In vitro assessment of the anti-diabetic activity of Sclerocarya birrea and Ziziphus mucronata

by

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Declaration

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Acknowledgements

“Holy, Holy, Holy is the Lord God Almighty, who was, and is, and is to come. With all creation I sing praise to the King of Kings. You are my everything and I will adore You”

Above all things, thank you Almighty God for every blessing, especially the knowledge that I am nothing without You! Thank you for Your unfailing love, Your grace, Your faithfulness and Your provision! I adore You and I give You all the glory and honour because You are worthy!

My parents, my sister and brothers. Thank you for your love, support and prayers. I love you all! “Behind every successful man there is a wise woman” - mummy thank you for being that wise woman in my life. I greatly appreciate the countless sacrifices you have made in order for me to succeed. I love you! Este trabalho é a realização de muito mais de que um sonho, mais sim a prova de que quando é Deus quem determina, não há nada que possa impedir “os que semeiam com lagrimas, com júbilo ceifarão” Salmos 126:5.

All my friends, with special mention - Marnes Fourie, Gareth Fluke, Tommie Sephton, Eathon Maritz, John and Tanya Webber - for their friendship, love, prayers, encouragement, accountability and support. Pastor’s Heinrich and Nikki Titus from Shofar Christian Church - for their love, friendship and unwavering prayers.

My supervisor’s, Professor Vanessa Steenkamp and Dr Jacob van Tonder, for their supervision, expertise, advice, patience and dedication to this project.

“I may not have much I can offer. Yet what I have is truly Yours. This is my offering Dear Lord. This is my offering to You God. And this is for You. For Your glory and Your fame”
Abstract

Diabetes mellitus is a growing threat to human health. Current pharmacological agents cause undesirable side-effects. Herbal remedies offer the potential for alternative treatment strategies that may prove more cost-effective and devoid of the undesirable side-effects. The purpose of this study was to evaluate the in vitro anti-diabetic activity of aqueous and methanol extracts of Sclerocarya birrea (A. Rich.) Hochst. (Anacardiaceae) and Ziziphus mucronata Willd. (Rhamnaceae), which are traditionally used for the treatment of diabetes mellitus in southern Africa.

Polyphenolic contents of extracts were quantified using the aluminium trichloride and Folin-Ciocalteau methods. The capacity of individual extracts to scavenge both the 2,2’-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) and 2,2-diphenyl-1-picrylhydrazyl radicals was used as a measure of antioxidant activity. The inhibitory activities of the crude extracts of both plants on the enzymes, α-amylase and α-glucosidase, were determined using colorimetric assays. The effects of the crude extracts on cell viability was assessed in C2C12 myotubes, HepG2 hepatocarcinoma cells, 3T3-L1 adipocytes and RIN-m5F pancreatic β-islet cells, using the Sulforhodamine B assay. Fluorescence detection was used to investigate the effects of the crude extracts on glucose uptake in C2C12, HepG2 and 3T3-L1 cells. Insulin secretion was assessed in RIN-m5F cells, using ELISA.

Crude extracts of both plants contained flavonoids and phenols, but flavonoid content was predominantly higher. All the extracts displayed antioxidant activity, with the methanol extract of S. birrea possessing the most potent free radical scavenging ability (IC$_{50}$ = 2.16 µg/ml). Aqueous and methanol extracts of S. birrea displayed significantly ($p < 0.05$) greater inhibition of α-amylase, than the positive control, acarbose. Only the methanol extract of Z. mucronata inhibited α-amylase activity. Furthermore, crude extracts of both plants also displayed potent α-glucosidase inhibitory activity. Most of the crude extracts had low toxicity, where concentrations of 100 µg/ml of crude extract of the plants did not induce 50% cell death.
Although no significant increase in insulin secretion from cultured RIN-m5F cells was noted, the crude extracts of both plants significantly \((p < 0.05)\) increased glucose uptake in C2C12, HepG2 and 3T3-L1 cells, with efficacy significantly \((p < 0.05)\) higher than the positive control, insulin.

From the results, the plant extracts appear to exert their hypoglycaemic effects independently of insulin, via an extra-pancreatic mechanism, possibly involving interactions with the different receptors. An additive hypoglycaemic effect originates from the inhibition of both \(\alpha\)-amylase and \(\alpha\)-glucosidase. The findings of the present study provide evidence that \textit{S. birrea} and \textit{Z. mucronata} possess \textit{in vitro} anti-diabetic activity. Further investigations are required to elucidate the mechanism(s) of action of the crude extracts using more targeted \textit{in vitro} assays.

\textbf{Key words:} \(\alpha\)-amylase, \(\alpha\)-glucosidase, diabetes mellitus, insulin, \textit{Sclerocarya birrea}, type 2 diabetes mellitus, \textit{Ziziphus mucronata}
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Glossary of Abbreviations

A

α  Alpha
ABTS  2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
ABTS\(^{++}\)  2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical
AdipoR1  Adiponectin receptor 1
AMPK  5' adenosine monophosphate-activated protein kinase
AOC  Total antioxidant capacity
APPL1  Adaptor protein containing pleckstrin homology domain, phosphotyrosine-binding domain and leucine zipper motif
ATCC  American Tissue Culture Collection
ATP  Adenosine triphosphate

