Metabolomic analysis of *Sclerocarya birrea* (A. Rich) Hochst: to determine the differences in chemical profile and anti-diabetic properties in relation to geographical distribution

By

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DECLARATION

I, Cynthia Kwena Marokane declare that the dissertation entitled “Metabolomic analysis of Sclerocarya birrea to determine the differences in chemical profile and anti-diabetic properties in relation to geographical distribution” is the results of my own research except as cited in the references, the dissertation has not been previously submitted at this or any other University.

Signature--------------------------------- Date---------------------------------
ABSTRACT

Metabolomics is a discipline where metabolites are assessed, identified and quantified in different samples. Metabolites are crucial components of the biological system and highly informative about its functional state due to the closeness to functional endpoints and to the organism's phenotypes. $^1$H NMR and LC-MS, the commonly used metabolomics analytical platforms were used to annotate the metabolites found in Sclerocarya birrea (S. birrea) leaves from five South African provinces, Limpopo (L), Gauteng (G), North West (NW), Mpumalanga (M) and KwaZulu-Natal (KZN). Supervised Orthogonal Partial Least Square – Discriminant Analysis (OPLS-DA) of the full spectra revealed a clear differentiation of S. birrea leaves from five provinces. In addition, the level of common metabolites were measured and compounds previously found to have anti-diabetes potential ((-)epicatechin 3-O-galloyl ester, myricetin-3-O-α-L-rhamnopyranoside, gallic acid and Kaempferol-3-O-α-L-rhamnopyranoside) were annotated in the samples. The samples from the five provinces showed anti-diabetic activity when exposed to an in-vitro glucose uptake assay, with the highest activity observed in male samples from M. The sample presented high concentrations of (--)epicatechin 3-O-galloyl ester, one of the metabolites with anti-diabetes activity. Overall $^1$H NMR and LC-MS metabolic profiling were successfully applied to discriminate all five sources of S. birrea leaves, and obtained qualitative information of many common metabolites.
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DEDICATION

Commitment, effort and dedication were fundamental elements for the completion of my Masters dissertation, but even more was the support of my family. To my brothers and sisters, and the two greatest projects of my life: My mother, Francinah Marokane, and my son, Ofentje Marokane. Today I dedicate them this important professional achievement because without their presence, support and understanding I would have not achieved my goal. I love you.
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Part of this work was presented at the 4th Prof. Humphrey memorial postgraduate student’s symposium, held at UNISA Science campus in 2013 and in 2015. For both the 2013 and 2015 presentations the award for best oral presentation and the one for best poster were obtained respectively.
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GLOSSARY OF TERMINOLOGIES

Antioxidant: Is a molecule that inhibits the oxidation of other molecules.

Data binning: A data pre-processing technique used to reduce the effects of minor observation errors.

Diabetes: A disease in which the body is unable to properly use and store glucose (a form of sugar). Glucose builds up in the bloodstream, causing an abnormal increase in blood glucose.

Diabetisane: Natural medicine for high blood sugar.

Glucogenic amino acid: An amino acid that can be converted into glucose through gluconeogenesis.

Glycosylation: The enzymatic process that attaches glycan to proteins, lipids or other organic molecules.

Hyperglycemic: A condition in which an excessive amount of glucose circulates in the blood plasma.

Hypoglycemic: A condition that involves abnormally diminished contents of the glucose in the blood.

In-vitro: Medical experiments that takes place in test tubes rather than in a living system

Insulin: A hormone produced in the pancreas by the islets of Langerhans, which regulates the amount of glucose in the blood.

Mass Spectrometry (MS): An analytical chemistry technique that helps identify the amount and type of chemicals present in a sample by measuring the mass-to-charge ratio and abundance of gas-phase ions.

Metabolites: Intermediates and products of metabolism.
Metabolism: The set of life sustaining chemical transformations within the cells of living organisms.

Metabolome: The complete set of small molecule metabolites found within a biological sample.

Metabolomics: The systematic study of the unique chemical fingerprints that specific cellular processes leave behind, the study of their small molecule profiles.

Metformin: A prescription drug used primarily in the treatment of type II diabetes. It is intended to help control the amount of sugar in the blood, and can be used on its own or combined with other medications.

Nuclear Magnetic Resonance (NMR): A research technique that exploits the magnetic properties of certain atomic nuclei.

Pharmacological: The branch of medicine and biology concerned with the study of drug action, where a drug can be broadly defined as any man-made, natural, or endogenous molecule which exerts a biochemical and/or physiological effect on the cell, tissue, organ, or organism.

Partial least squares discriminant analysis (PLS-DA): A partial least squares regression of a set Y of binary variables describing the categories of a categorical variable on a set X of predictor variables.

Principal component analysis (PCA): A statistical procedure that uses an orthogonal transformation to convert a set of observations of possibly correlated variables into a set of values of linearly uncorrelated variables called principal components.

Secondary metabolites: Organic compounds that are not directly involved in the normal growth, development, or reproduction of an organism.
Theoretical background and world problem statement

1.1 Introduction

Healing has always been a treasure for humankind no matter the race. Recently, there are bioprospecting studies initiated to determine medicinal properties particularly from plants. This has drawn many scientific studies to rely on traditional medicine accounts. *Sclerocarya birrea* is one of the African traditional trees with reported medicinal values (Mariod and Abdelwahab, 2012). The tree has been evaluated for antibacterial potential (Eloff, 2001), hypoglycemic potential (Ojewole, 2003), antiproliferative potential (Tonih and Ndip, 2013) and many other human aliments (Van Wyk et al., 2000). The use of *S. birrea* for the treatment of various diseases confirms the abundance of medicinal metabolites found in the tree. Moreover it was estimated that 25% of the commonly used medicines contain compounds isolated from plants (Kumar, 2008). Amongst all other uses of *S. birrea*, the tree is mostly used for its anti-diabetes potential.

Diabetes mellitus is a metabolic disorder characterized by a loss of glucose homeostasis with conflict of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action or both (WHO, 1999; Pietropopo, 2001; Defronzo, 2004). Without enough insulin, body tissues in particular, the liver, muscles and adipose tissues fail to take and utilize glucose from the blood circulation. This results in elevated blood glucose levels, a condition known as hyperglycemia. If blood glucose levels remains high over a long period of time, this can result in long term damage of organs such as kidneys, eyes, nerves, heart and blood vessels. Complications in some of these organs can lead to death (Hirch, 1995; Brownlee, 2001; Weiss and Sumpio, 2006).
An emerging field of metabolomics is aimed at uncovering sites of synthesis and accumulation of structurally diverse compounds by determining a metabolome for a plant which involved the profiling of the small molecules found throughout the plant and in response to a variety of growth conditions or treatments (medicinal plant metabolomics research). In the current study, NMR analysis combined with LC-MS were used for assessing the metabolite contents of methanol extracts of *S. birrea* leaves and addressed the differences in the samples from different geographical regions, this was performed in order to find out if the differences in the leaves from different regions affect the plant’s anti-diabetes activity.

1.2 Problem statement

*Sclerocarya birrea* is mostly studied for its anti-diabetic potential. Currently, Type II diabetes mellitus, the most common type of diabetes mellitus, is managed by a combination of diet, exercise, oral hypoglycemic drugs and sometimes insulin injections (Ojewole, 2003). However, synthetic oral hypoglycemic drugs, which are currently the main form of treatment for Type II diabetes mellitus have shown to have detrimental side effects and high secondary failure rates (Baily, 2000; Erasto et al., 2005; Dey et al., 2007).

In addition, these drugs are not affordable to the majority of people living in the rural communities of the developing countries such as South Africa because of their high cost (Baily, 2000). These limitations, of currently available anti-diabetic pharmacological agents have motivated researchers all over the world to investigate alternative anti-diabetic remedies. In particular, consideration was given to plants and herbs used by traditional healers and herbalists as anti-diabetic remedies with the hope of discovering new natural products that can be used or developed into safe, inexpensive and effective anti-diabetic remedies.

Numerous studies have established that the stem bark extract of *S. birrea* subspecies caffra possess anti-diabetic effects (Ojewole, 2003) but removing the stem bark can be detrimental to the plant. Furthermore, an in-vitro cell culture based assay (results unpublished) carried out recently at the Medical research council (MRC) in collaboration with the University of KwaZulu-Natal (UKZN), *S. birrea* leaf extract (a sustainable source) showed activity against Type II diabetes. In order to further study these properties in patients a significant quantity of *S. birrea* leaves will be required. Further, should these studies confirm the activity of *S.
**birrea** against Type II diabetes in patients, a sustainable source of *S. birrea* leaves will be required most likely from different regions of South Africa. For this reason, it is important to determine whether there are any differences in secondary metabolites in plants from different provinces. In this study the effect of geographical distribution of *S. birrea* was investigated and this was linked to the in-vitro anti-diabetes activity of the plant.

### 1.3 Aim, research question and objectives

#### 1.3.1 Aim

The Aim of the study was to investigate whether *S. birrea* from selected South African regions have identifiable differences in chemical composition which can affect the plant’s anti-diabetic activities.

#### 1.3.2 Research questions

- Does *S. birrea* from different regions differ in chemical composition and in medicinal properties?
- Are there any identifiable components responsible for differences between samples?
- How do the differences in chemical composition of the samples affect the in-vitro uptake of glucose by C2C12 cells.

#### 1.3.3 Objectives

- Perform NMR and MS analysis on the crude extract
- Conduct metabolomics analysis to identify the differences between the samples.
- Use metabolomics to construct the chemical profile of compounds.
- To compare the degree of glucose uptake by aqueous extracts of *S. birrea* from five different provinces.
1.4 Overview of the dissertation

Chapter 1 of this dissertation describes the general introduction of the study. This was then followed by the world problem statement, research questions and Aims. Chapter 2 begins with an overview on an introduction to metabolomics in medicinal plants and the analytical techniques used in metabolomics search. This was then followed by the description of *Sclerocarya birrea*, and its medicinal values. Chapters 3 and 4 we combined qualitative approach to investigate the differences in *Sclerocarya birrea* from different provinces and the metabolites responsible for the differences. Chapter 5 present the in-vitro glucose uptake of *Sclerocarya birrea* leaves from different provinces. The final chapters 6 and 7 deals with the general discussion and conclusion of the study.
1.5 LITERATURE CITED


2 Literature review

2.1 Introduction and aim of the literature review

Several analytical techniques have been developed in the characterization of plant materials. These techniques allow the detection of bio-activities from natural products, especially medicinal plants. Nuclear Magnetic Resonance (NMR), one of the techniques used in metabolomics, has the potential to provide evidence for the existence of clear Metabolomic differences among samples according to their places of origin. There has been a wide interest in the medicinal uses of S. birrea for centuries; this was advantaged by its potential to treat diseases (Street and Prinsloo, 2012). The tree has already demonstrated to present hypoglycemic effect and can therefore be used in the management of Type II diabetes mellitus (Ojewole, 2003). The aim of this literature review is to establish the current status of knowledge regarding different metabolites found in S. birrea and how they influence the plant’s medicinal values, in particular, consideration given to the treatment and management of Type II diabetes mellitus.

2.2 Introductions to metabolomics in medicinal plant

Natural products from plants serve as rich resources for drug development. Plant derived natural products have had a profound and a lasting impact on human health and includes compounds successfully used for decades such as digitalis, vincristine, taxol and morphine isolated from foxglove, periwinkle, yew, and opium poppy, respectively (Gurib-Fakim, 2006). The structural diversity and biological activity of plant-derived compounds suggest that additional, medicinally relevant compounds remain to be discovered in plants (Hur et al., 2013)
Providing the metabolome for medicinally relevant plant species is one means to reveal a deeper understanding of the metabolic potential of plants, and thus provide the opportunity to uncover sites of synthesis and accumulation of structurally diverse compounds (Wurtele et al., 2012). Determining the metabolome for a plant involves the profiling of small molecules found throughout the plant in all the tissues and organs, and in response to a variety of growth conditions and treatments (Hur et al., 2013). No single analytical method is sufficient to observe all the chemical diversity within the plant. In metabolomics studies, metabolic profiles are surveyed doing comprehensive measurements using mass spectrometry (MS) or Nuclear Magnetic Resonance (NMR) spectroscopy (Jantan, 2004). Studying a whole metabolic profile instead of only a few molecules provides a more comprehensive view of the complexity of the underlying biochemical processes (Van Der Greef et al., 2011). Metabolomics studies have the potential to provide in-depth answers to biological questions.

