimisation of tyrosinase activity of *G. radlkoferi* plant material as it showed increases towards autumn and winter seasons.

There are many factors affecting the availability of secondary metabolites in plants. These include, harvesting seasons, harvesting time, drying methods, extract preparation and others. In addition to this, the selection of the methods used for identification or isolation is of outmost importance as some instruments may discriminate certain metabolites. For example,

gallocatechin-(4 alpha->8)-epigallocatechin identified as a possible tyrosinase inhibitor in this study was not amongst the active anti-tyrosinase compounds which were isolated in *G. radlkoferi* leaves in a previous study by Lall and co-workers in 2016. An explanation for this could be that metabolites of the catechin group were either unavailable or probably available in small quantities to be identifiable using bioassay guided fractionation. This explanation could be true given the fact that the plant material used by these authors for tyrosinase inhibition assay was collected in summer. In the current study, metabolites of the catechin group were found to be highly associated with samples harvested in cold season (winter) rather than warm season (spring). Furthermore, from the bioactivity studies, it was clearly evident that the tyrosinase inhibition activity was higher in the colder seasons as compared to the warmer seasons. It is therefore not surprising that metabolites of the catechin group were not identified in the plant material harvested in summer meaning that they may have been absent or available in lower concentrations. Generally, TLC or HPLC methods are regarded as the narrowly focused techniques especially when it comes to quality control of herbal products. Untargeted metabolomics approach is commonly viewed as being more reliable and the advantage is that it does not discriminate certain compounds over the others as it analyses all the compounds in the sample, thus giving a holistic view of the extract. Furthermore, through loading plots, all compounds significantly contributing to the differences between plant material are highlighted and can be annotated/identified, tested for bioactivity thus providing an opportunity for discovering new active ingredients. NMR metabolomics technique successfully provided new information on tyrosinase inhibitors of *G. radlkoferi* leaf extracts, which could not be achieved by the TLC technique used by Lall and co-authors 2016.

The results of the anti-tyrosinase effects reported in the current study for *G. radlkoferi* are in agreement with those of the previous authors regarding the cosmeceutical value of this plant. The highest IC_{50} inhibition activity values obtained in drying studies (IC_{50}=17.80 µg/ml for air-dried samples) and winter harvested samples (IC_{50} =30.3 µg/ml) were comparable to the results obtained in a patented invention by De Cahna *et al*. (2015) and in a recent article by Lall and co-authors (2016). According to the invention, leaf extracts of *G. radlkoferi* displayed tyrosinase inhibitory activity by exhibiting 50% inhibitory concentrations (IC_{50}) ranging from 17, 96 µg/ml to 32.62 µg/ml when L-tyrosine and dihydroxyphenylalanine (DOPA) were used as substrates.

Similarly, Lall and co-authors (2016) reported IC_{50}=17.96 µg/ml when L-tyrosine was used as a substrate. The differences in IC_{50} values obtained in these *G. radlkoferi* studies can be attributed to batch-to-batch variations as the leaf material was collected at different times (as is evident from this study on seasonal variation) and from different areas for each of these studies. This stresses the importance of the effect of genotype as well as locality, and should be investigated in future studies.

The information generated in this study serves as a guide for a propagation method suitable for rapid establishment and multiplication of *G. radlkoferi*, a suitable harvesting time for optimum leaf yield, a suitable harvesting season and drying method that best preserve important metabolite constituents and thus increase their anti-tyrosinase activity. In particular, a valuable bioactive must be measurable, reproducible and linked to a relevant biochemical pathway. Therefore, to further assess the validity of the results presented here, isolation of gallocatechin-(4 alpha->8)-epigallocatechin from *G. radlkoferi* and testing for its ability to inhibit tyrosinase activity, as well as the two units (epigallocateching and gallocatechin) individually and in combination with previously isolated compounds are future research activities recommended for confirming increases in activity in winter harvested and air-dried leaves. The possibility of synergistic effects of indivivdual compounds, linked to variable concentrations of individual compounds as affected by external factors could yield valuable information for development, in general, of herbal formulations in future. This linked to studies investigating the genotype and locality interactions will provide clear guidelines on not only propagation, pre-and post-harvest effects, but influences that affect quality and batch-to-batch variability often experienced in plant extract preparations.