B

β  Beta

C

c  Concentration
\text{cm}^2  Centimeter squared
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>cAMP</td>
<td>3’-5’-cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular disease</td>
</tr>
<tr>
<td>°C</td>
<td>Degrees Celsius</td>
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<tr>
<td>df</td>
<td>Dilution factor</td>
</tr>
<tr>
<td>DM</td>
<td>Diabetes mellitus</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
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<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>DPPH</td>
<td>2,2-diphenyl-1-picrylhydrazyl</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<td>EMEM</td>
<td>Eagle’s Minimum Essential Medium</td>
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<td>FCS</td>
<td>Foetal calf serum</td>
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<td>Symbol</td>
<td>Term</td>
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<tr>
<td>G</td>
<td>g</td>
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<tr>
<td></td>
<td>Gram</td>
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<tr>
<td>g</td>
<td>Gravity</td>
</tr>
<tr>
<td>Y</td>
<td>Gamma</td>
</tr>
<tr>
<td>GAE</td>
<td>Gallic acid equivalents</td>
</tr>
<tr>
<td>GDM</td>
<td>Gestational diabetes mellitus</td>
</tr>
<tr>
<td>GLUT</td>
<td>Glucose transporter</td>
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<td>H</td>
<td>h</td>
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<td></td>
<td>Hour</td>
</tr>
<tr>
<td>H⁺</td>
<td>Hydrogen ion</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
</tr>
<tr>
<td>I</td>
<td>IBMX</td>
</tr>
<tr>
<td></td>
<td>3-isobutyl-1-methylxanthine</td>
</tr>
<tr>
<td>IC₅₀</td>
<td>Concentration that results in inhibition of 50% of biological activity</td>
</tr>
<tr>
<td>IRS</td>
<td>Insulin receptor substrates</td>
</tr>
<tr>
<td>L</td>
<td>LC₅₀</td>
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<tr>
<td></td>
<td>Concentration of compound that kills 50% of the test animals</td>
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<td><strong>M</strong></td>
<td><strong>Definition</strong></td>
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<td>----------------</td>
<td>--------------------------------------------------------</td>
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<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>m</td>
<td>Mass</td>
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<tr>
<td>mg/dL</td>
<td>Milligram per deciliter</td>
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<tr>
<td>mg/ml</td>
<td>Milligram per milliliter</td>
</tr>
<tr>
<td>min</td>
<td>Minute</td>
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<tr>
<td>ml</td>
<td>Milliliter</td>
</tr>
<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>mmHG</td>
<td>Millimeter of mercury</td>
</tr>
<tr>
<td>mmol/L</td>
<td>Millimole per liter</td>
</tr>
<tr>
<td>µg/ml</td>
<td>Microgram per milliliter</td>
</tr>
<tr>
<td>µl</td>
<td>Microliter</td>
</tr>
<tr>
<td>µm</td>
<td>Micrometer</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
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<tr>
<th><strong>N</strong></th>
<th><strong>Definition</strong></th>
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<tr>
<td>2-NBDG</td>
<td>2-[N-(7-nitrobenz-2-oxa-1,3-diaxol-4-yl)amino]-2-deoxyglucose</td>
</tr>
<tr>
<td>N</td>
<td>Normality</td>
</tr>
<tr>
<td>NC</td>
<td>Negative control</td>
</tr>
<tr>
<td>NCD</td>
<td>Noncommunicable disease</td>
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<tr>
<td>Acronym</td>
<td>Definition</td>
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<td>----------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>NEFA</td>
<td>Non-essential fatty acid</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometre</td>
</tr>
<tr>
<td>nM</td>
<td>Nanomolar</td>
</tr>
<tr>
<td>P</td>
<td>Percentage</td>
</tr>
<tr>
<td>p</td>
<td>Probability</td>
</tr>
<tr>
<td>p70S6K</td>
<td>Small ribosomal subunit protein 6 (p70) kinase</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PC</td>
<td>Positive control</td>
</tr>
<tr>
<td>PDK</td>
<td>Phosphatidylinositol-3,4,5-trisphosphate kinase</td>
</tr>
<tr>
<td>pH</td>
<td>Negative logarithm of the hydrogen ion concentration</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol-3-kinase</td>
</tr>
<tr>
<td>PIP₃</td>
<td>Phosphatidylinositol-3,4,5-trisphosphate</td>
</tr>
<tr>
<td>PKB</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PPAR</td>
<td>Peroxisome proliferator-activated receptor</td>
</tr>
<tr>
<td>PPRE</td>
<td>Peroxisome proliferator response element</td>
</tr>
<tr>
<td>R</td>
<td></td>
</tr>
<tr>
<td>RE</td>
<td>Rutin equivalents</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
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<td>-------------</td>
</tr>
<tr>
<td>RPMI-1640</td>
<td>Roswell Park Memorial Institute Medium-1640</td>
</tr>
<tr>
<td>S</td>
<td>SEM, Standard error of the mean</td>
</tr>
<tr>
<td></td>
<td>SRB, Sulforhodamine B</td>
</tr>
<tr>
<td></td>
<td>SUR, Sulfonylurea receptor</td>
</tr>
<tr>
<td>T</td>
<td>$t$, Test statistic</td>
</tr>
<tr>
<td></td>
<td>T1DM, Type 1 diabetes mellitus</td>
</tr>
<tr>
<td></td>
<td>T2DM, Type 2 diabetes mellitus</td>
</tr>
<tr>
<td></td>
<td>TEAC, Trolox equivalent antioxidant capacity</td>
</tr>
<tr>
<td>U</td>
<td>U/ml, Unit per milliliter</td>
</tr>
<tr>
<td></td>
<td>US, United States</td>
</tr>
<tr>
<td>V</td>
<td>$v$, Volume</td>
</tr>
<tr>
<td></td>
<td>$v/v$, Volume per volume</td>
</tr>
</tbody>
</table>
W

w/v    Weight per volume

WHO    World Health Organization
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Chapter 1: Literature Review

1.1 Noncommunicable diseases

Noncommunicable diseases (NCD’s) are non-infectious and non-transmissible diseases, which are of long duration and generally of slow progression\(^1\). The four most common NCD’s are: cardiovascular diseases (heart attacks and stroke), cancers, respiratory diseases (asthma and chronic obstructed pulmonary disease) and diabetes mellitus\(^1,2\). These diseases are largely due to, and linked by, preventable and modifiable risks factors such as: tobacco use, physical inactivity, excessive alcohol use, unhealthy diets, obesity, raised blood cholesterol and glucose\(^2-5\). Rapid urbanization is occurring worldwide, which is accompanied by shifts in the lifestyle patterns of populations, such as consumption of high calorie diets and lack of physical activity, contributing to an increased prevalence of NCD’s\(^3\).

NCD’s are the leading causes of death globally, killing more people each year than all other causes combined and affecting mostly the world’s low and middle income populations\(^5\). Over a third of cancers and up to 80\% of heart diseases, strokes and type 2 diabetes mellitus, could be prevented by eliminating the shared modifiable risk factors\(^6\). The World Health Organization (WHO) estimated that 36 million deaths of the 56 million deaths that occurred in 2008, were due to NCD’s\(^5\). In Africa alone, NCD’s are projected to cause approximately 3.9 million deaths annually by 2020\(^5\). Deaths by NCD’s are projected to increase by 15\% globally by 2030\(^5\) and by 20\% in Africa\(^5\). NCD’s are not only affecting human health, but are imposing an economic burden on health systems and national incomes, resulting in widening health gaps between and within countries\(^5,6\).

Currently, NCD’s represent the leading threat to human health and development, and remain one of the major challenges of the 21st century. Unless addressed, this epidemic will continue to grow.
1.2 Diabetes mellitus

The first case of diabetes mellitus (DM) was reported around 1500 B.C. in the Ebers Papyrus. DM is a chronic disease, characterized by a group of metabolic diseases associated with high blood sugar (hyperglycaemia), which occurs either when the pancreas does not produce enough insulin or when the body cannot effectively use the insulin it produces. DM, a globally prevalent NCD, is currently the fourth leading cause of death in most developed countries. Without effective prevention and control programs, the incidence of DM will continue to increase worldwide.