2.2.1 Analytical technologies commonly used in metabolomics research

Mass Spectrometry (MS)
MS has been extensively developed in the past few decades and holds a distinguished position in qualification and separation science (Dettmer et al., 2007). Recent advances in MS based metabolomics have created the potential to measure the levels of hundreds of metabolites that are the end products of cellular regulatory processes (Griffin et al., 2010). Due to its high sensitivity and wide range of covered metabolites, MS has become the technique of choice in many metabolomics studies (Griffin et al., 2010). The aims of developing MS for metabolomics range from understanding the structural characterization of important metabolites to biomarker discovery (Zhang, 2012). MS can be used to analyze biological samples either by direct-injection or following chromatographic separation (Griffin et al., 2010). Recent developments and improvements in mass accuracy have dramatically expanded the range of metabolites that can be analyzed by MS and have improved the accuracy of compound identification (Domon 2010).
Nuclear Magnetic Resonance spectroscopy (NMR)

In the past decade, metabolomics has been developed as an important field of plant sciences and natural products chemistry (Krishnan et al., 2004). The ultimate goal of metabolomics is to measure all the metabolites in an organism both qualitatively and quantitatively, which can provide a clear metabolic picture of a living organism under certain conditions, this is a very ambitious goal, as the plant metabolome is very complex (Kim et al., 2010). This makes the analysis of all metabolites in one single experiment extremely difficult. In view of this, instead of aiming to analyze all individual metabolites quantitatively and qualitatively, a more realistic and suitable approach will be to have a general view of all the metabolites present in an organism under certain conditions. NMR is a very suitable method to carry out such an analysis because it allows the simultaneous detection of diverse groups of secondary metabolites (flavonoids, alkaloids, terpenoids) besides abundant primary metabolites (sugars, organic acids and amino acids) (Doughari, 2012). NMR is a very useful technique for structure elucidation. Using various two-dimensional NMR measurements, many signals can be identified often without the need for further fractionation of the extract (Krishnan et al., 2005).

2.2.2 Experimental Procedures for NMR in metabolomics

Metabolomics is focused on the complete analysis of all metabolites of a certain sample. A major problem resides in the challenge to obtain a sample which permits the complete analysis of all metabolites contained in it, which is virtually impossible. If this sample is treated in some way, by extraction, separation or derivatisation, information will be lost (Kim et al., 2010). In an extraction only the soluble components are obtained and insoluble or partly soluble compounds are lost. When a separation is performed, certain parts of the sample are also lost.
2.3 Sample preparation for metabolomics research

In metabolomic studies, it is important that the reproducibility of the procedure is as best as possible (Defernez and Colquhoun, 2003). All sources of variation should be minimized. When leaves from a plant are collected, the position of the leaf, its age, exposure to sunlight and rain as well as the time of collection and the weather should be considered, all these factors potentially causes variation (Maher et al., 2007). After collection the sample treatment is important such as storage (room temperature or frozen) processing of the sample, artifacts might arise from interaction with solvents or by residual enzymatic activity (Kim et al., 2010). To analyze the metabolome as completely as reasonably possible, a single solvent will not be sufficient (Verpoorte et al., 2007). A reasonable coverage of all metabolites can be obtained with two solvents: one apolar extract (e.g. with chloroform) and a polar extract (e.g. with water or water–methanol) (Verpoorte et al., 2007). A problem with water extracts is often the high quantity of sugars (Schripsema, 2010). Lipids are generally the major components of the extract, when there are problems with superimposed signals, better results might be obtained by the use of two-dimensional NMR, e.g. J-resolved NMR (Viant, 2003; Liang et al., 2006; Widarto et al., 2006), TOCSY or HMBC (Widarto et al., 2006), or further fractionation of the samples should be undertaken.
Protein depletion of samples is generally not considered to be essential, but it should be considered that in water extracts, the catalytically active proteins might interfere in the spectrum (Schripsema, 2010). For example, sucrose in papaya extracts is rapidly converted by invertase activity into glucose and fructose (Schripsema et al., 2009). For serum samples a number of deproteinisation procedures were investigated by Daykin et al. (2002) and later by Tiziani et al. (2008). They found that the best results were achieved by ultrafiltration, which removes proteins quantitatively, yields good signal-to-noise, and is superior in reproducibility (Kim et al., 2008). If in the sample preparation procedure the sample is dried and re-dissolved in a deuterated solvent, volatile components should be completely or partially lost, e.g. ethanol (Tiziani et al., 2008) or salicylic acid (Kim et al., 2008). For extraction of biological material a suitable solvent mixture from the many available options should be selected. For this selection a number of parameters are considered (Kim et al., 2008):

1. The solvent strength of solvents as indicated in Table 2.1 gives a reasonable impression of the type of compounds which might be extracted. However, acid/base behavior should also be considered (Kim et al., 2008).

2. Boiling point is important if solvents need to be evaporated, which might lead to thermal decomposition or loss of compounds by evaporation (Kim et al., 2008). Water as a solvent, despite the relatively high boiling point, has the advantage that it can easily be removed by lyophilisation (Kim et al., 2008).

3. Toxicity and environmental considerations—for example, benzene should be avoided due to its carcinogenicity and can in extractions easily be substituted with toluene (Kim et al., 2008).

4. Possible contaminations in solvents that may interfere with the analysis, yielding unwanted signals in the spectrum, (Kim et al., 2008).

5. Possible contaminations that may cause artifact formation, e.g. peroxides (in ethers) and dichlorocarbene (in chloroform) (Kim et al., 2008)
Table 2.1: A series of solvents listed according to their eluting power on alumina adsorbent. The empirical solvent strength data were reported by Snyder (1968).

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Solvent strength</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>&gt;&gt;1</td>
</tr>
<tr>
<td>Methanol</td>
<td>0.95</td>
</tr>
<tr>
<td>Ethanol</td>
<td>0.88</td>
</tr>
<tr>
<td>2-Propano</td>
<td>0.82</td>
</tr>
<tr>
<td>Dimethyl sulfoxide</td>
<td>0.75</td>
</tr>
<tr>
<td>Pyridine</td>
<td>0.71</td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>0.65</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>0.58</td>
</tr>
<tr>
<td>Tetrahydrofuran</td>
<td>0.57</td>
</tr>
<tr>
<td>Acetone</td>
<td>0.56</td>
</tr>
<tr>
<td>Dichloromethane</td>
<td>0.42</td>
</tr>
<tr>
<td>Chloroform</td>
<td>0.40</td>
</tr>
<tr>
<td>Diethyl ether</td>
<td>0.38</td>
</tr>
</tbody>
</table>

2.4 NMR measurement

After preparation of the samples they should be submitted to NMR. Liquid samples in non-deuterated solvents can be submitted to NMR, but in that case a little deuterated solvent should be added to provide the lock signal for NMR and during the measurement, and solvent suppression should be applied (Ottiger and Bax, 1999). When the samples are dry they should be dissolved in deuterated solvents. The commonly available deuterated solvents are D$_2$O, methanol-D$_4$, DMSO-D$_6$, acetone-D$_6$, chloroform-D and benzene-D$_6$ (Hatada and Kitayama, 2004). The choice of solvent is determined by the nature of the sample and its preparation, for polar extracts usually D$_2$O, methanol-D$_4$ or a mixture of the two is used, with chloroform used for apolar extracts (Schripsema, 2010). Special care should be taken with the apparatus adjustments, such as shimming and temperature. Moreover, during processing care should be taken with the phasing, line broadening and zero filling (Defernez and Colquhoun, 2003).
2.5 Data processing

After the NMR spectra have been obtained, they should be processed to extract the data (Johnson and Blevins, 1994). Three methods to extract the data are: binning, peak-picking or deconvolution (Scalbert et al., 2009). One of these techniques is necessary, because the crude NMR data show artefacts due to physicochemical differences (Torgrip et al., 2008). Forshed et al. (2005) investigated two dedicated peak alignment methods and found that both produced better results than the bucketing approach. The best approach, however, is the deconvolution of the NMR spectra into the individual compound spectra, this approach has been described by Weljie et al. (2006) and the name ‘targeted profiling’ was proposed.
2.6 *Sclerocarya birrea*

2.6.1 Classification and description

*Sclerocarya birrea*, (A. Rich) Hochst, subspecies *caffra* (sond) kokwaro popularly known as the “cedar tree” or marula (English), maroela (Afrikaans), umgamu (Zulu) belongs to the plant family Anarcardiaceae, which encompasses 73 genera and about 600 plant species (Pretorius et al., 1985; van Wyk et al., 1997). *Sclerocarya birrea* is a medium sized, single stemmed, terrestrial, erect, perennial deciduous tree of about 10-15m in height (van Wyk et al., 1997). The stem bark of marula tree is flaky, with a grey mottled appearance due to contrasting gray and pale-brown pattern. The leaves are compound, about 10 cm long, with a varying number of pairs of ovate leaflets, but always one terminal leaflet. Only the female trees bear fruit while the male trees displays flowers (Figure 2.1A). The flowers are borne on small oblong clusters and are small with red sepals and yellow petals (Ojewole, 2003). Female *S. birrea* trees bear plum-like stony fruit (figure 2.1B) of about 30mm in diameter. The fruits are pale yellow when ripe (Figure 2.6C) (from late South African summer to mid-winter), fleshy and contain one kernel with 2 to 4 seeds (van Wyk, et al., 2000; Ojewole, 2003).

Figure 2.1: Structure of the male and female *S. birrea* flowers: left = male and right= female; (b) Structure of a *S. birrea* unripe fruits; (c) Structure of a *S. birrea* ripe fruit, (Van wyk et al., 2000).
2.6.2 Distribution and habitat

*Sclerocarya birrea* is widespread in Africa, from Ethiopia in the North to KwaZulu-Natal (South Africa) in the south, from Gambia in the west across to Nigeria and Cameroon in Central Africa, and to Kenya and Sudan in the East (van Wyk et al., 2000; Belemtougri et al., 2007). Its distribution in Africa is believed to have followed the Bantu people in their migrations, as it has been an important item in their diet since time immemorial (Van wyk et al., 2000). *Sclerocarya birrea* grows naturally in various types of woodlands on sandy soil or occasionally on sandy loam soils (van Wyk et al., 2000).

2.6.3 Phytochemical studies of *Sclerocarya birrea*

The ripe fruit of *S. birrea* is reported to have a high content of vitamin C (168 mg/100g), which is approximately three times higher than that of oranges and comparable to the amount found in guava (Wilson, 1980; Eromosele, 1991). According to Ogbobie, (1992) and Smith et al., (1996), the nuts found in the fruit of *S. birrea* contain crude oil (11.0%), carbohydrate (17.2%), crude protein (36.7%), fibre (3.4%) and crude saponinis (0.9%), minerals as well ascorbic acid. The gum from the tree is rich in tannin, and sometimes used in making an ink substitute (Venter and Venter, 1996).

Furthermore, Watt and Breyer-Brandwijk, (1962) and Dimo et al., (2007) found that both the stem bark and the leaf extracts of *S. birrea* contain alkaloids, flavonoids, tannins and saponosides. A condensed tannin, (-)-epicatechin-3-galloyl ester extracted from the stem bark of *S. birrea* has been shown to have insulin secretagogue activity (Galvez et al., 1992). In addition, Ojewole (2003) has discussed the possibility that arginine, glutamic acid, coumarins and/or terpenoids which are contained in the aqueous stem bark extract of *S. birrea* could be responsible for the hypoglycemic effect.
2.6.4 Pharmacological uses of Sclerocarya birrea

In South Africa and some African countries the stem-bark, roots and leaves of S. birrea are traditionally used in folk medicines for the treatment, management and control of a variety of human ailments, including malaria, diarrhea, hypertension and diabetes (Van wyk et al., 2000). The folk use of S. birrea for medicinal purposes have been supported by studies which have shown that stem-bark and leaf extract of S. birrea possess a number of pharmacological activities, including analgesic, anti-inflammatory, anti-diabetic and hypoglycemic (Ojewole, 2004; Dimo et al., 2007), antidiarrheal (Galvez et al., 1991; 1993) and antibacterial activity (Eloff, 2001).

2.6.5 Hypoglycemic and anti-diabetic research of Sclerocarya birrea

Type II diabetes affect many metabolic pathways in different tissues, many of which potential targets for drug treatment (Van de Venter et al., 2013). Unfortunately, this complicates the identification of new treatments, as most in-vitro screening models consider single cell type, metabolic pathway or enzyme, thus reducing the possibility to identify an anti-diabetic compound (Van de Venter et al., 2013). Type II diabetes is characterized by hepatic and peripheral (muscle, adipose tissue) insulin resistance (Van de Venter et al., 2013). The pancreas compensate by secreting more insulin (Figure 2.2), but eventually the beta cells will fail to sustain this (Cerasi, 2000), at which stage the patient requires insulin treatment (Van de Venter et al., 2013). During the time when insulin is still produced, various other drugs in combination with alteration in life style can be used to manage the disease (Matthaei et al., 2000). These drugs work through a number of different ways to reduce blood glucose level (Cheng and Funtus, 2005) (Figure 2.3). In this study S. birrea was selected based on literature reports for its anti-diabetic activity.
Figure 2.2: The role of the pancreas in glucose homeostasis (Cheng and Fantus, 2005).
Figure 2.3: Major target organs and actions of orally administered anti-hyperglycemic agents in Type II diabetes mellitus. TZD=thiazolidinedione; FFA= free fatty acid; AGI= glucosidase inhibitor (Cheng and Funtus, 2005).