South Africa, realizing the value of its biodiversity, invests intensively in development of the bio-economy, by progressing research leads to the commercial market. This study provides answers to many of the gaps and questions, currently not answered, in development of the production chain of a new cosmeceutical product to reach the commercial market. This study pioneers in-depth analysis of exogenous effects on the bioactivity and chemical profiles of an indigenous cosmeceutical species, and the effects of pre-and-post-harvesting methods on the quality of material.

REFERENCES

Lall, N. et al., 2016. Insights into tyrosinase inhibition by compounds isolated from Greyia radlkoferi Syzsyl using biological activity, molecular docking and gene expression analysis. *Bioorganic & Medicinal Chemistry*.

De Cahna, M.N. *et al*., 2015. Extract of Greyia radlkoferi and use thereof. Available at: www.google.com/patents/WO2013171720A1?cl=en.

CHAPTER 8

APPENDIX

8.1 ANTI-TYROSINASE ASSAY REAGENTS PREPARATION

The following reagents were prepared and used for conducting the anti-tyrosinase inhibition assay. The volumes given are only for testing a sub-set of samples but the same concentrations are used for making sufficient amounts for more samples.

Buffer preparation

Potassium phosphate buffer (pH 6.5) was made by mixing 250 ml of 50 mM potassium hydrogen phosphate (K_2HPO_4) with 250 ml of 50 mM potassium dihydrogen phosphate (K_2HPO4). Using the pH meter, (K_2HPO_4) was added (K_2HPO_4) while stirring the mixture with a magnetic stirrer until pH of 6.5 was obtained.

Preparing 250 ml of 50mM (K_2HPO_4) solution:

m = C x V x Mr

= (0.05 M) x (0.250 L) x (174.18 g/mol)

= 2.1773 grams (K_2HPO_4) in 250 ml distilled water

Preparing 250 ml of 50mM (K_2HPO_4) solution:

m = C x V x Mr

= (0.05 M) x (0.250 L) x (136.09 g/mol)

= 1.7011 grams (K_2HPO_4) in 250 ml distilled water

Substrate preparation

Approximately 2 mM of L-tyrosine was prepared in 10 ml of potassium buffer as follows:

m = C x V x Mr

= (0.002 M) x (0.010 L) x (181.19 g/mol)

= 0.0036 grams of L-tyrosine in 10 ml potassium phosphate buffer

Tyrosinase enzyme preparation

For each stock solution, 10ml of 3330units of tyrosinase was prepared. Each milligram (1mg) of tyrosinase powder contains 2130 units, therefore 1.5633 mg (3271.68 units) of tyrosinase powder was added to 9.824 ml of potassium phosphate buffer.

Samples preparation

The samples (2 mg of plant extracts and 2 mg of kojic acid) were dissolved in 100 µl of DMSO to make the stock solutions. 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 (A= 600 µg/ml) to the lowest (H= 437.5 µg/ml) after dilutions. The negative control was prepared by adding 30 µl of DMSO into 970 µl of the prepared potassium buffer.