1.3 The global burden of diabetes mellitus

In 2011, 366 million people were diagnosed with DM and this number is projected to increase to 552 million by 2030. It is estimated that an additional 183 million people have DM, but remain undiagnosed. There is an explosion of DM worldwide and developing countries are the worst-hit. The International Diabetes Federation reported that 14.7 million people in Africa were identified as having DM, in 2011. Furthermore, Africa has the second highest incidence of undiagnosed diabetes in the world. It is estimated that approximately 4 - 6 million people in South Africa have DM and the majority of these are undiagnosed. South Africa has the second highest number of people with DM in Africa, second to Nigeria. The WHO predicts that, in South Africa, the numbers will triple in the next 15 years.
Figure 1: Population percentage (%) in a country diagnosed with diabetes mellitus, in 2011, amongst 20 - 79 year old individuals \cite{15}.
Figure 2: Population percentage (%) in a country with undiagnosed diabetes mellitus, in 2011, amongst 20 - 79 year old individuals\textsuperscript{16}.
Of all deaths worldwide in 2011, 4.6 million are attributable to DM and its complications (Figure 3). This figure is of similar magnitude to the combined deaths from several infectious diseases including: human immunodeficiency virus, malaria and tuberculosis, that are major public health priorities, and equates to one death every seven seconds. In Africa alone, 344 000 deaths were attributed to DM in 2011.

DM, not only inflicts an extensive economic burden on its victims, but also on healthcare systems. In 2011, healthcare expenditures related to DM accounted for 11% of the world’s total healthcare expenditures. A global estimate revealed that, in 2011, 465 billion US dollars was used to treat DM and prevent its complications, which equates to 1274 US dollars per person. This figure is expected to surpass 595 billion US dollars by 2030. In Africa alone, DM imposed an economic burden of 2.8 billion US dollars on the healthcare systems during 2011.

### 1.4 Classification of diabetes mellitus

An appropriate classification of DM is an important requirement for proper, orderly epidemiologic and clinical research on DM and its management. The process of understanding the aetiology of a disease and studying its natural history, entails the ability to identify and discriminate between its different forms and place them into a rational aetiopathologic framework. DM is currently classified on the basis of the pathogenic process that leads to hyperglycaemia, as opposed to earlier criteria such as age of onset or type of therapy.

DM can be classified into four distinct types, namely:

- Type 1 diabetes mellitus (T1DM)
- Type 2 diabetes mellitus (T2DM)
- Gestational diabetes mellitus (GDM)
- Non-classical causes of DM

The classification of DM not only illustrates its heterogeneity, but also has important implications for both clinical management and biomedical research.
**Figure 3:** Number of deaths in a country attributable to diabetes mellitus, in 2011, amongst 20 - 79 year old individuals\textsuperscript{17}.\n
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1.4.1 Type 1 diabetes mellitus

T1DM, previously known as insulin-dependent DM, immune-mediated diabetes or juvenile/childhood-onset diabetes, is a catabolic disorder caused by an auto-immune reaction where the body’s defence system incorrectly recognises beta (β) cells (the pancreatic insulin-producing cells) as foreign, resulting in selective β-cell destruction. This produces severe or absolute insulin deficiency due to the inability of the β-cells to respond to any insulinogenic stimuli\textsuperscript{11,21–23}. It accounts for 5 - 10% of all cases of DM\textsuperscript{23}. Although the disease can occur at any age, it usually occurs in children or young adults. Common symptoms of T1DM include: fatigue, weight loss, vision disturbances, constant hunger, thirst (polydipsia) and excessive urine excretion (polyuria)\textsuperscript{10,11}.

It is postulated that auto-immune, genetic and environmental factors, and possibly viruses, contribute to the development of T1DM\textsuperscript{24}. There is an inherited predisposition, with a 10-fold increased incidence in first-degree relatives\textsuperscript{21}, and strong associations with particular human leukocyte antigens (HLA)\textsuperscript{21,25}. However, only 10 - 15% of type 1 diabetics have a positive family history\textsuperscript{22}. From genetic studies, it is evident that the two loci that contribute the most to the risk of T1DM are the HLA region and the insulin gene locus\textsuperscript{23}. Viral infections may also cause damage to the β-cells and expose antigens that initiate a self-perpetuating autoimmune cascade\textsuperscript{21}. An individual is considered explicitly diabetic when more than 90% of their β-cells have succumbed\textsuperscript{21}.

1.4.2 Type 2 diabetes mellitus

T2DM, previously known as non-insulin-dependent DM or maturity/adult-onset diabetes, accounts for at least 90% of all cases of DM\textsuperscript{26}. T2DM is a heterogeneous group of disorders characterized by insulin resistance, where the cells in the body do not respond to insulin, and a relative insulin secretion deficiency\textsuperscript{21,22,27}.
People with the following risk factors have a high chance of developing T2DM\textsuperscript{28,29}:

- Excess body weight (especially around the waist)
- High density lipoprotein cholesterol below 35 mg/dL
- Blood levels of triglycerides greater than 250 mg/dL
- Hypertension (greater than or equal to 140/90 mmHg)
- A condition called acanthosis nigricans, characterized by dark and thickened skin around the neck or armpits
- Physical inactivity (less than three times a week)
- Older than 45 years
- Family history of DM
- Ethnicity (Africans are five times more likely to develop T2DM)

T2DM is marked by an impaired insulin secretion, excessive hepatic glucose production and abnormal glucose transport in adipose tissue and skeletal muscle\textsuperscript{20}. This abnormal regulation of carbohydrate metabolism leads to hyperglycaemia. The pancreas compensates for this abnormality by secreting more insulin. However, as the disorder progresses, \( \beta \)-cells will eventually deteriorate in function\textsuperscript{27} and fail to maintain normal blood glucose levels, after which insulin therapy is required\textsuperscript{30}.

Symptoms similar to those of T1DM are seen in T2DM, however, they are often less prominent. As a result of this, T2DM is often diagnosed several years after onset, once secondary complications have already arisen. The diagnosis is usually made from associated complications or incidentally through a routine blood or urine glucose test\textsuperscript{26}. Although T2DM was originally only seen in adults, it is now also occurring in children\textsuperscript{10}.

### 1.4.3 Gestational diabetes mellitus

GDM is characterized by hyperglycaemia with onset or first recognition during pregnancy\textsuperscript{10,31}. Insulin resistance is created by the placenta and placental hormones, and becomes more evident during the last trimester\textsuperscript{22}.
Symptoms similar to those of T2DM are seen in GDM. However, GDM is mostly diagnosed through prenatal screening rather than reported symptoms, as is the case with T1DM and T2DM\(^\text{10}\).

GDM occurs in 1 out of every 25 pregnancies, and is associated with complications in the period immediately before and after birth\(^\text{26}\). Although GDM usually disappears after pregnancy, postpartum women and their offspring are susceptible to develop T2DM later in life\(^\text{24,26}\). Approximately half of women with a history of GDM develop T2DM within 5 to 10 years after delivery\(^\text{26}\). The reason why these women and their offspring are more susceptible to develop T2DM is still not fully understood. Dysfunction of the β-cells has been implicated as a possible cause for why these women develop T2DM\(^\text{32}\), whereas excess intrauterine exposure to glucose has been suggested as a possible reason for why their offspring develop T2DM\(^\text{33}\).