The blood lowering effect of *S. birrea* stem-bark extract has been studied and confirmed using experimental animal models of diabetes. Recent studies have shown that administration of 300 mg/kg stem-bark methanol/methylene chloride extract of *S. birrea* exhibited at termination, a significant reduction in blood glucose, increased plasma insulin levels, and reduced plasma cholesterol, triglyceride and urea levels (Dimo et al., 2007).

2.7 Secondary herbal metabolites with reported medicinal properties

The medicinal and pharmacological actions of medicinal herbs are often dependent on the presence of bioactive compounds called secondary herbal metabolites (Bruneton, 1999; Henrichet al., 2004). Unlike the ever-present macromolecules of primary metabolism (e.g. monosaccharides, polysaccharides, amino acids, proteins, nucleic acids and lipids) which are present in all plants, secondary metabolites with medicinal properties are found only in a few species of plants (Henrich et al., 2004). Some of these secondary metabolites serve as defensive compounds against herbivores and pathogens. Others function in mechanical support, in attracting pollinators and fruit dispersers, in absorbing harmful ultraviolet radiation, or reducing the growth of nearby competing plants (Cheynier, 2005; Gurib-Fakim, 2005). Secondary herbal metabolites with reported medicinal properties in particular anti-diabetes properties include, alkaloids, flavonoids, tannins and saponosides (Dimo et al., 2007). These metabolites are briefly discussed below.
2.7.1 Alkaloids

Alkaloids often contain one or more rings of carbon atoms, usually with a nitrogen atom in the ring. Many have declared pharmacological activity (Harborne, 1998). Most alkaloids have a strong bitter taste and are very toxic, for these reasons they are used by plants to protect themselves against herbivory, and attacks by microbial pathogens and invertebrate pests (Harborne, 1998). Several alkaloid containing medicinal herbs are reported to have been used by the early man as pain relievers, as recreational stimulants or in religious ceremonies to enter a psychological state to achieve communication with ancestors or God (Heinrich et al., 2004; Gurib-Fakin, 2005). Alkaloids are classified into several groups either on the basis of their basic ring system (e.g. atropine, indole, quinoline, isoquinoline, imidazole, piperidine alkaloids), plant sources (e.g. opium, belladonna, vinca, cinchona and ergot alkaloids) or their pharmacological properties (e.g. analgesic, stimulant or anti-malarial alkaloids) (Kinghorn and Balandrin, 1993; Bruneton, 1999, Harborne, 1998; Henrich et al., 2004).

2.7.2 Phenolics

Phenolics is a class of herbal secondary metabolites that are characterized by the presence of one or more hydroxyl (-OH) groups attached to a benzene ring or to other complex aromatic ring structures (Bruneton, 1999, Harborne, 1998 and Henrich et al., 2004). Phenolic herb secondary metabolites are widely distributed in herbs and are responsible for color development, pollination and protection against UV radiation and pathogens (Bruneton, 1999; Henrich et al., 2004). They also contribute to the color and astringency of some foods. On the basis of their structure, phenolic compounds can be classified into two broad classes: the non-flavonoids and the flavonoid phenolic compounds (Bruneton, 1999; Henrich et al., 2004).

2.7.2.1 Non-flavonoid phenolic compounds

Non-flavonoid phenolic compounds include simple phenols (eugenol, catechol, hydroquinone, phloroglucinol hydroquinone, and p-anisaldehyde) (Jadhav et al., 2004), the C6-C1 benzoic acids (vanillic acid, gallic acid and protocatechuic acid), the C6-C3 phenyl propanoids and their derivatives (cinnamic acid, caffeic acid, ferulic acid myristicin and sinapyl alcohol), coumarins
(scopoletin; warfarin and dicoumarol), hydrozable tannis (gallotannins and ellagitannins) and lignans and related compounds (Kumar et al., 2010).

2.7.2.2 Flavonoid phenolic compounds

Flavonoids are a large and complex group of compounds containing a three ring structure with two aromatic centers and a central oxygenated heterocyclic ring (Bohm, 1998; Hollman and Katan, 1999). The six major classes of flavonoids are flavones, flavonols, flavonones, catechins (flavanols) anthocyanidins and isoflavones (Bohm, 1998; Bruneton, 1999; Pieta, 2000; Scalbert et al., 2005; Goutam and Dilip, 2006). Flavonoids have several proven medicinal properties, such as anti-inflammatory, anti-oxidant, anti-cancer, antibacterial and antiviral properties (Hollman and Katan, 1999; Harborne and Williams, 2001; Cheynier, 2005; Manach et al., 2004).

2.7.3 Glycosides

Glycosides are secondary metabolites made up of two components, a carbohydrate component known as the glycone and a non-carbohydrate component known as the aglycone. The glycone component usually consists of one or more glucose units whereas the aglycone may be any one of the secondary metabolites discussed above (Bruneton, 1999; Heirich et al., 2004; Gurib-Fakim, 2005). Aglycones tend to be soluble in organic solvents and sugar part in aqueous solvents. In general, glycosides can be extracted with acetone, ethanol or an aqueous/ethanol mixture (Jones and Kinghorn, 2005). Medicinally important glycosides consist of anthraquinone glycosides, coumarin glycosides and steroidal (cardiac) glycosides.
2.7.3.1 Anthraquinone glycoside

Herbs such as Cassia senna, rhubarb (Rheum palmentum), cascara (Rhamnus purshiana) and Aloe vera have long been known for their laxative properties (Bruneton, 1999; Heinrich et al., 2004; Gurib-Fakim, 2005). This property has been attributed to the presence of anthraquinone and enthrones glycosides present in these plants (Heinrich et al., 2004). When ingested anthraquinone glycosides hydrolyze in the large intestine (colon) to liberate the aglycones which stimulate peristalsis and increase water retention in the colon (Bruneton, 1999).

2.7.3.2 Coumarin glycosides

Coumarins glycosides are phytoalexins, and are synthesized by the plant in response to bacterial or fungal infection, physical damage, chemical injury, or a pathogenic process (Gurib-Fakim, 2005). For example, scopoletin is synthesized by the potato (Solanum tuberosum) following fungal infection. Coumarin glycosides are very fragrant. They are the source, for instance, of freshly-mown hay scents (Heinrich et al., 2004). Medicinally, coumarin glycosides have been shown to have hemorrhagic, anti-fungicidal, and antitumor activities (Bruneton, 1999).

2.7.3.3 Steroidal glycosides

Steroidal (cardiac) glycosides are naturally occurring drugs whose actions include both beneficial and toxic effects (at higher doses) on the heart (Bruneton, 1999; Gurib-Fakim, 2005; Heinrich et al., 2004). Herbs containing cardiac glycosides include Digitalis purpurea (foxglove) and Strophanthus. Foxglove is the source of two potent glycosides used as heart stimulants; digoxin and digitoxin. Both digoxin and digitoxin are widely used in the modern treatment of congestive heart failure, atrial fibrillation and flutter (Heinrich et al., 2004).

2.8 Selection of herbal species

Any plant species and herb part collected randomly can be investigated using available phytochemical methods. However, a more targeted approach is often preferred to a random
selection (Kinghorn and Balandrin, 1993; Harborne, 1998). Extracts prepared from herbs used as traditional remedies to treat certain diseases are more likely to contain biologically active compounds of medicinal interest (Yuan and Lin, 2000). Literature early in the selection process can provide some preliminary information on the type of natural products already isolated from the plant and the extraction methods employed to isolate those (Heinrich et al., 2004). Another approach known as the information driven approach, utilizes a combination of ethnobotanical, chemotaxonomic and random approaches together with a data base that contains all relevant information concerning a particular plant species (Kinghorn and Balandrin, 1993; Harborne, 1998). The database is used to prioritize which herbs should be extracted and screened for biological activity.

2.8.1 Collection and identification of plant material

The whole plant or a particular part can be collected depending on where the metabolites of interest (if they are known) accumulate. Hence aerial (e.g. leaves stems, flowering tops, fruit, seed, and bark) and underground (e.g. tubers, bulbs, roots) parts can be collected separately. Collection of herb materials can be influenced by factors such as the age of the plant and environmental conditions (e.g. temperature, rainfall, amount of daylight, soil characteristics and altitude) (Williams et al., 1996; Harborne, 1998). Thus, it is important to take this into consideration for the re-collection purpose, in order to ensure a reproducible profile (nature and amount) of metabolites (Satyajit et al., 2006). The plant from which the material is collected must also be identified correctly. A plant taxonomist or a botanist should be involved in the detailed authentication of the plant (i.e. classification into its class, order, family, genus and species) (Satyajit et al., 2006). Any feature related to the collection, such as the name of the plant, the identity of the parts collected, the place and date of collection, should be recorded as part of the voucher deposited in a herbarium for future reference (Harborne, 1998; Satyajit et al., 2006).

2.8.2 Extraction of plant materials

Herbal materials are commonly extracted by means of liquid solvents in what is known as the “solid-liquid solvent extraction” (Starmans and Nijhuis, 1996). Typical solid-liquid solvent extraction processes for herbal materials involve drying and grinding of the herbal material, choosing a suitable extraction solvent and extraction procedure (Starmans and Nijhuis, 1996; Cheng et al.,
2001; Jones and Kinghorn, 2005).

2.8.3 Drying and grinding the plant material

Once the herbal material has been collected, it needs to be dried as soon as possible. A common practice is to leave the sample to dry on trays at ambient temperature and in a room with adequate ventilation (Heinrich et al., 2004; Satyajit et al., 2006). Dry conditions are essential to prevent microbial fermentation and subsequent degradation of metabolites. Herbal materials should be sliced into small pieces and distributed evenly to facilitate homogeneous drying. Protection from direct sunlight is advised to minimize chemical reactions induced by ultraviolet rays (Satyajit et al., 2006). To facilitate the drying process, the material can be dried in an oven. This can also minimize reactions (e.g. hydrolysis of glycosides) that can occur as long as there is some residual moisture present in the herbal material. The dried material should be stored in sealed containers in a dry and cool place. Storage for prolonged periods should be avoided as some constituents may be decomposed (Heinrich et al., 2004; Jones and Kinghorn, 2005). After drying, herbal materials are commonly grounded into a fine powder (Figure 2.4). Grinding of plant materials into smaller particles facilitates subsequent extraction procedures by rendering the sample more homogeneous, increasing the surface area, and facilitating the penetration of solvents into cells (Harborne, 1998; Satyajit et al., 2006). Mechanical grinders (e.g. hammer and cutting mills) are employed to shred the herbal material into various particle sizes.
2.8.4 Choice of a suitable extraction solvent

The choice of the extraction solvent depends mainly on the polarity and hence the solubility of the bioactive compounds of interest. Although water is usually applied as a solvent in many traditional protocols, organic solvents of varying polarities are often used (either alone or in different combinations) in modern methods of extraction to exploit the solubility of herbal ingredients (Lapornik et al., 2005). The polarity and chemical profiles of most of the common extraction solvents have been determined (Ayaffor et al., 1994; Eloff, 2001; Cowan, 1995) and are summarized in table 2.2.
Table 2.2: Polarity and chemical profiles of most of the common extraction solvents

<table>
<thead>
<tr>
<th>Polarity</th>
<th>Solvent</th>
<th>Extracted chemical profile</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>Chloroform</td>
<td>Fatty acids, waxes and terpenoids</td>
<td>Perett et al., (1995)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cowan, (1999)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Bruneton, (1999)</td>
</tr>
<tr>
<td>Medium</td>
<td>Acetone</td>
<td>Less polar and polar flavonoids, tannins, terpenoids, and glycosides</td>
<td>Eloff, (1998)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Bruneton, (1999)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Scalbert et al., 2005</td>
</tr>
<tr>
<td>High</td>
<td>Methanol</td>
<td>Carbohydrates, lecitin, amino acids, polypeptides, phenolic acids, phenylpropanoids, polar flavonoids, glycosides and alkaloids</td>
<td>Bruneton, (1999)</td>
</tr>
<tr>
<td></td>
<td>Ethanol</td>
<td>Polar flavonoids, tannins and glycosides (saponins)</td>
<td>Scalbert et al., 2005</td>
</tr>
<tr>
<td></td>
<td>Aqueous acid or base</td>
<td>Alkaloid</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cowan, (1999)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Bruneton, (1999)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Bruneton, (1999)</td>
</tr>
</tbody>
</table>
Thus, if the polarity or the solubility of the compounds of interest is known, the information in
the above table can be used to select a proper extractor solvent or a mixture of two or more
solvents of different polarity (Kaul et al., 1985). Alternatively, a solvent such as acetone, which has
the capacity to extract both polar and non-polar substances, and has been recommended by Eloff
(2001) for the extraction of most polar and nonpolar compounds can be used. If the polarity
of the compounds of interest is not known, the powdered herbal material can be extracted
simultaneously with a mixture of different proportions of two or more solvents of different
polarity (Bruneton, 1999, Cowan, 1995). Alternatively, the powdered herbal material can be
extracted sequentially with solvents of different polarity in what is known as a sequential
extraction procedure (Bruneton, 1999).