8.2 NMR SPECTRUM OF *G. RADLKOFERI* LEAF EXTRACTS UNDER VARIOUS CONDITIONS

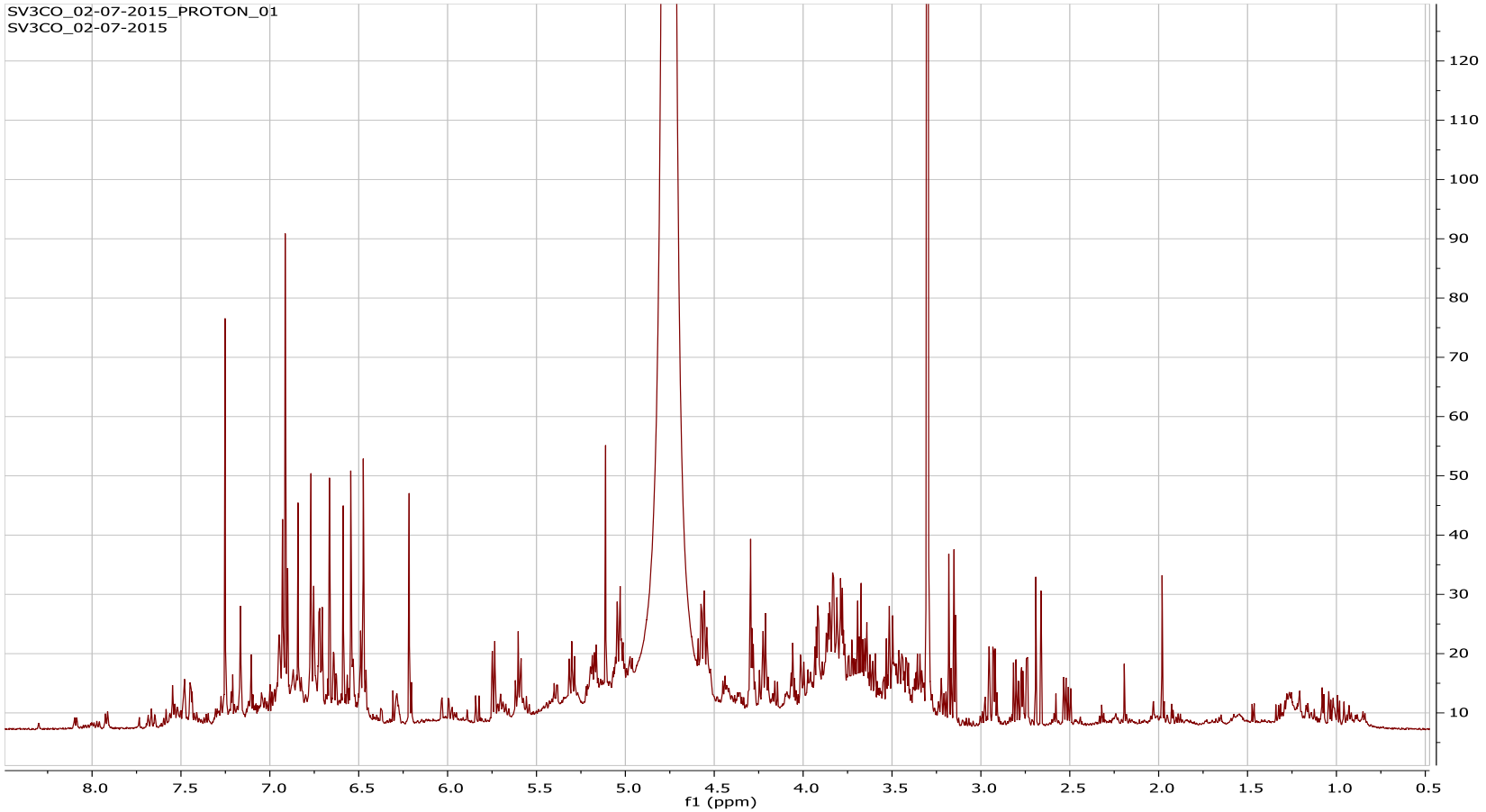

Figure 8.2.1 Representative ^{1}H-NMR spectrum of *G. radlkoferi* leaf extracts in spring season