### 1.4.4 Non-classical causes of diabetes mellitus

Despite its low prevalence, this scientific category was created to accommodate diabetic states that are due to clearly established, non-classical causes that can be either genetic or acquired\(^\text{31}\). These causes include the following subtypes: pancreatic diseases, drugs and chemicals, other endocrine diseases, abnormalities of insulin or its receptor and other genetic conditions\(^\text{31}\). Pancreatic diseases such as pancreatitis and haemochromatosis, may result in the onset of DM. Certain drugs and chemicals have been shown to trigger the onset of DM. The latter includes: glucocorticoids, corticotrophin, diuretics, β-blockers, β\(_2\)-agonists, phenytoin, cyclosporine, nicotinic acid, diazoxide and vacor (rodenticide)\(^\text{31}\). DM is classified as an endocrine disease, however, other endocrine diseases such as: Cushing’s syndrome, acromegaly, thyrotoxicosis, phaeochromocytoma, hyperaldosteronism and glucagonoma, may also actuate the development of DM\(^\text{31}\). Abnormalities of insulin or its receptor include: insulinopathies, receptor defects and circulating anti-receptor antibodies, all of which may also trigger the onset of DM\(^\text{31}\). Various genetic conditions have been shown to result in the development of DM. These conditions include: wolfram syndrome, myotonic dystrophy and other muscle disorders, type 1 glycogen storage disease and cystic fibrosis\(^\text{31}\).
1.5 Complications of diabetes mellitus

One of the common effects of uncontrolled DM is hyperglycaemia, which, over time, increases the risk of microvascular and macrovascular complications\textsuperscript{34}. The severity of these complications is determined by the magnitude and duration of hyperglycaemia in DM\textsuperscript{35}.

i. Diabetic eye disease

Diabetes-related eye complications are very frequent\textsuperscript{36} and usually occur together with hypercholesterolaemia and hypertension\textsuperscript{11}. If the complications are not detected at an early stage, or left untreated, visual impairment and blindness can occur\textsuperscript{8,10,11}. About 2\% of people with DM become blind and approximately 10\% develop severe visual impairment\textsuperscript{10}. Common eye diseases that occur in diabetics are: diabetic retinopathy, macular oedema, cataracts and glaucoma\textsuperscript{36,37}.

Diabetic retinopathy is a non-inflammatory, degenerative disease of the retina\textsuperscript{38}, which occurs as a result of damage to the small blood vessels of the retina, in the back of the eye\textsuperscript{36}. About 74\% of the diabetic population develop diabetic retinopathy\textsuperscript{36}. Macular oedema, which occurs in 29\% of people with DM, results from fluids that leak from the vessels in eye and build up in the macula\textsuperscript{36}. This causes the macula to swell and cease to function\textsuperscript{36}. Cataracts occur due to complete or partial opacity of the ocular lens\textsuperscript{38}, and develop at an early stage in diabetic patients\textsuperscript{37}. Glaucoma is associated with increased intraocular pressure, excavation and atrophy of the optic nerve, which results in loss of vision and may result in blindness\textsuperscript{37,38}. A diabetic patient is twice as likely to develop glaucoma than a non-diabetic patient\textsuperscript{37}.

ii. Diabetic nephropathy

Diabetic nephropathy is an inflammatory and degenerative disease\textsuperscript{38}. It is characterised by hyperglycaemia, which causes damage to the glomeruli, affecting the filtration capacity of the kidneys\textsuperscript{39}. 

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Diabetic nephropathy is marked by: increased levels of albumin in the urine (microalbuminuria), elevated blood pressure and deteriorating kidney function\textsuperscript{39}. This condition occurs in a third of the diabetic population\textsuperscript{39} and literature provides evidence that DM is the leading cause of end-stage renal failure\textsuperscript{8,40}.

iii. Diabetic neuropathy

Diabetic neuropathy affects the nervous system\textsuperscript{38,41} and occurs as a result of prolonged exposure to hyperglycaemia and hypertension\textsuperscript{11}. About 60 - 70\% of diabetics have some form of neuropathy\textsuperscript{41}. Diabetic neuropathy can be classified as peripheral, autonomic, proximal or focal. Peripheral neuropathy is the most common type and is characterised by pain, tingling or numbness in the extremities (especially the toes, feet, legs, arms and hands)\textsuperscript{11,41}.

Diabetic foot commonly occurs in diabetics, as the nerves to the feet are the longest in the body and are the ones most often affected by neuropathy\textsuperscript{41}. It is for this reason that diabetic patients have to take special care of their feet and examine them daily. In diabetics, normal sweat secretion and oil production that lubricates the skin of the foot is impaired\textsuperscript{42}. This impairment puts added strain on the skin of the foot during walking, which can cause breakdown of the skin covering the foot, resulting in the formation of sores. These sores are normally unnoticed at the initial stages because of the numbness that diabetics experience. Damage to the blood vessels and impairment of the immune system, all complications arising from DM, result in failure of these sores to heal\textsuperscript{42}. Bacterial infections of the skin, connective tissue, muscles and bones, can also occur. These infections usually develop into gangrene. Poor blood flow to the lower limbs, occurring as a result of the damage to the blood vessels, results in the failure of drugs, such as antibiotics, to reach the site of infection, thereby decreasing their efficacy\textsuperscript{42}. Often, the only treatment for diabetic foot is amputation. Diabetics carry a risk of amputation that is 25 times greater than that of non-diabetics\textsuperscript{41}.
iv. Cardiovascular disease

DM is well established as a major risk factor for cardiovascular disease (CVD)\textsuperscript{43,44}. Patients who have both hypertension and DM, which is a common combination, are twice as likely to develop CVD\textsuperscript{45}. Hyperglycaemia increases the risk for heart attacks, stroke, angina and coronary artery disease\textsuperscript{46}. About 50\% of the diabetic population die of CVD\textsuperscript{10}, which includes myocardial infarction, stroke and congestive heart failure\textsuperscript{11,44}.

The pathophysiology of the link between DM and CVD is complex and multifactorial\textsuperscript{47}. Evidence suggests that although hyperglycaemia, the hallmark of DM, contributes to CVD, it is not the only factor\textsuperscript{47}. Other factors that arise from DM, in combination with other complications include\textsuperscript{47–52}:

- Increased expression of the small dense form of low-density lipoprotein, which easily penetrates the arterial walls and forms stronger attachments.
- Accumulation of foam cells in the subendothelial space by increasing the production of leukocyte adhesion molecules and pro-inflammatory mediators. This eventually leads to endothelial dysfunction.
- Increased levels of endothelin-1, which causes vasoconstriction, induces vascular smooth muscle hypertrophy and activates the renin-angiotensin system.
- Reduced prostacyclin and nitric oxide activity, which enhances platelet aggregation and promotes adhesion.
- Increased coagulation activity, by stimulating production of pro-coagulants, which decreases fibrinolysis, increases thrombus formation and accelerates plaque formation.

The major cause of morbidity and mortality from DM is CVD\textsuperscript{53}. As the prevalence of DM is on the rise, the development of new treatment strategies targeting both the primary prevention of DM and the prevention of diabetic complications like CVD, are currently important research and clinical objectives\textsuperscript{53}. 