2.8.5 Choice of the extraction procedure

The choice of the extraction procedure depends on the nature of the source material and the
compound to be isolated. Solvent extract procedures applied to herbal products include but are
not limited to maceration, percolation, soxhlet extraction, steam distillation and sequential solvent
extraction (1998; Jones and Kinghorn, 2005).

2.8.5.1. Maceration

This simple, but still widely used procedure involves leaving the pulverized plant to soak in a
suitable solvent in a closed container at room temperature (Harborne, 1998). Occasional or
constant stirring of the preparation (using mechanical shakers or mixers) can increase the speed
of the extraction. Maceration involves soaking the herbal material in a suitable solvent, filtering
and concentrating the extract (Harborne, 1998; Jones and Kinghorn, 2005). The use of a cold
solvent reduces decomposition, but the process takes longer and uses larger amounts of solvent.

2.8.5.2. Percolation

This is similar to the maceration process, but hot solvent is refluxed through the herbal
material. It is quicker and uses less solvent, but decomposition due to heat may occur (Jones and
Kingham, 2005; Satyajit et al., 2006).
2.8.5.3. Soxhlet extraction

Soxhlet extraction is a form of continuous percolation with fresh solvent, which uses special glass ware. In this procedure, the herbal material is separated from the extract by encasing it in a paper cap beneath the dropping condensed solvent. When full, the solvent in the thimble siphons off into the main vessel containing the extractant, and the process continues (Jones and Kinghorn, 2005). The advantage of this procedure is that fresh solvent continually extract the herbal material more effectively with minimum solvent, however, heating and hence decomposition of compounds is again a disadvantage (Nikhal et al., 2010).

2.8.5.4. Steam distillation

There is a special apparatus for distilling volatile oils which are immiscible with water. If compounds being extracted are water soluble, the method is less useful because a large volume of aqueous extract is produced. However, in some cases a partition system may be used to concentrate the extract (Jones and Kinghorn, 2005; Satyajit et al., 2006).

2.8.5.5. Sequential solvent extraction

If the polarity and solubility of compounds that are isolated is not known, a convenient and frequently used procedure is sequential solvent extraction. In sequential solvent extraction, the herbal material is extracted with a series of solvents of different polarity (Starmans and Nijhuis, 1996). The usual way is to start with a non-polar solvent and exhaustively extract the herbal material followed by a series of more polar solvents until several extracts are obtained of increasing solute polarity. For example, a first step, with dichloromethane, will extract terpenoids, less polar flavonoids (flavones, flavonols, flavonones) and other less polar materials (Jones and Kinghorn, 2005, Okwu, 2001). A subsequent step with acetone or ethyl acetate will extract flavonoid glycosides and other medium polar constituents. A subsequent extraction with an alcohol or water will extract highly polar constituents (Jones and Kinghorn, 2005). Once the extraction is complete, the extractant is usually concentrated under vacuum, for large volumes of solvents and blown down under nitrogen for small volumes, ensuring at the same time that volatiles are not lost. Aqueous extracts are generally freeze-dried and stored at -20°C as this low temperature reduces the degradation of the bioactive natural products (Starmans
and Nijhuis, 1996). Extraction protocols may sometimes be modified depending on the type of mole cules being extracted, for example, acids may be added to extract alkaloids as their salts (Jones and Kinghorn, 2005).

2.8.6 Bioassay guided fractionation and isolation of active compounds

Active fractions are fractionated using bioassay guided fractionation. In bioassay-guided fractionation, a crude mixture is fractionated into its fraction components using chromatographic procedures, followed by biological evaluation (bioassay) of each fraction (Vaidya and Antarkar, 1994). Only fractions which display biological activity in the bioassay are selected for further fractionation. The cycle of fractionation and testing and further fractionation is repeated until a pure compound with the desired activity is isolated (Rimando et al., 2001). The general scheme for carrying out a bioassay guided fractionation is summarized in figure 2.5 below.

![Diagram of bioassay guided fractionation](image)

Figure 2.5: General scheme for bioassay guided isolation of active compounds (Rimando et al., 2000)
2.8.7 Characterization and structure elucidation of isolated compounds

Once the biological evaluation has been performed and the separation of the natural product has been achieved, the chemist will try to attempt the elucidation of the compound. Structure elucidation depends on classical spectroscopic techniques such as: Nuclear Magnetic Resonance (NMR) 1-D and 2-D HNMR as well as C-13 NMR, Infra-Red (IR), Mass Spectrometry (MS) and X-Ray analysis (Harborne, 1998).
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3 NMR based geographical characterization of *Sclerocarya birrea* methanol extracts

### 3.1 Abstract

Nuclear Magnetic Resonance (NMR) is frequently used as a technological platform for metabolomics applications. In this study the metabolic profile of *S. birrea* methanol leaf extracts from five different provinces were recorded by $^1$H NMR. When multivariate data analysis was performed on $^1$H NMR results, the supervised OPLS-DA showed the samples having separated in three groups. The samples from Gauteng and those from KwaZulu-Natal separated away from those from Mpumalanga, Limpopo and North West. The samples from Mpumalanga, Limpopo and North West formed one large group, representing some similarities between the provinces. The contribution plots presented sugars to be metabolites important in the samples from Gauteng, and the phenolic metabolites as the ones important in the samples from KwaZulu-Natal. The samples from the other three provinces (Limpopo, Mpumalanga and North West) presented very few sugar metabolites as the most important ones in these samples. The applied approach appears to be a promising strategy in extending the analytical capacities of these metabolomics techniques with regard to the discovery and identification of biomarkers and yet unknown metabolites.
3.2 Introduction

Humans have relied for ages on natural products for relief of pain and improvement of health. Specialized metabolites from plants serve as a rich resource for drug development. The molecular and physiological effects of medicinal plant extracts and components are often characterized in research studies of mammalian systems; almost 100 plant-derived compounds were included in clinical trials in 2007, and as of 2008, 68% of all pharmaceuticals were plant derived or plant inspired (Zhu et al., 2012).

The stem bark, roots and leaves of *S. birrea*, has been used for decades as a traditional medicine. *Sclerocarya birrea* is mostly called “Marula” because of the stone found inside the fleshy fruit. The tree has been evaluated for antibacterial potential (Eloff, 2001), hypoglycemic potential (Ojewole, 2003), antiproliferative potential (Ndip and Tonih, 2013) and many other human aliments (Van Wyk et al., 2000). Amongst all the medicinal uses presented above, *S. birrea* is mostly studied for its anti-diabetic potential. Currently, type II diabetes mellitus, the most common type of diabetes mellitus, is managed by a combination of diet, exercise, oral hypoglycemic drugs and sometimes insulin injections (Bailey, 2000). However, synthetic oral hypoglycemic drugs, which are currently the main form of treatment for type II diabetes mellitus have been shown to have detrimental side effects and high secondary failure rates (Bailey, 2000; Erasto et al., 2005; Dey et al., 2007).

In part because they lack mobility, plants have evolved chemically-based strategies for defense and attraction (Weng et al., 2012; Mithöfer and Boland, 2012). As a consequence, even low levels of thousands of metabolites that are synthesized across the plant kingdom interact with mammalian signaling networks via variety of molecular mechanisms. However, metabolic diversity is poorly characterized for most species that are used medicinally and indeed for plants in general. In addition, understanding of the molecules and metabolic pathways that lead to the formation of already-known plant-derived medicinal compounds is still incomplete. Modeling of metabolism requires computational technologies integrated with informed biological understanding of metabolites and pathways. In the case of medicinal plants (i.e., non-model-species), such data are scarce and difficult to integrate into a meaningful biological framework.
One feature that can facilitate studies of plant metabolites and the corresponding pathways is that the content and profile of metabolite accumulation vary widely with developmental stage, cell and tissue type, genotype, and environmental perturbation (Balandrin and Klocke, 1988; Crispin and Wurtele, 2013; Luca et al., 2012). A metabolomics-based analysis of natural products across multiple conditions is a first step towards elucidating the associated metabolic pathways and identifying metabolites found in the sample. In this study the differences in the chemical profiles of *S. birrea* from different regions were investigated, and this was linked to the anti-diabetes potential of the extracts.

### 3.3 Materials and methods

#### 3.3.1 Study sites and sample collection

Leaf samples from 12 *S. birrea* trees were collected in October 2013, from each of the five sampling areas Limpopo (L), Gauteng (G), North West (NW), Mpumalanga (M) and KwaZulu-Natal (KZN) (Figure 3.1). From each province, two regions were identified for collection, and in each region samples were collected from three different trees with two samples per tree. The origins of samples are summarized in table 3.1. The leaves were harvested from trees of almost the same height (9.5 m). The height of a tree was determined by implementing a method described by the Teachers notes on how to determine the height of the tree. In brief the method relies on trigonometry and the fact that if you view a tree top at 45 degree angle then the height of the tree is equivalent to the distance you are from that tree.
Figure 3.1: A geographical map of Limpopo, Gauteng, North West, Mpumalanga and KwaZulu-Natal showing areas where *S. birrea* leaves were sampled.
Figure 3.2: Determination of a tree’s height

Table 3.1. List of *S. birrea* leaf samples analyzed

<table>
<thead>
<tr>
<th>Sample</th>
<th>Gender</th>
<th>Region</th>
<th>Province</th>
</tr>
</thead>
<tbody>
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<td>Male</td>
<td>Ga-sekgoboko</td>
<td>L</td>
</tr>
<tr>
<td>Skb 1-2</td>
<td>Male</td>
<td>Ga-sekgoboko</td>
<td>L</td>
</tr>
<tr>
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<td>Male</td>
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</tr>
<tr>
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<td>Male</td>
<td>Ga-sekgoboko</td>
<td>L</td>
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</tr>
</tbody>
</table>
3.3.2 Sample preparation for NMR spectroscopy

3.3.2.1 Methanol extracts preparation

NMR analysis was done on a Bruker 600 MHz spectrometer (Wageningen University, The Netherlands) operating at a proton NMR frequency of 600.13 MHz. 15mg of the dried leaf material was dissolved in 400µl methanol-D4. The mixture was sonicated for 20 minutes and then centrifuged for 10 minutes at 10 000 rpm, room temperature, and 200 µl of the supernatant was transferred to standard 3-mm NMR tubes. Gradient shimming was used to improve the magnetic field homogeneity prior to all acquisition. All spectra were Fourier- transformed, phase-corrected and baseline- corrected manually.

3.3.2.2 Multivariate data analysis

The $^1$H NMR spectra were reduced to ASCII files using Mestrenova 9.0. (Mestrelab Research). The region of 0.00- 10.00 ppm was reduced to bins 0.04 ppm in width. The region ranging from 3.28-3.36 ppm (residual MeOH) and 4.60-5.00 ppm (residual water) were removed prior to statistical analyses. The ASCII files generated were then imported into Microsoft Excel 2010 for secondary variable labeling after which the files were imported into Simca version 13.0 (Umetrics Umea, Sweden). The data was Pareto scaled before being subjected to PCA and OPLS-DA analysis.
3.4 Results and Discussion

3.4.1 $^1$H NMR analysis of *S. birrea* methanol extracts

For the metabolomics analysis, all the extracts were analyzed under the same condition to limit technical errors in the data generated. All the 60 samples as mentioned in table 3.2.1 above were subjected to Metabolomic analysis.

The NMR spectra appeared as a complex ensemble of resonances, indicating the presence of multitude of metabolites (Figure 3.3). In addition, given the presence of resonances throughout the whole spectra width (0-8 ppm), variable chemical features ranging from aliphatic to aromatic groups were detected in the extracts. In particular, intense signals in the sugar region mostly in the samples from Gauteng were observed, indicating the presence of glycosylated metabolites and free sugars (Figure 3.4). From the visual comparison of the spectra, there is an obvious similarity between all spectra, suggesting similar metabolic profiles between *S. birrea* from different provinces.