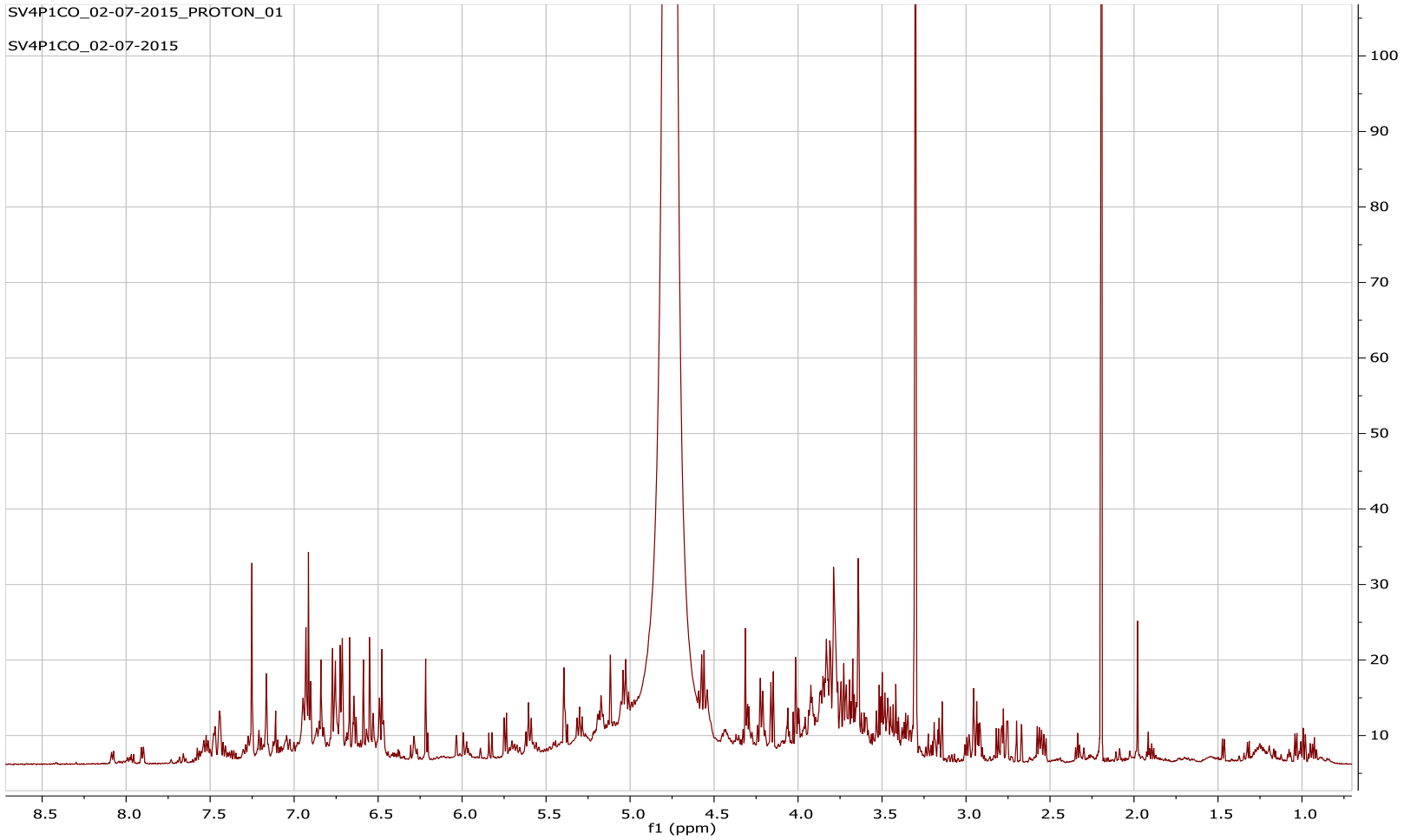

Figure 8.2.2 Representative ^{1}H-NMR spectrum of *G. radlkoferi* leaf extracts in winter season