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1.6 Factors involved in the pathogenesis of T2DM

1.6.1 Insulin

Insulin was discovered in 1921 by Frederick Banting and Charles Best\textsuperscript{11}. In 1955, the amino acid sequence of insulin was determined by Sanger’s group in Cambridge\textsuperscript{21,54}, making it the first protein ever sequenced\textsuperscript{21}. It is a small protein that contains 51 amino acids arranged in two chains (A and B), linked by disulfide bridges\textsuperscript{9,22,54}. The two chains form a highly complex structure.

Insulin is synthesised as a precursor (preproinsulin) in the rough endoplasmic reticulum and is thereafter transported to the Golgi apparatus, where it undergoes proteolytic cleavage to form proinsulin\textsuperscript{21,55}. Proinsulin is processed within the Golgi apparatus and packaged into granules, where it is hydrolyzed into insulin and a residual fragment called C-peptide, by the removal of four amino acids\textsuperscript{21,22,55} (Figure 4). Insulin co-precipitates with zinc ions to form microcrystal’s due to its low water solubility\textsuperscript{55}. Proinsulin is believed to possess mild hypoglycaemic action\textsuperscript{22}, whereas C-peptide has no known physiological function\textsuperscript{21,22}. Insulin and C-peptide are stored in granules in the β-cells and are co-secreted via exocytosis in equimolar amounts together with smaller and variable amounts of proinsulin\textsuperscript{21,55}. About 95% of the secreted hormone product is insulin, while the remaining 5% is proinsulin\textsuperscript{55}.

Insulin is secreted from the β-cells at a steady basal rate and at a much higher rate in response to a variety of stimuli\textsuperscript{21,22}. This response consists of two phases: an initial rapid phase reflecting release of stored hormone and a slower delayed phase, reflecting both continued release of stored hormone and new synthesis\textsuperscript{21}. This biphasic response is significantly diminished and defective in diabetics\textsuperscript{56,57}.

Glucose is the main factor that regulates the secretion and synthesis of insulin. The β-cells respond to both the absolute glucose concentration and to the rate of change of blood glucose\textsuperscript{21}. Blood glucose levels > 3.9 mmol/L stimulate insulin synthesis primarily by enhancing protein translation and processing\textsuperscript{20}.
Figure 4: Illustration of the chemical structures of proinsulin and insulin\textsuperscript{58}.
Glucose stimulation of insulin secretion begins with its transport into the β-cells by a facilitative glucose transporter\(^{20}\). Glucose is metabolized into glucose-6-phosphate by the enzyme glucokinase. Glucose phosphorylation by glucokinase is the rate-limiting step that controls glucose-regulated insulin secretion\(^{20}\). Further metabolism of glucose-6-phosphate causes increased production of adenosine triphosphate (ATP), which results in membrane depolarization and closure of the ATP-dependant potassium channels (Figure 5). Decreased potassium efflux, leads to membrane depolarization and opening of the voltage-gated calcium channels. This causes the calcium levels in the β-cells to rise that, in turn, triggers the cells to secrete insulin via exocytosis (Figure 5).

Insulin secretory profiles reveal a pulsatile pattern of hormone release, with small secretory bursts occurring approximately every 10 minutes, superimposed upon greater amplitude oscillations of 80 - 150 minutes\(^{20}\). Certain drugs, fatty acids, amino acids (arginine and leucine), the parasympathetic nervous system and gut peptide hormones can also stimulate the release of insulin\(^{21}\).

The liver and kidney are the organs responsible for the degradation of insulin. The liver clears approximately 60% of insulin circulating in the blood, while the kidney removes 35 - 40% of the hormone\(^{22}\). This ratio is reversed in diabetics receiving insulin replacement therapy\(^{22}\). The half-life of circulating insulin is 3 - 5 minutes\(^{22}\).
Figure 5: A model depicting the control of insulin secretion from pancreatic β-cells by glucose\textsuperscript{59}. 
i. The insulin receptor

After being secreted by the pancreas, insulin enters circulation where it binds to specialized receptors. Insulin binds to these receptors, which have only been identified in its major target tissues (liver, adipose tissue and skeletal muscle), with high specificity and affinity\(^{22}\). The insulin receptor is composed of two covalently-linked heterodimers, each compromising an α-subunit, which is located extracellularly and constitutes the recognition site, and a β-subunit, that spans the membrane and contains a tyrosine kinase\(^9,22\) (Figure 6). When insulin binds to the α-subunits on the extracellular surface of the cell, it activates the receptor and brings the catalytic loops of the opposing cytoplasmic β-subunits in close proximity of each other. This results in autophosphorylation of the tyrosine residues and stimulation of tyrosine activity in the β-subunits. The insulin receptor substrates (IRS) are the first proteins to be phosphorylated by the activated receptor tyrosine kinases. After these secondary messengers are phosphorylated, they bind to and activate other kinases. The two main pathways activated through this phosphorylation are the mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3-kinase (PI3K) pathways (Figure 6).

A network of phosphorylations occur within the cell, eventually leading to the translocation of glucose transporters (GLUT’s) (Table 1), especially GLUT-4, to the cell membrane. The latter leads to an increase in glucose uptake, glycogen synthase activity and glycogen formation, and can exert various effects on protein synthesis, lipolysis and lipogenesis, as well as activation of transcription factors that enhance DNA synthesis, and cell growth and division\(^{22}\).
Figure 6: Illustration of how insulin binds to the insulin receptor resulting in the activation of the MAPK and PI3K pathways\textsuperscript{60}. IRS= insulin receptor substrates; MAPK= mitogen-activated protein kinase; P= phosphorylation; PI3K= phosphatidylinositol-3-kinase.
**Table 1:** The various glucose transporters, their tissue distribution and functions.

<table>
<thead>
<tr>
<th>TRANSPORTER</th>
<th>TISSUES</th>
<th>FUNCTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLUT-1</td>
<td>All tissues, especially red blood cells and the brain</td>
<td>Basal uptake of glucose; transport across the blood-brain barrier</td>
</tr>
<tr>
<td>GLUT-2</td>
<td>Liver, kidney, gut and β-cells of pancreas</td>
<td>Regulation of insulin release and other aspects of glucose homeostasis</td>
</tr>
<tr>
<td>GLUT-3</td>
<td>Brain, kidney, placenta and other tissues</td>
<td>Uptake into neurons and other tissues</td>
</tr>
<tr>
<td>GLUT-4</td>
<td>Skeletal muscle and adipose tissue</td>
<td>Insulin-mediated uptake of glucose</td>
</tr>
<tr>
<td>GLUT-5</td>
<td>Gut and kidney</td>
<td>Absorption of fructose</td>
</tr>
</tbody>
</table>
ii. Effects of insulin on its major target tissues

Insulin is an anabolic hormone that controls intermediary metabolism and exerts its actions on three key target tissues: the liver, adipose tissue and skeletal muscle. The hormone suppresses glucose output from the liver and stimulates glucose uptake and metabolism in adipose tissue and skeletal muscle. The effects of insulin on its main target tissues are summarised in Table 2.