The analysis of *S. birrea* leaf extracts from five provinces by $^1$H NMR allowed the detection of essentially primary (polar) metabolites such as sugars, amino acids, and organic acids, and the abundance of the corresponding resonances indicated the high natural concentration of these metabolites in the leaves. The relatively low abundance of secondary metabolites and the large amount of resonances in the spectrum made the detection of secondary metabolites, such as phenolic acids, flavonoids and alkaloids, more difficult as compared to the detection of the primary metabolites. Based on these NMR profiles, the relative differences between the *S. birrea* from different provinces were visualized by plotting the scores of a principal component analysis.
Figure 3.3: $^1$H NMR spectra for sixty methanol extracts of *S. birrea*

Figure 3.4: Average spectra of sixty methanol extracts of *S. birrea*
3.4.2 Metabolomic analysis of Sclerocarya birrea extracts from different provinces

To provide comparative interpretation and visualization of the fundamental variations and metabolite differences in the samples from different origins, Principal Component Analysis (PCA) and Orthogonal Partial Least square- Discriminant Analysis (OPLS-DA) were applied to the $^1$H NMR spectrum dataset. The PCA showed more significant differences between KZN, L, NW and M, than between KZN and G (Figure 3.5). The PCA model for distinguishing S. birrea samples obtained from different origins was established using seven components and revealed R2X and Q2 values of 0.85 and 0.78 respectively.

Figure 3.5: Score plot of PCA-X performed by considering all 60 methanol samples. The red dots represent samples from KZN; blue represent samples from G; yellow represent samples from NW; green represent samples from L and light blue represent samples from M.
To improve the clustering and to identify the metabolites responsible for the differences between the provinces, OPLS-DA model was constructed (Figure 3.6), and revealed RY2 and Q2 values of 0.73 and 0.62 respectively. The samples from five provinces were clearly separated in the OPLS-DA score plot (Figure 3.6), which means that the non-correlated variation in X metabolites to Y metabolites was removed, resulting in maximum separation. This maximum separation also indicates that the levels of *S. birrea* metabolites were strongly influenced by the various growing conditions in each region.

**Figure 3.6:** Score plot of OPLS-DA performed by considering all 60 methanol sample. The red dots represent samples from KZN; blue represent samples from G; yellow represent samples from NW; green represent samples from L and light blue represent samples from M.

Clustering into five groups were observed, according to the provinces indicates that there are some metabolites unique between the provinces. The samples from Gauteng and KwaZulu-Natal presented the greatest differences in that they were both further apart from the others. When the contribution plots were constructed on the samples from Gauteng, it was clear that the sugars contributed to the differences between Gauteng and other provinces. The sugar region in the peaks in the positive bars of
the contribution plots of Gauteng (Figure 3.7) is more abundant than the aliphatic and the aromatic regions indicating the importance of sugars in samples from Gauteng than in the others. Using Chenomx, the metabolites important in Gauteng were annotated in table 3.2.

Figure 3.7: Contribution plots generated by comparing samples from Gauteng to those from Limpopo, North West, KwaZulu-Natal and Mpumalanga. The buckets (positive bars) represent the specific regions of the $S. \text{birrea}$ NMR spectra responsible for the differences. The negative bars represent the areas important in the other four provinces while the positive bars represent the ones important in Gauteng.
Figure 3.8: Contribution plots generated by comparing samples from KwaZulu-Natal to those from Limpopo, North West, Gauteng and Mpumalanga. The buckets (positive bars) represent the specific regions of the *S. birrea* NMR spectra responsible for the differences. The negative bars represent the areas important in the other four provinces while the positive bars represent the ones important in KwaZulu-Natal.

In addition to the sugars presented by the contribution plots in Gauteng, the samples from KwaZulu-Natal showed very few sugars, few aliphatic and more aromatics to be important in the samples from this province (Figure 3.8), the metabolites important to KwaZulu-Natal are annotated in table 3.2. Visual inspection of the contribution plots from both KwaZulu-Natal and Gauteng confirms that the samples from Gauteng are rich with sugars while those from KwaZulu-Natal are rich in aromatic metabolites.

It can therefore be concluded that the separation of samples from Gauteng to those from KwaZulu-Natal, Limpopo, Mpumalanga and North West is influenced by the sugars while the separation of samples from KwaZulu-Natal to those from the other four provinces is mostly influenced by aromatics, some aliphatic, and some sugars. In the OPLS-DA scores (figure 3.6), the three provinces (Mpumalanga, Limpopo, and North West) showed the closest similarity in that their separation was not clear (Figure 3.6). To confirm that in deed the samples are similar, the samples from Gauteng and those from KwaZulu-Natal were excluded from the dataset and the comparison was only between Limpopo, Mpumalanga and North West (Figure 3.9). The, OPLS-DA model constructed between the three Provinces (Limpopo, Mpumalanga and North West), revealed RY2 and Q2 values of 0.73 and 0.63 respectively.
Figure 3.9: OPLS-DA scores performed by considering samples from Limpopo (green), North West (yellow) and Mpumalanga (Light blue).

When the contribution plots were performed on the samples in Figure 3.9, Mpumalanga appeared to have more aromatics as the metabolites of importance in the province (Figure 3.10). The other two provinces (Limpopo, and North West) presented aliphatic as the important metabolites as opposed to aromatics in Mpumalanga. The metabolites are annotated in table 3.2. All the phenolic metabolites annotated in this chapter were previously identified in the cultivated and wild *Sclerocarya birrea* leaves by Braca et al (2003). The metabolites annotated in table 3.2 are shown in figure 3.12 below. There is a great difference in the levels of metabolites between the provinces; therefore the medicinal properties of this plant from different provinces can be expected to differ.

Figure 3.10: Contribution plots generated by comparing samples from Mpumalanga to those from Limpopo, and North West. The buckets (positive bars) represent the specific regions of the *S. birrea* NMR spectra responsible for the differences. The
negative bars represent the areas important in Limpopo and in North West provinces while the positive bars represent the ones important in Mpumalanga

Table 3.2 Metabolites with the highest intensities from the contribution plots and their $^1$H NMR chemical shifts

<table>
<thead>
<tr>
<th>Province</th>
<th>Metabolite</th>
<th>Chemical shift(multiplicity$^a$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gauteng</td>
<td>Lactate</td>
<td>1.32(d) 4.05(q)</td>
</tr>
<tr>
<td></td>
<td>Glutamate</td>
<td>2.04(m) 2.34 (m)</td>
</tr>
<tr>
<td></td>
<td>*Sucrose</td>
<td>3.44(t) 3.76(t) 3.54(dd) 5.39(d) 4.17(d)</td>
</tr>
<tr>
<td></td>
<td>Fructose</td>
<td>3.94(m) 3.51(d) 4.01(dd) 3.65-3.72(m) 3.77-3.87(m)</td>
</tr>
<tr>
<td></td>
<td>Alanine</td>
<td>1.46(d) 3.70(q)</td>
</tr>
<tr>
<td>KwaZulu-Natal</td>
<td>*Lactate</td>
<td>1.32(d) 4.05(q)</td>
</tr>
<tr>
<td></td>
<td>Alanine</td>
<td>1.46(d) 3.70(q)</td>
</tr>
<tr>
<td></td>
<td>Sucrose</td>
<td>3.44(t) 3.76(t) 3.54(dd) 5.39(d)</td>
</tr>
<tr>
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<td></td>
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<td>7.04(s)</td>
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<td>Gallic acid</td>
<td>7.04(s)</td>
</tr>
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</table>

$^a$ Letters parenthes denotes the peak multiplicity: s, singlet; d, doublet; t, triplet; dd, doublet of doublet; q,quarted; and m, multiplet

$^*$ denotes the peak with the highest intensity in the contribution plots

Overall all provinces presented the metabolites as unique to each. The sugar region in Gauteng was so unique in that the differences were observed even on the spectra itself before the statistical analysis with Simca. To confirm that the sugar region was mostly important in the samples from Gauteng, the region was excluded from the dataset and the OPLS-DA scores (Figure 3.11) with four components with R2X and Q2 values of 0.61 and 0.47 respectively was observed. The samples from KwaZulu-Natal are furthest away from the rest (Figure 3.11). No clustering of KwaZulu-Natal, Gauteng, Limpopo or North West is observed, supporting the importance of the sugars in clustering of the samples. Very few samples from KwaZulu-Natal grouped with the samples from Limpopo, North West, Mpumalanga and Gauteng. This is mostly because of the lower concentration of sugars present in the samples which was
also observed in the contribution plots from KwaZulu-Natal.

Figure 3.11: The score plot of samples from five provinces with exclusion of the sugar region. (3-4 ppm)
Figure 3.13: Metabolites annotated by NMR from *S. birrea* leaves; A= Lactate, B=Glutamate, C=Sucrose, D=Alanine, E=Malate, F= Myricetin, G= Quercetin, H= Kaempferol and I=Gallic acid, (chemspider)
3.5 Concluding remarks

In this study, methanol extracts of *S. birrea* from different provinces were compared and characterized based on multivariate statistical analysis of $^1$H NMR –based metabolomics data. Many of the primary metabolites (sugars) and few secondary metabolites (phenolics) were identified. *Sclerocarya birrea* from different provinces exhibited significant differences in their metabolite profiles confirming that several metabolites can be used as markers for origin. The samples also showed some close similarities, in that some metabolites (sucrose and lactate) were found to be important in the samples from all five provinces, as confirmed by the contribution plots. The most significant difference was with regard to the primary metabolites, specifically the sugar region influenced by glycosylation in the samples from Gauteng. KwaZulu-Natal presented a relatively high amount of phenolic metabolites. The removal of the sugar region resulted in the Gauteng samples mixing with the samples from Mpumalanga, North West and Limpopo with no clustering. Therefore, although there are close similarities, there are also compounds which are unique from these provinces. For better understanding of the differences and similarities, a more sensitive technique like LC-MS should be used for better detection of relatively low abundance secondary metabolites which can be responsible for the differences observed. In this chapter we demonstrated that $^1$H NMR-based profiling provides a fast and efficient method to fingerprint metabolic differences between same species of *S. birrea* grown in different regions. This analytical approach can be used to discriminate among origin of *S. birrea* and to identify primary and secondary metabolites responsible for discrimination. The next step in this study was to measure the same samples used in this study, with LC-MS with the aim to detect more secondary metabolites.
3.6 LITERATURE CITED


KIM, H.K., CHOI, Y.H. and VERPOORTE, R., 2011. NMR-based plant metabolomics: where do we stand,


4 LC-MS based geographical characterization of *Sclerocarya birrea* methanol extract

4.1 Abstract

The goal of metabolomics is to measure all the metabolites in an organism both qualitatively and quantitatively, which can provide a clear metabolic picture of a living organism under certain conditions. This is a very ambitious goal, as the plant metabolome is very complex and therefore no single technique is enough to study the whole metabolome of a plant. The same samples used for $^1$H NMR were use also for LC-MS measurements. The base peak chromatogram in LC-MS showed some similarities between the samples. The metabolite that can either be Myristicin or ‘$^1$ Hydroxychavicol acetate annotated from Human metabolome database was present in all the samples with a m/z ratio of 191. The compounds m/z 381, 171, 289, 261, 453, 418 contributed to the differences observed with PLS-DA in samples from five provinces. The multivariate data analysis with PLS-DA confirmed the samples to have some differences in metabolite profiles, in that they separated into five groups according to their places of origin.
4.2 Introduction

LC-MS and NMR are distinct analytical techniques, concerning detection and sensitivity. LC-MS is a fast and sensitive technique when compared to NMR. However, the separation of metabolites is dependent on the chromatographic column used, the detection is dictated by the ionization ability of the analytes and the molecular elucidation has some intrinsic limitations, such as the resolution of isomers (Moco et al., 2008). NMR is indiscriminative towards matrix properties, given that the analytes are soluble. NMR is a highly selective technique for distinguishing molecular structures, but has a lower sensitivity compared to MS (Moco et al., 2007a). The statistical combination of metabolomics data from profiling equivalent samples by NMR and LC-MS opens opportunities to relate spectrometric and spectroscopic properties for single metabolites. These statistical strategies have been applied to large-scale analytical analyses of urine (Crockford et al., 2006; Forshed et al., 2007a, b).

Beyond the analytical acquisition of data, the interpretation of metabolomics data is highly dependent on the performed data analysis. Due to the production of large datasets and complexity of untargeted metabolic fingerprinting techniques, appropriate conclusions arise only after preprocessing and statistical validation of metabolomics datasets (Moco et al .,2008). Multivariate analyses methods are useful in discriminating information, dealing with the redundancy often present in metabolomics data (Trygg et al., 2007). One of the statistical methods to establish relationships between metabolite signals belonging to a biological system is correlation analysis. The presence of high correlations in metabolomics data can be attributed to several phenomena which might not be directly related to pathway proximity (Camacho et al., 2005; Steuer, 2006). It has been proposed that the highest correlations belong to either an equilibrium situation (positive correlations) or to a mass conservation situation in which there is a moiety conserved cycle (negative correlations) (Camacho et al. 2005; Steuer, 2006). Both MS (Fraser et al., 2007; Moco et al., 2006a; Schauer et al., 2005; Tikunov et al.,2005) and NMR (Le Gall et al., 2003; Mattoo et al., 2006; Mounet et al. 2007) have previously been used for the profiling of metabolites in various plant species. In the present study, we used LC-QTOF-MS techniques to record the metabolic profiles of 60 *S. birrea* leaf samples from five different provinces.
4.3 Materials and method

4.3.1 Study site and sample collection

The same samples as the ones used in chapter 3 where used for LC-MS measurements and analysis.