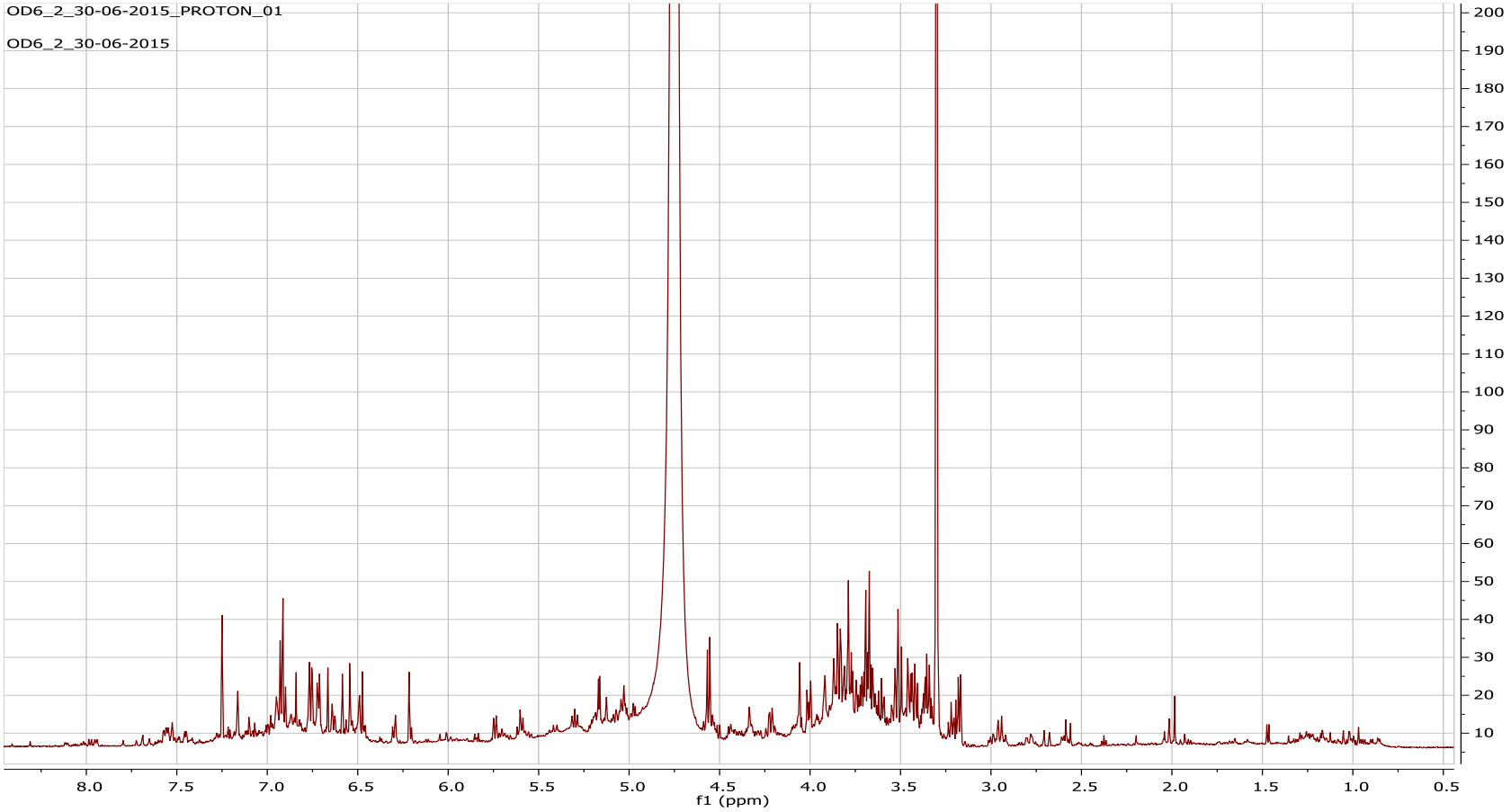

Figure 8.2.3 Representative ^{1}H-NMR spectrum of oven-dried *G. radlkoferi* leaves