In T2DM, the major target tissues of insulin are resistant to its effects, resulting in a reduction in glucose uptake in adipose tissue and skeletal muscle, and an increase in gluconeogenesis in the liver. This causes hyperglycaemia. Furthermore, prolonged exposure to hyperglycaemia causes damage to the β-cells, resulting in reduced insulin secretion. Combined, these disturbances result in a defective control of carbohydrate metabolism and glucose homeostasis.

1.6.2 α-Amylase and α-glucosidase

α-Amylase and α-glucosidase are key enzymes involved in carbohydrate metabolism. α-Amylase cleaves the α-1,4-glucosidic bonds in starch and is responsible for the hydrolysis of starch to a mixture of smaller oligosaccharides consisting of maltose, maltotriose and oligoglucans. These products are further degraded to glucose by α-glucosidase, which acts on the terminal α-1,4-linked glucose residues of these products. Thereafter, glucose is absorbed into the bloodstream (Figure 7).

Post-prandial hyperglycaemia is a feature of T2DM, which can cause damage to the β-cells, resulting in impaired insulin secretion and reduced glucose uptake. The high density of these enzymes in the jejunum and a high content of refined carbohydrates in the diet, are the main reasons why the digestive process usually takes place very rapidly in the small intestine.
Table 2: The metabolic effects of insulin in the liver, on adipose tissue and skeletal muscle\textsuperscript{21}.

<table>
<thead>
<tr>
<th>TYPE OF METABOLISM</th>
<th>LIVER</th>
<th>ADIPOSE TISSUE</th>
<th>SKELETAL MUSCLE</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Carbohydrate metabolism</strong></td>
<td>↓ Gluconeogenesis</td>
<td>↑ Glucose uptake</td>
<td>↑ Glucose uptake</td>
</tr>
<tr>
<td></td>
<td>↓ Glycogenolysis</td>
<td>↑ Glycerol synthesis</td>
<td>↑ Glycolysis</td>
</tr>
<tr>
<td></td>
<td>↓ Glycolysis</td>
<td></td>
<td>↑ Glycogenesis</td>
</tr>
<tr>
<td></td>
<td>↓ Glycogenesis</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Fat metabolism</strong></td>
<td>↑ Lipogenesis</td>
<td>↑ Synthesis of triglycerides</td>
<td></td>
</tr>
<tr>
<td></td>
<td>↓ Lipolysis</td>
<td>↑ Fatty acid synthesis</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>↓ Lipolysis</td>
<td></td>
</tr>
<tr>
<td><strong>Protein metabolism</strong></td>
<td>↓ Protein breakdown</td>
<td></td>
<td>↑ Amino acid uptake</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>↑ Protein synthesis</td>
</tr>
</tbody>
</table>
Figure 7: The roles of α-amylase and α-glucosidase in carbohydrate metabolism. A high density of these enzymes in the jejunum results in rapid carbohydrate digestion, which causes hyperglycaemia (modified from Bischoff\textsuperscript{63}).
A rapid carbohydrate digestion rate leads to an accentuated post-prandial rise in blood glucose (Figure 7). The inhibition of these enzymes delays digestion of carbohydrates and prolongs overall carbohydrate digestion time, which results in a reduction in the rate of glucose absorption and post-prandial blood glucose levels\(^63\)–\(^65\). Hence, retardation of carbohydrate digestion by inhibition of \(\alpha\)-amylase and \(\alpha\)-glucosidase, significantly contributes to the management of T2DM.

### 1.6.3 Oxidative stress

Oxidative stress is defined as excessive production of reactive oxygen species (ROS) in the presence of diminished antioxidant substances\(^66\), resulting in lipid peroxidation, as well as DNA and protein damage\(^67,68\) (Figure 8). Furthermore, oxidative stress can lead to a toxic state, in which cellular function is altered and macromolecules are damaged\(^69\).

During lipid peroxidation damage to the membranes occurs. DNA damage entails breakage of the DNA strands and modification of the base pairs. Protein damage entails damage to enzymes, ion channels and receptors. These events result in cell death and tissue damage, and contribute to the development of DM\(^67,68\). \(\beta\)-Cells are particularly susceptible to the destructive effects of ROS, possibly due to low expression of endogenous antioxidant enzymes, in comparison to other tissues\(^70–76\).

Oxidative stress plays an important role in the aetiology and pathogenesis of various diseases, including DM\(^77,78\). In DM, oxidative stress and ROS production is greatly increased due to prolonged exposure to hyperglycaemia\(^71,74,79\), which co-exists with a reduction in antioxidant capacity\(^80\). Oxidative stress is a known pathogenic mechanism in diabetic complications like diabetic retinopathy, nephropathy and microangiopathy\(^81\), and a trigger for insulin resistance\(^79,82,83\). Furthermore, an increase in oxidative stress results in a reduction in the expression of GLUT-4\(^84\), impairment in glucose uptake in skeletal muscle\(^85\) and adipose tissue\(^84\), and damage to \(\beta\)-cells, which in turn results in impaired insulin secretion\(^86,87\).
Figure 8: Flow diagram indicating the negative effects of oxidative stress, leading to the development of diabetes mellitus (modified from Irshad and Chadhuri\textsuperscript{67} and Nirmala \textit{et al.}\textsuperscript{68}).
Antioxidants include: vitamins (A, C, and E), antioxidant minerals (copper, zinc, manganese and selenium), glutathione, α-lipoic acid, mixed carotenoids, co-enzyme Q10, several bioflavonoids, co-factors (folic acid, vitamins B1, B2, B6, B12) and the enzymes: superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase. Antioxidants decrease the formation of radicals, neutralize existing radicals and limit their harmful effects. Scientific evidence shows that antioxidants may delay the onset and decrease the incidence of DM, reduce elevated serum glucose levels, as well as the secondary complications of DM. Antioxidants have also been shown to prevent the development of insulin resistance, although the mechanisms by which this occurs are still not fully understood. Therefore, more targeted antioxidant therapies, based on the mechanisms of diabetes-induced oxidative stress, might be worth considering as part of the therapeutic strategy to prevent diabetic complications.

1.7 Treatment of diabetes mellitus

Patients with DM are treated by the administration of either oral or subcutaneous medications.