4.3.2 Sample preparation for LC-MS spectroscopy

4.3.2.1 Methanol extracts preparation.

The remaining 200 µl from samples used in chapter 3 was used in carrying out LC-MS measurements. The operating parameters were; end plate offset, -v; capillary voltage, 3.5kv; dry gas, 8.0L min⁻¹; dry temperature, 200°C. The samples were analyzed in negative mode.

4.3.3 Multivariate data analysis

Quantification of *S. birrea* metabolite profiles of LC-MS analyses was performed using XCMS online data analysis software, which can be accessed freely from the Scripps Centre For Metabolomics (https://xcmsonline.scripps.edu). This software approach employs peak alignment, matching and comparison (Smith et al., 2006). The raw LC-MS files were first converted to Mzxml files using MS convert. Peaks were subsequently extracted using XCMS. The final statistical analysis where performed by employing MetaboAnalyst 3.0- a comprehensive tool suite for metabolomic data analysis, which also can be accessed freely from www.metaboanalyst.ca/MetaboAnalyst. This software employs data normalization, multivariate analysis methods (PCA and PLS-DA), clustering analysis and many more analysis approaches. All the samples were auto scaled prior to the analysis.
4.4 Results and discussions

4.4.1 LC-MS base peak chromatogram in negative mode of *S. birrea* extracts

Representative LC-MS base peak chromatograms of methanol *S. birrea* extracts from five provinces (L, G, NW, KZN and M) are shown in Figure 4.1 to 4.5. As to be expected, MS is more sensitive than NMR, which was made clear by the fact that visual inspection of the chromatograms did not give clear similarities as was observed with NMR. Visual inspection of the chromatograms showed that most metabolites in KZN are also available in G. The three other chromatograms for L, NW and M also showed some similarities in metabolites. This relationship in similarities complements the OPLS-DA obtained with NMR. When the samples are compared, L compares to both NW and MP and KZN compares well to G.

Figure 4.1: Base peak chromatogram of *Sclerocarya birrea* leaves from KwaZulu-Natal.
Figure 4.2: Base peak chromatogram of *Sclerocarya birrea* leaves from Gauteng.

Figure 4.3: Base peak chromatogram of *Sclerocarya birrea* leaves from Limpopo.
Figure 4.4: Base peak chromatogram of *Sclerocarya birrea* leaves from North West.

Figure 4.5: Base peak chromatogram of *Sclerocarya birrea* leaves from Mpumalanga.
More than 25 compounds were resolved, of which 13 were annotated; m/z 191 accounted for the highest abundance amongst the species (figure 4.1-4.5). Due to time limitation and scope of this study, it was not possible to annotate all the metabolites. The presence of the metabolite at m/z 191 annotated as either Myristicin or ‘1Hydroxychavicol acetate in all the samples, confirms that this metabolite is one of the marker compounds in *S. birrea* species, the metabolites annotated with LC-MS are provided in table 4.1 below, and their chemical structures are shown in figure 4.6. Samples from Mpumalanga lacked the metabolite Myricetin 3-0-α-L-rhamnopyranoside (m/z 463). This metabolite was previously reported to have anti-diabetes potential; the absence of this metabolite might have an effect on the activity of the leaves in the treatment of diabetes. Therefore, the anti-diabetic activity of the leaves should be performed on samples from individual provinces. Should the samples from Mpumalanga not present any activity, then that will indicate that the metabolite Myricetin 3-0-α-L-rhamnopyranoside is not the key metabolite in anti-diabetes activity of the leaves and that other compounds might be more important contributors to the activity.

**Table 4.1: Compounds annotated in *Sclerocarya birrea* methanol leaf extract by LC-MS**

<table>
<thead>
<tr>
<th>Province</th>
<th>rt (min)</th>
<th>Annotation</th>
<th>[M-H]- (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KwaZulu-Natal</td>
<td>27</td>
<td>Myricetin 3-0-α-L-rhamnopyranoside</td>
<td>463.1138</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>2,3-dehydroxy 6,3-linolenic acid</td>
<td>479.1078</td>
</tr>
<tr>
<td></td>
<td>34</td>
<td>Docosahexaenolic acid</td>
<td>327.351</td>
</tr>
<tr>
<td></td>
<td>54.5</td>
<td>Unknown</td>
<td>265.1619</td>
</tr>
<tr>
<td></td>
<td>8.7</td>
<td>Myristicin/‘1Hydroxychavicol acetate</td>
<td>191.0669</td>
</tr>
<tr>
<td></td>
<td>63</td>
<td>Unknown</td>
<td>174.9653</td>
</tr>
<tr>
<td>Limpopo</td>
<td>25.9</td>
<td>Unknown</td>
<td>647.1024</td>
</tr>
<tr>
<td></td>
<td>25.7</td>
<td>Myricetin 3-0-α-L-rhamnopyranoside</td>
<td>463.1049</td>
</tr>
<tr>
<td></td>
<td>38.3</td>
<td>Unknown</td>
<td>287.2322</td>
</tr>
<tr>
<td></td>
<td>55.4</td>
<td>Atenolol</td>
<td>265.1574</td>
</tr>
<tr>
<td></td>
<td>8.7</td>
<td>Myristicin/‘1Hydroxychavicol acetate</td>
<td>191.0666</td>
</tr>
<tr>
<td></td>
<td>63.6</td>
<td>Unknown</td>
<td>174.9632</td>
</tr>
<tr>
<td>Gauteng</td>
<td>25.2</td>
<td>Myricetin 3-0-α-L-rhamnopyranoside</td>
<td>463.1101</td>
</tr>
<tr>
<td></td>
<td>34.9</td>
<td>Ent-16-kauren-19-ol-acetate</td>
<td>329.2477</td>
</tr>
<tr>
<td></td>
<td>54.6</td>
<td>Atenolol</td>
<td>265.1604</td>
</tr>
<tr>
<td></td>
<td>8.7</td>
<td>Myristicin/‘1Hydroxychavicol acetate</td>
<td>191.0689</td>
</tr>
<tr>
<td></td>
<td>64.3</td>
<td>Unknown</td>
<td>174.9646</td>
</tr>
<tr>
<td>North West</td>
<td>13.6</td>
<td>3,4,5-Trihydroxy-37 dimethoxylavone/3,5,7-Trihydroxy-46 dimethoxylavone</td>
<td>331.0865</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>Diospyrin</td>
<td>483.1049</td>
</tr>
<tr>
<td></td>
<td>25.6</td>
<td>Myricetin 3-0-α-L-rhamnopyranoside</td>
<td>463.1165</td>
</tr>
<tr>
<td></td>
<td>35</td>
<td>Ent-16-kauren-19-ol-acetate</td>
<td>329.2529</td>
</tr>
<tr>
<td></td>
<td>54.5</td>
<td>Unknown</td>
<td>265.1629</td>
</tr>
<tr>
<td></td>
<td>8.7</td>
<td>Myristicin/‘1Hydroxychavicol acetate</td>
<td>191.0689</td>
</tr>
<tr>
<td></td>
<td>63.5</td>
<td>Unknown</td>
<td>174.9669</td>
</tr>
<tr>
<td>Mpumalanga</td>
<td>27.9</td>
<td>Unknown</td>
<td>433.1029</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>Abietinol</td>
<td>287.2379</td>
</tr>
<tr>
<td></td>
<td>54.7</td>
<td>Unknown</td>
<td>265.1629</td>
</tr>
<tr>
<td></td>
<td>8.7</td>
<td>Myristicin/‘1Hydroxychavicol acetate</td>
<td>191.0683</td>
</tr>
<tr>
<td></td>
<td>62.9</td>
<td>Unknown</td>
<td>174.967</td>
</tr>
</tbody>
</table>
Figure 4.6: Metabolites annotated by LC-MS from *S. birrea* leaves; A=2,3 dehydrosilybin, B=Docosahexaenolic acid, C=Myristicin and D=myricetin, (Chemspider)
4.4.2 MS data processing and PCA analysis

Although different metabolite patterns were observed by visual inspection of LC-MS chromatograms in the samples from different provinces, we attempted to analyze the results more holistically using PCA to explore the relative variability in the samples from different origins. Extracts were analyzed only in negative ion electrospray ionization HPLC-(CSI) MS mode. All the sixty samples were analyzed and no clear separation was achieved for both 2D and 3D (Figures 4.7 and 4.8) PCA plots. The samples did not separate, indicating that they are related in composition.

Figure 4.7: 2D score plot between selected PCs. The explained variables are shown in bracket
Figure 4.8: 3-D PCA Score plot between the selected PCs. The explained variances are shown in brackets.

4.4.3 MS data processing and PLS-DA analysis

The 2D score plots (Figure 4.9) did not show a clear separation although the samples from the provinces showed some clustering. The 3D plot (Figure 4.10) showed that five distinct clusters are formed, distributed over three opposing regions on PLS-DA plots corresponding to five different provinces researched. L, G and KZN were placed on the left side of the vertical line, representing PC1, whereas M and NW samples were placed on the right. Discrimination between samples from L, from G and KZN is also possible; with KZN samples on the lower left part of the score plots (Figure 4.11). Mpumalanga (M) and NW are in the same quadrant, i.e. related in composition but still distinct. The separation observed in PLS-DA can further be explained in terms of the identified compounds using loading plots. When the loading plots are performed, the unknown compounds with the masses, 381.19, 289.18, 171.01, 261.05, 453.11, and 418.14 are the most prominent compounds in the plot (Figure 4.10).
Figure 4.9: 2-D PLS-DA Score plot between the selected PCs. The explained variances are shown in brackets.

Figure 4.10: 3-D PLS-DA Score plot between the selected PCs. The explained variances are shown in brackets.
Figure 4.11: The loading plot for the PLS-DA.

After unsupervised cluster analysis (Figure 4.7) and supervised PLS-DA analysis (Figure 4.9), fifteen metabolites (the total VIP value of the principal components of every metabolite was above 1.0) were obtained as differential metabolites (table 4.2). All the metabolites annotated in table 4.2 were present in high concentrations in the samples from North West, Mpumalanga and KwaZulu-Natal respectively.

Figure 4.12: Important features identified by PLS-DA. The colored boxes on the right indicate the relative concentrations of the corresponding metabolite in each group under study.
**Table 4.2: Metabolites annotated from VIP scores**

<table>
<thead>
<tr>
<th>[M-H]- m/z</th>
<th>Annotation</th>
</tr>
</thead>
<tbody>
<tr>
<td>463.0531</td>
<td>Myricetin 3-0-α-L-rhamnopyranoside</td>
</tr>
<tr>
<td>441.0723</td>
<td>(−)-Epicatechin 3-0-galloyl ester</td>
</tr>
<tr>
<td>447.1466</td>
<td>Quercetin 3-0-α-L-rhamnopyranoside</td>
</tr>
<tr>
<td>231.0232</td>
<td>Unknown</td>
</tr>
<tr>
<td>209.0407</td>
<td>Unknown</td>
</tr>
<tr>
<td>285.0954</td>
<td>Unknown</td>
</tr>
<tr>
<td>210.0454</td>
<td>Unknown</td>
</tr>
<tr>
<td>565.1498</td>
<td>Unknown</td>
</tr>
<tr>
<td>423.0952</td>
<td>Unknown</td>
</tr>
<tr>
<td>489.291</td>
<td>Unknown</td>
</tr>
<tr>
<td>179.0649</td>
<td>Unknown</td>
</tr>
<tr>
<td>615.1344</td>
<td>Quercetin 3-0-β-D(6''-galloyl)galactopyranoside</td>
</tr>
<tr>
<td>426.1108</td>
<td>Unknown</td>
</tr>
<tr>
<td>439.101</td>
<td>Unknown</td>
</tr>
<tr>
<td>534.1929</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

**4.5 Concluding remarks**

Metabolic profiling and metabolomic techniques are reliable and well-studied investigative tools. In this study, methanol extracts of *S. birrea* leaves from different provinces (L, KZN, G, NW and M) were compared and characterized based on multivariate statistical analysis of LC-MS data. Because of its great sensitivity, LC-MS detected mostly secondary metabolites. Many of the metabolites could not be accurately annotated as further analysis is necessary. For a more detailed annotation of all metabolites, MS/MS will be carried out in which fragmentation of a parent ion mass will be made. The metabolites annotated in the VIP score were previously reported to have anti-diabetes potential, which supports the use and application of the technique. It was however important that the anti-diabetes potential of the samples be determined on samples from individual provinces in order to confirm the importance of these compounds in the samples and to determine the effect of the concentration differences of these metabolites. The next step in this study was to screen the samples for blood glucose uptake activity, to determine if the difference in compounds and the difference in concentration would affect the anti-diabetic potential of the samples from the five provinces. Overall LC-MS was sensitive enough to detect the less abundant secondary metabolites from *S. birrea* leaves which could not be easily detected by NMR.
4.6 LITERATURE CITED