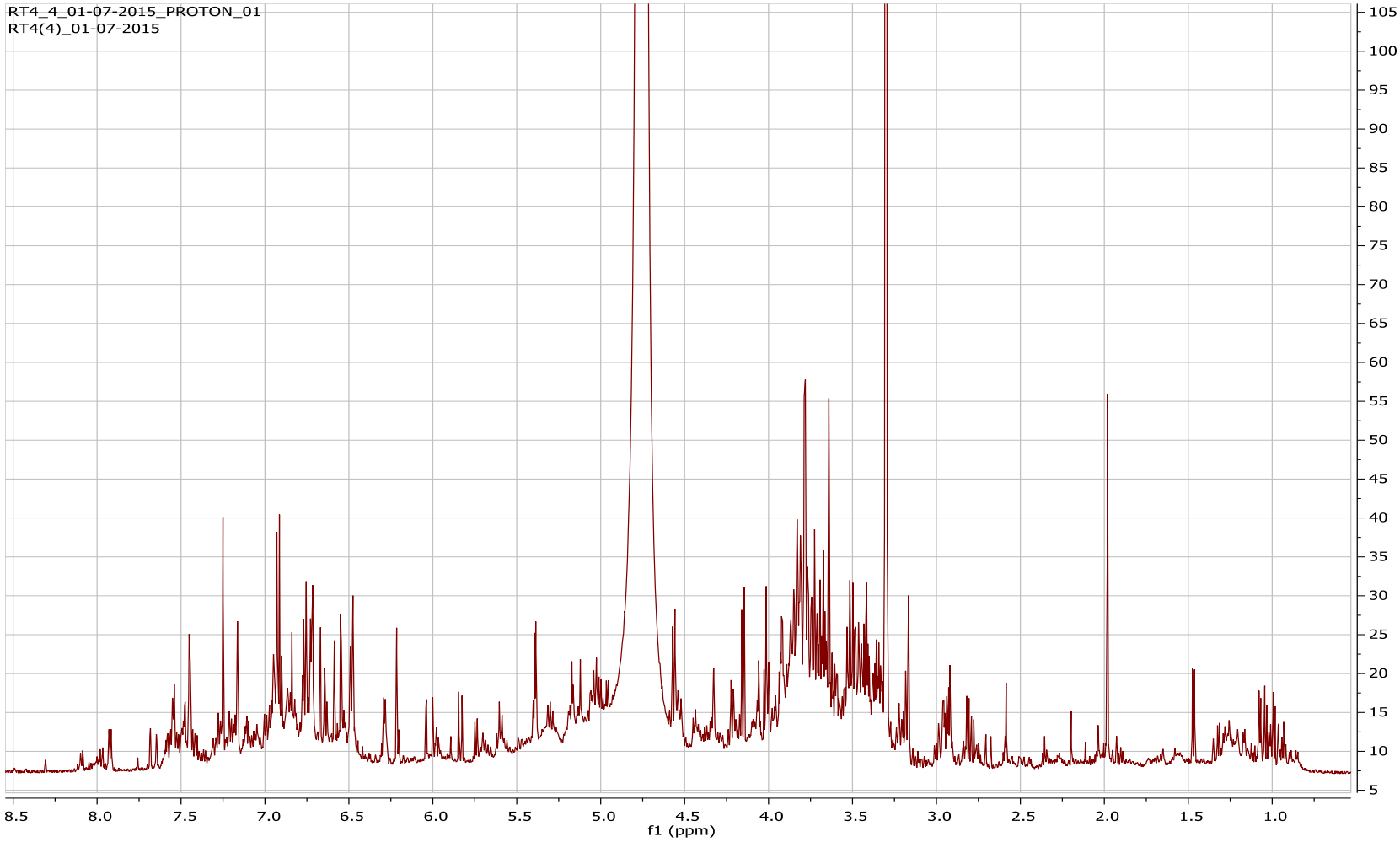

Figure 8.2.4 Representative ^{1}H-NMR spectrum of air-dried *G. radlkoferi* leaves