1.7.1 Oral medications

Four categories of oral anti-diabetic agents are available: thiazolidinediones, α-glucosidase inhibitors, biguanides and sulfonylureas.

i. Thiazolidinediones

Thiazolidinediones are a class of oral anti-diabetic drugs that enhance insulin sensitivity by stimulating peroxisome proliferator-activated receptor-gamma (PPAR-γ). Although PPAR-γ receptors are found in adipose tissue, skeletal muscle and the liver, they are more abundant in adipose tissue.
These receptors modulate the expression of the genes involved in glucose and lipid metabolism, are involved in insulin signal transduction, and play a role in the differentiation of adipocytes and other tissues\textsuperscript{9,21,90}. Thiazolidinediones enhance glucose uptake via GLUT-4 and also stimulate lipogenesis resulting in a reduction of circulating non-essential fatty acid (NEFA) concentrations\textsuperscript{90,91}. A reduction in plasma NEFA concentrations is associated with increased glucose utilization and reduced gluconeogenesis\textsuperscript{90}. Thiazolidinediones also reduce the production and activity of the pro-inflammatory cytokines, tumour necrosis factor-\(\alpha\) and interleukin-6, which have been implicated in the development of impaired insulin action\textsuperscript{90,91}, and increase the concentrations of adiponectin\textsuperscript{91}. Adiponectin is an adipose tissue-derived protein (adipocytokine), which inhibits inflammatory pathways, increases insulin sensitivity and improves glucose tolerance\textsuperscript{92}. Its insulin sensitizing effect appears to be due to enhanced suppression of glucose production\textsuperscript{92}. Genetic variants in the adiponectin gene, resulting in reduced levels of adiponectin, have been shown to contribute to the development of insulin resistance and T2DM\textsuperscript{92,93}.

Two commercially available thiazolidinediones are: rosiglitazone and pioglitazone. Weight gain and fluid retention are the most common side-effects.

\textbf{ii. \(\alpha\)-Glucosidase inhibitors}

\(\alpha\)-Glucosidase inhibitors are competitive reversible inhibitors of pancreatic \(\alpha\)-amylase and membrane bound intestinal \(\alpha\)-glucosidase enzymes\textsuperscript{9}. They competitively bind to the oligosaccharide binding site of the \(\alpha\)-glucosidase enzymes, thereby preventing enzymatic hydrolysis\textsuperscript{9}. These inhibitors reduce post-meal glucose excursions by delaying the digestion and absorption of starch, and other oligosaccharides and disaccharides. These compounds are able to lower post-prandial glucose and improve glycaemic control without increasing the risk for weight gain or hypoglycaemia\textsuperscript{9}. \(\alpha\)-Glucosidase inhibitors also indirectly alter glucose-dependant release of intestinal hormones that enhance nutrient-induced insulin secretion\textsuperscript{90}. Overall, \(\alpha\)-glucosidase inhibitors reduce post-prandial insulin concentrations through the attenuated rise in post-prandial glucose levels\textsuperscript{90}.
Acarbose and miglitol, are examples of commercially available α-glucosidase inhibitors. Side-effects include: abdominal pain, bloating, flatulence, loose stools and/or diarrhoea. Hypoglycaemia may occur if an α-glucosidase inhibitor is used in combination with a sulphonylurea or insulin\textsuperscript{90}.

iii. Biguanides

Biguanides lower blood glucose levels by mechanisms that are still not fully understood\textsuperscript{21}. The main therapeutic mechanism is a decrease in hepatic glucose production through activation of the 5′ adenosine monophosphate-activated protein kinase (AMPK) pathway. These drugs also cause: impairment of renal gluconeogenesis, slowing of glucose absorption from the gastrointestinal tract with increased glucose-to-lactate conversion by enterocytes, direct stimulation of glycolysis in tissue, increased glucose removal from blood and reduction of plasma glucagon levels\textsuperscript{22}. At a cellular level, biguanides improve insulin sensitivity via post-receptor signalling pathways\textsuperscript{90}.

The most commonly used biguanide is metformin. Frequent side-effects include: dose-related gastrointestinal disturbances (anorexia, diarrhoea and nausea) and the risk of lactic acidosis\textsuperscript{90}.

iv. Sulfonylureas

Sulphonylureas reduce plasma glucose levels by stimulating the β-cells, which results in the release of insulin. The drug binds to the sulfonylurea receptor-1 (SUR-1), expressed on the β-cell membranes. This results in closure of the ATP-dependant potassium channels leading to depolarization of the plasma membrane and the opening of the voltage-dependant calcium channels. This, in turn, results in calcium influx, granule exocytosis and insulin release\textsuperscript{9}. Sulphonylureas stimulate both the first and second phase of insulin release\textsuperscript{90}.  

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First-generation sulphonylureas include: tolbutamide, chlorpropamide and tolazamide. Second-generation sulphonylureas include: glyburide, glipizide and glimepiride. The latter are prescribed more regularly because they have fewer side-effects and drug interactions\textsuperscript{22}. Hypoglycaemia, which can be severe, prolonged and occasionally life threatening, is a common side-effect\textsuperscript{21,90}.

### 1.7.2 Subcutaneous medications

The objective of subcutaneous insulin therapy is to replicate normal physiologic insulin secretion by replacing the basal overnight fasting between meal and prandial insulin\textsuperscript{22}. However, an exact replication of the normal glycaemic profile is not possible\textsuperscript{22}. The four main types of parenteral insulin available are\textsuperscript{22}:

1. Rapid-acting, with very fast onset and short duration
2. Short-acting, with rapid onset of action
3. Intermediate-acting
4. Long acting, with slow onset of action

The common side-effect of subcutaneous insulin therapy is hypoglycaemia, which can cause brain damage, depending on its severity\textsuperscript{94}.

### 1.7.3 Herbal medicines

People have been using herbal medicines for the control, management and treatment of a variety of ailments, since pre-historic times\textsuperscript{95}. The WHO estimates that approximately 80% of the world’s population routinely makes use of herbal medicines\textsuperscript{96}.

DM was discovered as early as 700 - 200 B.C.\textsuperscript{9} and was managed principally by traditional practices, using medicinal plants\textsuperscript{9,97}. Inadequacies in the current treatments of DM, has led to people increasingly using complementary and alternative medicines\textsuperscript{98}. 

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Worldwide, approximately 400 herbs and plant preparations have been documented to possess beneficial effects for the treatment of DM\textsuperscript{97,98}, but only a small number of these have received medical and scientific evaluation to assess their efficacy\textsuperscript{97,99,100}. The explosion of interest in herbal medicines has prompted the WHO to recommend further investigations into traditional methods for treatment of DM\textsuperscript{101}.

The commonly prescribed first-line anti-diabetic drug for T2DM is the biguanide, metformin\textsuperscript{102,103}. This highly prescribed anti-diabetic drug is derived from the French lilac plant, *Galega officinalis* L. (Fabaceae). It has been known as an anti-diabetic agent since the Middle Ages and is rich in guanidine, a compound which possesses hypoglycaemic effects\textsuperscript{98,103}.

In South Africa, a number of plants are used in traditional medicine for the treatment of DM. Plants for which scientific data could be obtained to support their *in vitro* hypoglycaemic effects are listed in Table 3. However, for the majority of plants there is no or limited scientific evidence for their efficacy in the treatment of DM. A lack of such data also exists for the two plants, *Sclerocaya birrea* and *Ziziphus mucronata*, investigated in this study\textsuperscript{104,105}. 


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Table 3: Plants used traditionally in South Africa with documented *in vitro* anti-diabetic activity.