5 Assessment of the glucose uptake activity of aqueous *Sclerocarya birrea* leaves

### 5.1 Abstract

*Sclerocarya birrea* (*S. birrea*) is a well-known traditional plant. Its geographical origin can be determined by its biochemical composition. In this study the metabolic pattern in *S. birrea* from Limpopo, KwaZulu-Natal, North West, Gauteng and Mpumalanga was measured on $^1$H NMR, followed by multivariate data analysis. The samples clearly separated according to five groups, representing their places of origin. The separation was also observed between male and female samples. To investigate the anti-diabetes potential of *S. birrea*, the aqueous extracts from five different provinces were screened against C2C12 myocytes. Since metabolomics revealed the difference between male and female samples, an investigation on the male and female samples was conducted to determine the differences between the samples. All the extracts were active in C2C12 myocytes. The male samples from Mpumalanga gave the best overall results with the activity higher than that of insulin, the positive control. This screening system increases the likelihood of identifying drug candidates using in-vitro anti-diabetic screening of crude plant extracts. The highest activity in the male samples is believed to be encouraged by the highest intensity of (-)-epicatechin-3-galloyl ester, a secretagogue compound found in the male samples. The results of this experimental tissue culture study indicate that aqueous extract of *S. birrea* possesses hypoglycemic activity, and therefore confirms the suggested folkloric use of the plant in the management and/or control of adult-onset, type-II diabetes mellitus in some African communities.
5.2 Introduction

Diabetes mellitus is a metabolic disorder characterized by a loss of glucose homeostasis with conflict of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action or both (WHO, 1999; Pietropopo, 2001; Deffronzo, 2004). Without enough insulin, body tissues in particular, the liver, muscles and adipose tissues fail to take and utilize glucose from the blood circulation. The stem bark of *S. birrea* has previously been found to possess anti-diabetes potential (Ojewole, 2008). The study by Ojewole 2008 opened doors for more studies on other parts of *S. birrea*, especially the ones with no detrimental effect on the plant during harvesting. In the current study the in-vitro glucose uptake was assessed on the leaves of *S. birrea*. The use of in-vitro studies to evaluate the glucose uptake in cell lines following stimulation with insulin and other active compounds and plant extracts, is a direct and sensitive method of determining the glucose lowering effect of these agents (Van de Venter et al., 2008). It allows for rapid screening of compounds in terms of their efficacy and toxicity in an ethically acceptable manner (Van de Venter et al., 2008).

Other cell lines, not tested in this study include C3A and 3T3-L1 clonal mouse embryonic fibroblastic cells. C3A cells are a clonal derivative of HepG2 cells that are used for a wide application of in-vitro screening due to strong contact inhibition of growth, high albumin production, and high production of alpha fetoprotein (AFP), oxygen dependent gluconeogenesis and the ability to grow in a glucose deficient medium (Kelly 1994; Iyer et al., 2010). These characteristics contribute to these cells expressing a wide range of liver-specific functions. 3T3-L1 clonal mouse embryonic fibroblastic cells are chemically transformed into lipid-accumulating adipocytes. These cells are representative of adipose tissue, an insulin-sensitive tissue type involved in peripheral glucose clearance and maintenance of glucose homeostasis (Van de Venter et al., 2008).

The C2C12 muscle cells are derived from mouse myoblasts. C2C12 cells differentiate rapidly to form myotubules, thereby expressing muscle proteins such as GLUT4, the insulin responsive glucose transporter. These cells have been used as in-vitro models to study myogenesis and cell differentiation, glucose metabolism and insulin signaling (Van de Venter et al., 2008; Brunetti et al., 1989). In this study five *S. birrea* extracts were exposed to C2C12 muscle cells in order to assess the in-vitro glucose uptake of the extracts by these muscle cells.
5.3 Materials and methods

5.3.1 Preparation of plant extracts for anti-diabetic screening

Plant leaves from five provinces L, G, NW, M male (M) and female (F), and KZN were separated and air dried in a room with adequate ventilation. Dried plant leaves were ground to a course powder using a hammer mill and stored at an ambient temperature prior to extraction. For each extraction procedure 150g of powdered plant material was extracted with 1, 5 L purified water for 24 hours. The aqueous extracts were concentrated by freeze-drying and stored at -20°C.

5.3.2 In-vitro Cell Culture Models

C2C12 in-vitro Glucose Uptake Model.

During culture C2C12 myocytes were exposed to the aqueous extracts (L, M (f), M (m), KZN, NW and G) prepared in Krebs-ringer bicarbonate HEPES buffer (KRHB) containing 8 mM glucose for one hour at concentrations ranging from 100, 10, 1, 0.1, 0.01 µg/mL. After the one hour incubation, glucose uptake in C2C12 cells was determined using pulse-labeling with [3H]-2-deoxy-D-glucose (3H-2-DOG) in glucose-free KRHB buffer containing the extracts for fifteen minutes. 2-deoxy-D-glucose is a fierce competitor with D-glucose for glucose transport across the cell membrane and can therefore be used to estimate the rate of D-glucose uptake. Once transported into the cell, labeled 2-deoxy-D-glucose is phosphorylated by hexokinase to 2-deoxy-D-glucose-6-phosphate but unlike D-glucose, due to a missing hydroxyl group, cannot be metabolized further and remains as an inert intracellular metabolite. Liquid scintillation counting was used to measure intracellular 3H-2-DOG. Insulin (1µM) was included as a positive control and metformin (1µM) as a reference drug control.

Activity was determined as fmol (3H-2-DOG)/mg (protein) and expressed below as 100% relative to the aqueous control. The extracts, insulin (1µM) a positive control and metformin (1µM) as a reference control are expressed as % relative to the aqueous control.
5.3.3 Data analysis

Means and SEM are from three replicates in a single experiment. One way ANOVA was performed with a Dunnet’s post hoc test if \( p \leq 0.05 \) that was deemed to be statistically significant.

5.3.4 Preparation of extracts for NMR spectroscopy

Six samples used for in-vitro glucose uptake assays were used again for NMR spectroscopy. NMR analysis where done on a Varian 600 MHz spectrometer (CSIR) operating at a proton NMR frequency of 599.74 MHz where 50mg of the dried leaf material was dissolved in 750 µl Deuterium oxide and 750 µl potassium phosphate buffer in 0.1% TSP. The mixture was sonicated for 20 minutes and then centrifuged for 10 minutes at 13 000 rpm, room temperature, and 600 µl of the supernatant were transferred to standard 5-mm NMR tubes. Gradient shimming was used to improve the magnetic field homogeneity prior to all acquisition. All spectra were Fourier- transformed, phase-corrected and baseline- corrected manually.
5.4 Results and discussions

From all the 60 samples used for NMR, only six samples were used for glucose uptake assays due to the high cost of anti-diabetic screening procedures. Figure 5.1 shows the glucose uptake in C2C12 myocytes. Since *S. birrea* has separate male and female trees, which might contain different chemical profiles, it was of great importance to determine the anti-diabetes potential of the male and female samples separately. From each province a representative sample consisting of both male and female was used. Exception was made for Mpumalanga samples; Mpumalanga samples showed to have anti-diabetes activity in the experiments carried out previously at the University of KZN (results unpublished). The samples were labeled A-F, representing Limpopo, Mpumalanga (female), Mpumalanga (male), KwaZulu-Natal, North West and Gauteng respectively (Figure 5.1).

Various glucose uptake assays exist, employing different methodologies (Soumyanath and Srijayanta, 2005; Zou et al., 2005). In the present study, glucose uptake was monitored in C2C12 cells, using insulin as a positive control. Aqueous extracts of *S. birrea* from the five provinces (Limpopo, Mpumalanga, Gauteng, KwaZulu-Natal and North West) showed activity, which indicated the presence of compound/s that possesses insulin mimetic properties. The male extracts from Mpumalanga (Figure 5.1 c) caused a significantly (p<0.01) higher increase in glucose uptake in C2C12 cells in comparison to the positive control, insulin. The latter may implicate that this crude extract may be developed into a potent therapy in preventing and alleviating hypoglycemia.

The male extract showed better results than the female extract which support the statement from Shackleton and co-workers with interviews in Namibia that “The medicinal power of the male tree is greater than that of the female tree” (as cited in Shackleton et al., 2002). At 100 µg/mL the activity was higher than insulin the positive control. The extract from KZN increased glucose uptake at 0.1µg/mL; however appears to be cytotoxic at 100 µg/mL as glucose uptake is reduced to ca. 64% of the control.

The NW extract significantly improved glucose uptake at 1 µg/mL. The activity was comparable to that of insulin. Similar activity was observed for extract G at 1 µg/mL however at 100 µg/mL glucose uptake was reduced to ca. 83% of the control. The extracts from L, NW and G demonstrated almost identical activity in the assay. This is in agreement with the LC-MS results that showed almost identical profiles and clustering of samples from L and NW (Figures 4.3 and 4.4). A table with glucose uptake data is shown in table 8.1 appendix.
Figure 5.1: Glucose uptake activity % in C2C12 myocytes exposed to aqueous 7 extracts of Limpopo (Fig 5A), Mpumalanga (Female) (Fig 5B), Mpumalanga (Male) (Fig 5C), KwaZulu-Natal (Fig 5 D), North West (Fig 5E) and Gauteng (Fig 5F). The percentage is expressed relative to the control at 100.
In the study conducted at MRC in collaboration with UKZN (Results unpublished), the assessment of cytotoxicity using the MTT assay showed no extract-induced cytotoxicity at any of the concentrations tested in the C2C12 cells (Figure 5.2), and the same cell lines and extracts were used in this study. Therefore, the extracts showed significant enhancement of glucose uptake in C2C12 myotubules without showing any form of toxicity.

**Figure 5.2.** Percentage cell viability of C2C12 cells.
5.4.1 Metabolomic analysis of the male and female *S. birrea* aqueous extracts

When the sixty samples extracted with methanol (in chapter 3), were extracted with water, the separation between the male and the female extracts was clear. The supervised OPLS-DA model for distinguishing male and female *S. birrea* samples from different provinces was established using six components and revealed R2X and Q2 values of 0.940 and 0.640 respectively. Although there is a slight overlap between the male and female samples, which may be a result of the metabolic variation between the two classes not related to gender, the scores still showed separation of compounds according to their genders (Figure 5.3). The methanol extracts couldn’t separate based on genders (appendix 8.5). In the contribution plot (Figure 5.4) the aliphatic and the sugar regions were found to be important in the female samples as opposed to the males. The male samples were found to be rich in aromatic metabolites (negative bars of Figure 5.4), which support the greater anti-diabetes activity presented by the male samples.

![Figure 5.3: OPLS-DA score plots for female and male *S. birrea* aqueous extracts. The green scores represent the females while the blue represent the male extracts.](image-url)
A significant difference in activity was observed between the male and female samples of Mpumalanga indicating a difference in chemical profile between the male and female samples. To investigate which metabolites have contributed to the activity in all the six samples, $^1$H NMR was carried out on the six aqueous extracts used for glucose uptake assays (Figure 5.5). Visual inspection of the six extracts, confirmed them to be rather similar. Compounds such as Gallic acid were also found in all the samples. In the study by Gopalakrishnapillai et al (2010), induction of glucose uptake activity with Gallic acid was performed in 3T3-Li cells, and their results showed that only 10 micromolars of Gallic acid caused 90% increase in glucose uptake activity compared to untreated cells. In the present study the presence of Gallic acid will then also contributes to the anti-diabetes activity of the samples.

The male samples from Mpumalanga province presented a maximum activity without showing any forms of toxicity. The female samples from the same province showed some signs of toxicity at a concentration of 10µg/ml (Figure 5.1b), in that the activity decreased. For further understanding on why the samples from Mpumalanga had the different activities, the NMR spectra of the two samples (males and females) were compared. Visual inspection showed that the two spectra are rather similar (Figure 5.6), and they also showed generally equal intensities
in the levels of metabolites. Nevertheless, the difference in the levels of (-)-Epicatechin 3-O-galloyl ester was observed (Figure 5.7). This metabolite was previously found to have secretagogue activity in the study by Galvez et al., 1991. At this point in this study, it can be appreciated that the higher concentrations of this metabolite in the male samples from Mpumalanga will also contribute to the greater activity presented in this extract. The possibility of other compounds that might become more toxic at high concentrations should be considered, especially in commercial development of the extracts and will warrant future studies.