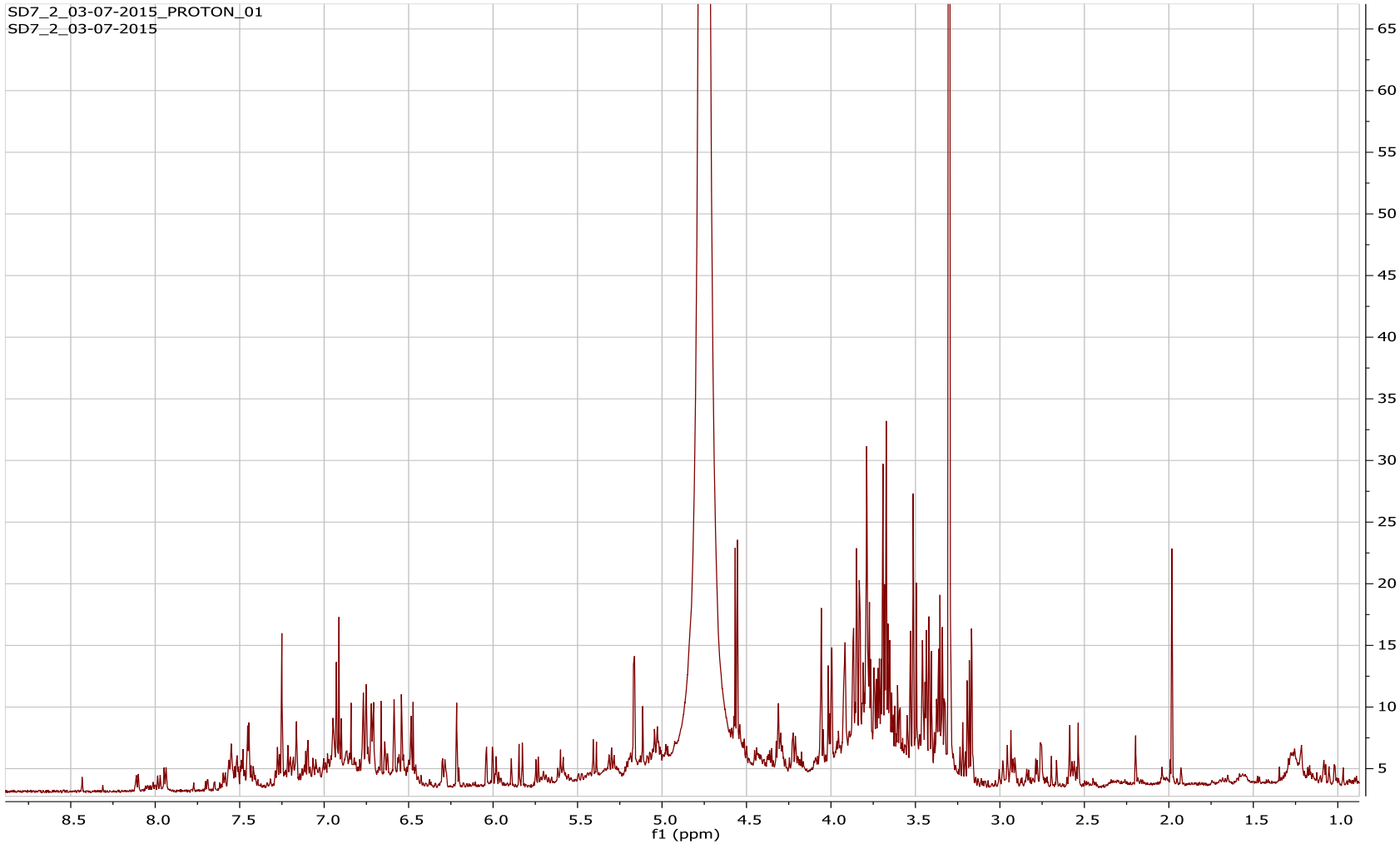

Figure 8.2.5 Representative ^{1}H-NMR spectrum of sun-dried *G. radlkoferi* leaves