Figure 5.5: Spectra of the queues Sclerocarya birrea leaves extracts used for blood glucose assay.
Figure 5.6: NMR spectrum of the male and female samples from Mpumalanga. The blue spectrum represents the female samples and the red represent the male samples showing the similarities between the samples.

Figure 5.7: NMR spectrum of the male and female sample from Mpumalanga. The
blue spectrum represents the female and the red represent the males. The difference in the concentration of (-) - epicatechin 3-0-galloyl ester is indicated at 6.90 ppm.

5.5 Concluding remarks

In this chapter it was clearly illustrated that *S. birrea* causes improvement in blood glucose uptake in C2C12 muscle cells, and this can be accounted for, by the blood glucose reduction through glucose utilization by muscle tissue. The effect(s) therefore of *S. birrea* on the blood glucose uptake may be useful for treating patients with insulin insufficiency or insulin resistance, however the nature and mechanism of these effects needs to be investigated. It is important to note that from the results it is confirmed that the leaves of *S. birrea* are effective in uptake of glucose from blood. Furthermore the results also indicate that the male *S. birrea* leaves are more effective as compared to the female samples. The mechanism of action of the male extract in lowering blood glucose is not known; therefore isolation of compounds from the active extracts will be ideal to further understand the differences in activities presented by the extracts from different provinces. The presence of (-)-Epicatechin 3-0-galloyl ester in higher concentrations in the male sample might be a contributing factor in the increased activity of the male sample. The increased concentration in combination with other anti-diabetic compounds such as Gallic acid explains the positive results obtained in the assay.
5.6 LITERATURE CITED


plants traditionally used in South Africa. Journal of Ethnopharmacology (119), pp.81-86.

General discussion

In this study, methanol extracts of *S. birrea* from different provinces were compared and characterized based on multivariate statistical analysis of $^1$H NMR–based metabolomics data. Visual inspection of the spectra from five provinces looked similar confirming the similarities within the *S. birrea* samples from different provinces. A number of the primary metabolites (sugars) and few secondary metabolites (phenolics) were identified. Although there were similarities, the differences were also clear, which can allow the use of some of the metabolites as biomarkers. *Sclerocarya birrea* from different provinces exhibited significant differences in their metabolite profiles with closer investigation. Intense signals in the sugar region mostly in the samples from Gauteng were observed, indicating the presence of glycosylated metabolites and free sugars.

The OPLS-DA scores revealed three different groups. The first group represented samples from Gauteng, the second group represented samples from KwaZulu-Natal and the third group represented the samples from Limpopo, North West and Mpumalanga. The third group which contained samples from three different provinces confirmed that these provinces had more similarities than the samples from other provinces. When the contribution plots were performed, indeed Gauteng presented the sugars as important metabolites in this province.

Liquid Chromatography-Mass Spectroscopy confirmed both the similarities and the differences between the samples from different provinces. The supervised and unsupervised scores showed that the samples were from different places, by separating them into five groups representing the place of origin for each. The metabolites in the VIP scores analysis were mostly annotated with Myricetin 3-0-α-L-rhamnopyranoside, (−)-Epicatechin 3-0-galloyl ester, Quercetin 3-0-α-L-rhamnopyranoside and Quercetin 3-0-β-D(6''-galloyl)galactopyranoside important compounds in samples from North West, Mpumalanga and KwaZulu-Natal.
The application of analysis of untargeted metabolomics data, obtained by LC-MS and $^1$H NMR based profiling of exactly the same biological material, appears to be a powerful strategy in discovering and linking metabolite information which can be used in biomarker discovery and annotation. From the metabolites annotated, some metabolites observed in NMR were not identified in LC-MS and vice versa; this was due to the differences in the degree of sensitivities between the two techniques. Nevertheless, the two techniques still complement each other in that it was also able to detect the common metabolite at 191 m/z (Myristicin or ‘1 Hydroxychavic acetate) as well as myricetin 463 m/z, quercetin 447 m/z and epicatechin glycosides 441 m/z. We compared the performance of OPLS-DA and PLS-DA from $^1$H NMR and LC-MS spectra respectively, to better evaluate the classification potential of both technologies. When compared to the OPLS-DA of NMR data (Figure 3.6), it is apparent that the LC-MS results were in general agreement in both plots, G and KZN samples clustered separately from the other samples.

The similarities between both plots are remarkable given to the observed divergence in metabolites detected. Therefore, it can be proposed that metabolic differences, which separate S. birrea leaves from five provinces belongs to both primary (mostly detected by NMR) and secondary (mostly detected by LC-MS) metabolism. It might also indicate that there are specific metabolic pathways that are responsible for the chemical differences observed for both primary and secondary metabolites resulting in a similar clustering pattern for the LC-MS and NMR analysis. An attempt to identify more metabolites in the samples from five provinces will be done as part of the next stage in this study, for better understanding the similarities and differences between the samples. This shall be done using metabolomics techniques in which the obtained molecular mass from MS will be splitted; a technique known as MS-MS fragmentation. Having the similarities and the differences confirmed by LC-MS and $^1$H NMR, the in-vitro glucose uptake was done on samples from individual provinces. The activity for each province was different, supporting the metabolomics analysis of the samples from five provinces. With regard to Mpumalanga samples, male and female samples in this study were screened separately. The resulting blood glucose uptake assays confirmed the statement by Shackleton et al., 2002 which reported that male S. birrea plants have stronger medicinal values than females. Previous studies showed that aqueous and methanol extracts of the stem bark and roots of S. birrea were found to enhance glucose utilization in cheng liver, C2C12 and 3T3-L1 cells (Van de Venter et al., 2008). The glucose lowering effect of S. birrea using the in-vitro model is known and also supports the C2C12 results of the present study in which the cells were treated with aqueous leaf extracts of S. birrea (Gondwe et al., 2008; Makom et al., 2010; Ojewole, 2003; Dimo et al., 2007). Aqueous
stem bark extracts have been reported to lower blood glucose levels in diabetic rats (Ojewole, 2004; Ojewole 2003). A methanol/methylene chloride extract (150 and 300 mg/kg) has been found to cause a reduction in blood glucose levels and increase plasma insulin levels in diabetic rats (Dimo et al., 2007).

A similar hypoglycemic effect with increased plasma insulin was reported in diabetic rats treated with 300µg/ml aqueous stem bark extract (Makom et al., 2010). Contradicting findings were reported in diabetic rats treated with ethanol stem bark extract (60,120 and 240mg/kg), in which a reduction in blood glucose levels without significantly affecting pancreatic insulin secretion was observed (Gondwe et al., 2008). These reports suggest that the plant extracts exerts their hypoglycemic effect independently of insulin, which supports the findings of the present study.

In the study by Gopalakrishnapillai et al (2010), induction of glucose uptake activity with gallic acid was performed in 3T3-Li cells, and their results showed that only 10 micromolars of gallic acid caused 90% increase in glucose uptake activity compared to untreated cells. On cellular and molecular levels, kaempferol improves glycolysis, glucose uptake, glycogen synthesis, AMPK activity, and Glut4 expression in skeletal muscle, and dietary supplementation of kaempferol significantly ameliorated hyperglycemia and preserved functional islet mass in old adult obese diabetic mice (Alkhalidy et al., 2015). The presence of this metabolite in our extracts may explain the ability of the extracts to take up glucose and therefore the anti-diabetic potential of *S. birrea* leaves. It is evident that not only one metabolite in *S. birrea* leaves possess hypoglycemic potential, therefore as part of the activities in this ongoing project, an attempt to identify more metabolites using metabolomics techniques will be made. This shall be followed by fractionation of active compounds using chromatography, with the aim to isolate metabolite/s with anti-diabetes potential, and if possible identify the most effective compound. The activity presented by the male samples from Mpumalanga, suggests that (-)-Epicatechin 3-O-galloyl ester, contribute significantly to the activity expressed by these samples, since it was present in higher concentrations as compared to the females. According to literature epicatechins displays antidiabetic activities and is one of the most active antioxidant constituents (Berregi et al., 2003). Cho et al. (2006) found that catechins enhanced the expression and secretion of adiponectin, an adipocyte-specific secretory hormone that can increase insulin sensitivity and promote adipocyte differentiation. They also found that catechin treatment increased insulin-dependent glucose uptake in differentiated adipocytes and augmented the expression of adipogenic marker genes. In search of the molecular mechanism responsible for the inducible effect of (-)-catechin on adiponectin expression they found that catechin suppressed
the expression of Kruppel-like factor 7 protein. This protein inhibited the expression of adiponectin and other adipogenesis related genes that play an important role in the pathogenesis of type 2 diabetes. Zaid et al. (2002) found that treatment with epicatechin (1mM) resulted in a significant increase in the activity of erythrocyte Ca\(^{++}\)-ATPase in both normal and type 2 diabetic patiences. According to Jalil et al. (2009) the intake for 4 weeks of a cocoa extract supplemented with polyphenols (2.17 mg epicatechin, 1.52 mg catechin, 0.25 mg dimmer and 0.13 mg trimer g-1 cocoa extract) and methylxanthines (3.55 mg caffeine and 2.22 mg theobromine g-1 cocoa extract) significantly (P, 0.05) reduced the plasma total cholesterol, triglycerides and low-density lipoprotein cholesterol of obese-diabetic rats compared to non-supplemented animals. A study done by Kobayashi et al. (2000) using a rat everted sac showed that tea polyphenols consisting mainly of catechins, epicatechin gallate, epigallocatechin and epigallocatechin gallate inhibited sodium-dependent glucose transporters. This indicated that tea polyphenols interacts with sodium-dependent glucose transporters, possibly playing a role in controlling dietary glucose uptake in the intestinal tract.
6.1 LITERATURE CITED


Journal of endocrinology, 205(1), pp. 79-86.


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Conclusion

Despite the widespread medicinal use of *S. birrea*, the levels of most phytocontituents in leaf extracts are not all standardized and several compounds responsible for the pharmacological properties are not yet fully elucidated. An unbiased multiplex approach combining NMR and MS techniques was adopted to reveal compositional differences in primary and secondary metabolites among *S. birrea* from different geographical regions using multivariate data analysis. Interesting and meaningful differences between the various samples and detection method were identified.

To the best of our knowledge, this study provides the first approach to reveal metabolite compositional differences among *S. birrea* leaves from different provinces and between male and female trees. LC-MS appears to be a suitable method to determine species relationships, whereas NMR is ideal for quantification of metabolites and it is easy to perform. When compared to OPLS-DA plots of NMR data (Figure 3.6) it is apparent that the LC-MS PLS-DA results were in general agreement. In both OPLS-DA and PLS-DA plots, G and KZN samples were clustered separately from the other samples. NMR mostly detected the primary metabolites while LC-MS detected secondary metabolites.

Very few non-flavonoid phenolic compounds were detected, but we could not detect any of the complex flavonoid phenolic compounds. This is probably due to their complexity, and they were probably unable to dissolve in methanol at a very short time used for the extraction. Although many metabolites still need to be identified, the metabolites annotated in this study, provide support for the anti-diabetic activity presented by the samples. The six extracts will be further screened for glucose uptake activity in-vitro with other cell line types (3T3-Li, and C3A) and in-vivo. The samples will also be subjected to chromatographic fractionation, and fractions which show the best biological activity in the bioassay will be selected for further fractionation and subsequent identification of the compounds.
Appendix

Figure 8.1: OPLS-DA plots for 60 *S. birrea* samples with the exclusion of the aromatic region (6.5-8ppm)
Figure 8.2: OPLS-DA plots for 60 *S. birrea* samples with the exclusion of the aliphatics (1-2.5ppm)

Figure 8.3: Important features identified by two-way ANOVA

Figure 8.4: OPLS-DA plots for sixty aqueous *S. birrea* samples.
Figure 8.5: OPLS-DA of the male and female plots of methanol *S. birrea* samples
Table 8.1: C2C12 glucose uptake data.

<table>
<thead>
<tr>
<th>Glucose</th>
<th>Metformin</th>
<th>Insulin</th>
<th>Limpopo</th>
<th>Gauteng</th>
<th>North West</th>
<th>Mpumalanga male</th>
<th>Mpumalanga female</th>
<th>KwaZulu-Natal</th>
</tr>
</thead>
<tbody>
<tr>
<td>1µM</td>
<td>1µM</td>
<td>0.01</td>
<td>0.1</td>
<td>1</td>
<td>10 100</td>
<td>0.01 0.1 1 10 100</td>
<td>0.01 0.1 1 10 100</td>
<td>0.01 0.1 1 10 100</td>
</tr>
<tr>
<td>Activity %</td>
<td>100 120 140</td>
<td>90 110 140 90</td>
<td>90 100 110 120 100</td>
<td>90 100 110 100 98</td>
<td>100 110 110 120 190</td>
<td>96 97 100 95 96</td>
<td>110 120 110 100 60</td>
<td></td>
</tr>
</tbody>
</table>