8.3 ANALYSIS OF *G. RADLKOFERI* LC-MS DATA ON MAGMa

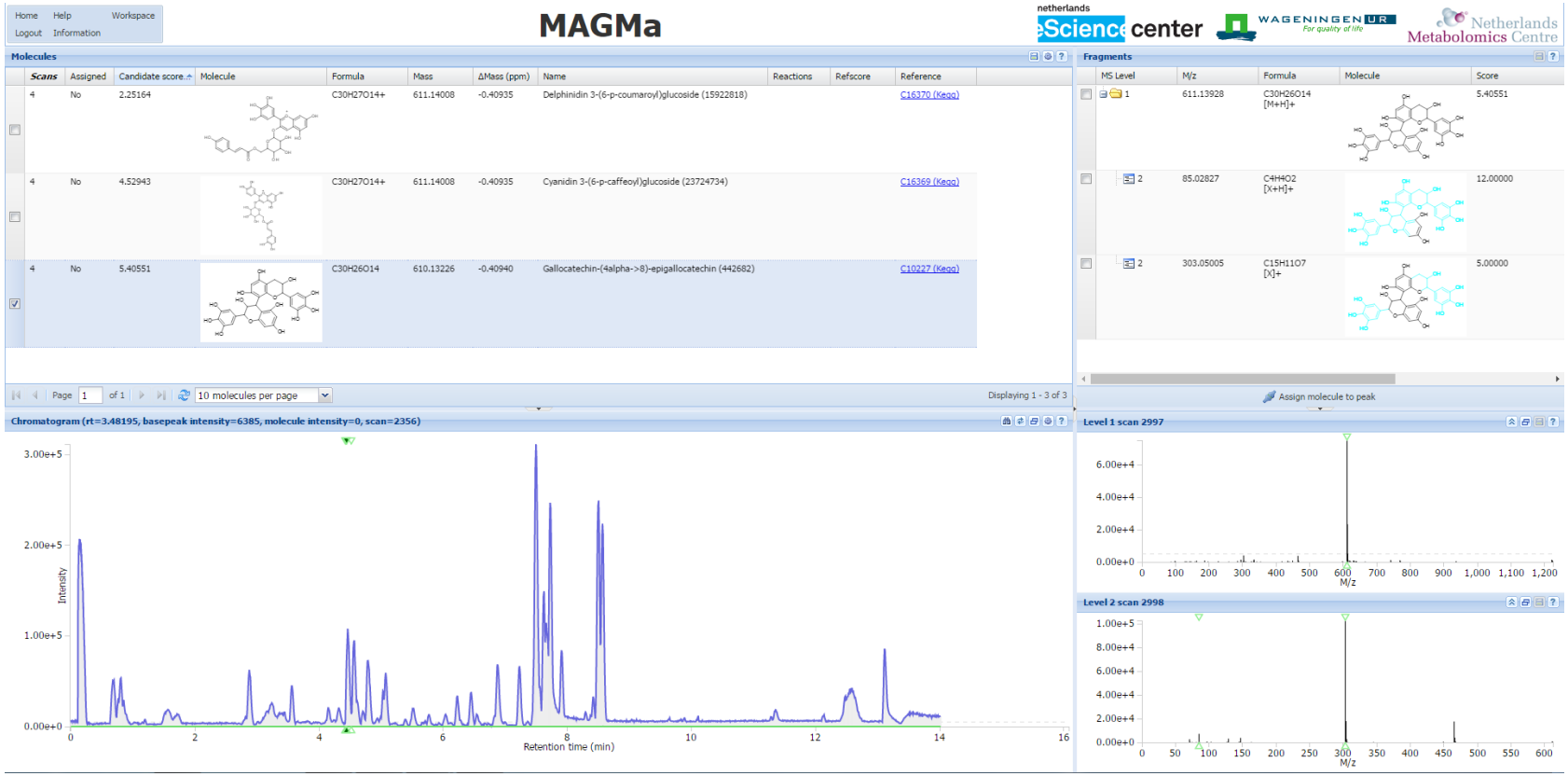

Figure 8.3.1: Magma showing some of the possible compounds present in *G. radlkoferi* leaf extracts (left)and the spectral trees (far right) responsible for the identification of gallocatechin-(4alpha->8)-epigallocatechin

8.4 STRUCTURE OF GALLOCATECHIN-(4ALPHA->8)EPIGALLOCATECHIN

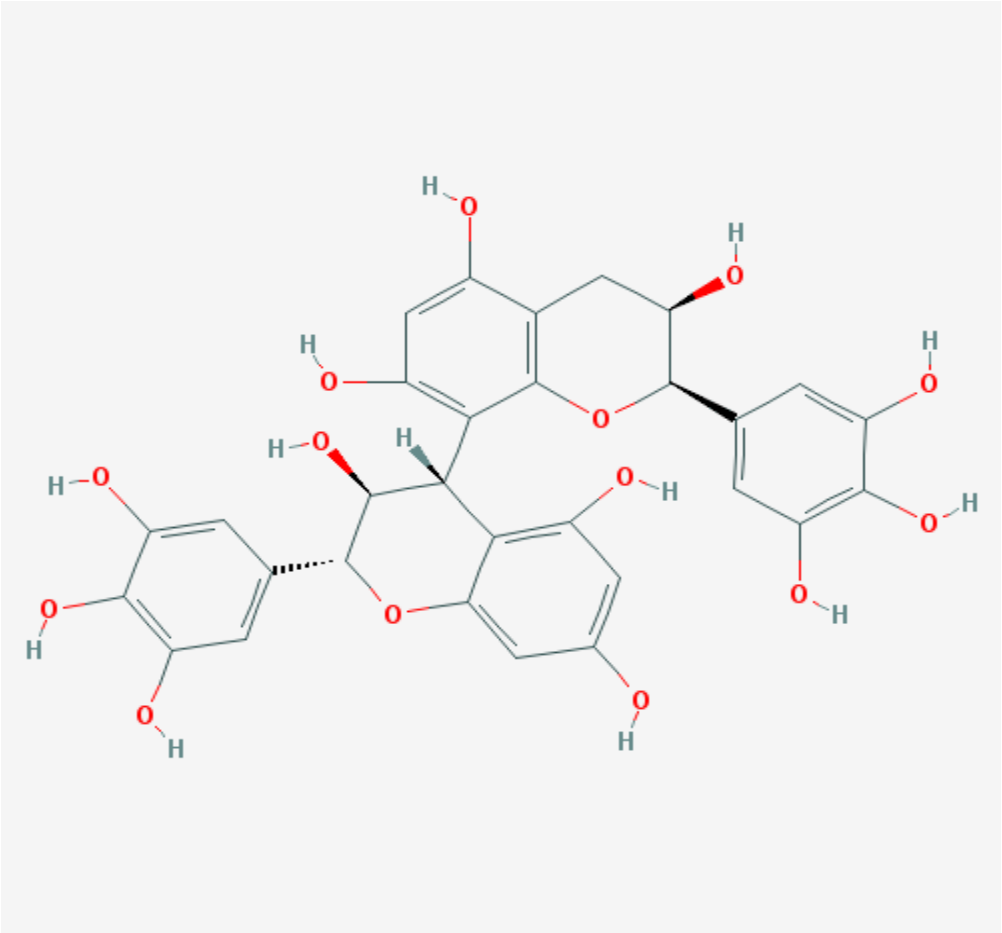

Figure 8.4.1: 2D structure of gallocatechin-(4alpha->8)-epigallocatechin (from PubChem)