<table>
<thead>
<tr>
<th>PLANT AND FAMILY</th>
<th>PLANT PART/REMEDY</th>
<th>RESULTS</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Aloe ferox</em> Mill.</td>
<td>Leaves</td>
<td>Increased adiponectin secretion from 3T3-L1 cells<em>16</em></td>
</tr>
<tr>
<td>Asphodelaceae</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Artemisia afra</em> Jacq. ex Willd.</td>
<td>Leaves</td>
<td>Displayed inhibitory activity against α-amylase and α-glucosidase<em>17</em></td>
</tr>
<tr>
<td>Asteraceae</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Brachylaena discolor</em> (DC.)</td>
<td>Leaves, roots and stems</td>
<td>Increased glucose utilization in Chang liver, C2C12 and 3T3-L1 cells<em>16</em></td>
</tr>
<tr>
<td>Asteraceae</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Bulbine frutescens</em> (L.) Willd.</td>
<td>Whole plant</td>
<td>Increased glucose utilization in Chang liver and C2C12 cells<em>18</em></td>
</tr>
<tr>
<td>Asphodelaceae</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Cannabis sativa</em> (L.)</td>
<td>Leaves</td>
<td>Increased glucose utilization in 3T3-L1 cells<em>17</em></td>
</tr>
<tr>
<td>Cannabaceae</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Catha edulis</em> (Vahl) Endl.</td>
<td>Leaves and stem</td>
<td>Increased glucose utilization in C2C12 and 3T3-L1 cells<em>18</em></td>
</tr>
<tr>
<td>Celastraceae</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Catharanthus roseus</em> (L.) G. Don</td>
<td>Leaves and twigs</td>
<td>Increased glucose utilization in Chang liver, C2C12 and 3T3-L1 cells<em>16</em></td>
</tr>
<tr>
<td>Apocynaceae</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Chironia baccifera</em> (L.)</td>
<td>Whole plant</td>
<td>Increased glucose utilization in Chang liver, C2C12 and 3T3-L1 cells<em>16</em></td>
</tr>
<tr>
<td>Gentianaceae</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Cinnamomum cassia</em> (Nees &amp; T.Nees) J.Presl</td>
<td>Leaves and bark</td>
<td>Increased insulin secretion from INS-1 cells<em>10</em> and displayed inhibitory activity against α-amylase and α-glucosidase<em>10</em></td>
</tr>
<tr>
<td>Lauraceae</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Cissampelos capensis</em> (L.)</td>
<td>Leaves</td>
<td>Increased glucose utilization in 3T3-L1 cells<em>18</em></td>
</tr>
<tr>
<td>Menispermaceae</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Clausena anisata</em> (Willd) Hook</td>
<td>Leaves</td>
<td>Displayed inhibitory activity against α-amylase and α-glucosidase<em>11</em></td>
</tr>
<tr>
<td>Rutaceae</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Cucurbita pepo</em> L.</td>
<td>Leaves and bark</td>
<td>Displayed inhibitory activity against α-amylase and α-glucosidase<em>11</em></td>
</tr>
<tr>
<td>Cucurbitaceae</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Cymbopogon citratus</em> (DC.) Stapf</td>
<td>Leaves and bark</td>
<td>Displayed inhibitory activity against α-amylase and α-glucosidase<em>11</em></td>
</tr>
<tr>
<td>Poaceae</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Elaeodendron transvaalense</em> (Burtt Davy) R.H. Archer</td>
<td>Stem-bark and root-bark</td>
<td>Increased glucose uptake in 3T3-L1 cells and displayed inhibitory activity against α-amylase and α-glucosidase<em>12</em></td>
</tr>
<tr>
<td>Celastraceae</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Euclea natalensis</em> A,DC.</td>
<td>Root-bark</td>
<td>Displayed inhibitory activity against α-amylase and α-glucosidase<em>10</em></td>
</tr>
<tr>
<td>Ebenaceae</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Euclea undulata</em> Thunb. var. myrtina (Burch.)</td>
<td>Stem-bark and root-bark</td>
<td>Increased glucose utilization in Chang liver, C2C12 and 3T3-L1 cells and displayed inhibitory activity against α-amylase and α-glucosidase<em>12</em></td>
</tr>
<tr>
<td>Ebenaceae</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Hypoxis hemerocalidea</em> Fisch., C.A.Mey. &amp; Avé-Lali.</td>
<td>Leaves and bark</td>
<td>Displayed inhibitory activity against α-amylase and α-glucosidase<em>11</em></td>
</tr>
<tr>
<td>Hypoxidaceae</td>
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<td></td>
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</tbody>
</table>
Table 3: Continued.

<table>
<thead>
<tr>
<th>PLANT AND FAMILY</th>
<th>PLANT PART/REMEDY</th>
<th>RESULTS</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Momardica balsamina</em> (L.)</td>
<td>Stems and flowers</td>
<td>Increased glucose utilization in Chang liver, C2C12 and 3T3-L1 cells&lt;sup&gt;50&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cucurbitaceae</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Momardica foetida</em> Schumach.</td>
<td>Whole plant</td>
<td>Increased glucose utilization in C2C12 cells&lt;sup&gt;77&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cucurbitaceae</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Nuxia floribunda</em> Benth.</td>
<td>Leaves and bark</td>
<td>Displayed inhibitory activity against α-amylase and α-glucosidase&lt;sup&gt;177&lt;/sup&gt;</td>
</tr>
<tr>
<td>Buddlejaceae</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Ornithogalum longibracteatum</em> Jacq.</td>
<td>Bulbs</td>
<td>Increased glucose utilization in Chang liver and C2C12 cells&lt;sup&gt;184&lt;/sup&gt;</td>
</tr>
<tr>
<td>Liliaceae</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Psidium guajava</em> (L.)</td>
<td>Leaves and roots</td>
<td>Increased glucose utilization in C2C12 and 3T3-L1 cells&lt;sup&gt;90&lt;/sup&gt;, and displayed inhibitory activity against α-amylase and α-glucosidase&lt;sup&gt;187&lt;/sup&gt;</td>
</tr>
<tr>
<td>Myrtaceae</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Pteronia divaricata</em> (P.J. Bergius) Less.</td>
<td>Whole plant</td>
<td>Displayed inhibitory activity against α-amylase and α-glucosidase&lt;sup&gt;172&lt;/sup&gt;</td>
</tr>
<tr>
<td>Compositae</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Ruta graveolens</em> L.</td>
<td>Arial parts</td>
<td>Increased glucose utilization in Chang liver and Chang liver cells&lt;sup&gt;108&lt;/sup&gt;</td>
</tr>
<tr>
<td>Rutaceae</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Schkuhria pinnata</em> (Lam.) Kuntze ex Thell.</td>
<td>Whole plant</td>
<td>Increased glucose uptake in 3T3-L1 and Chang liver cells&lt;sup&gt;172&lt;/sup&gt;</td>
</tr>
<tr>
<td>Asteraceae</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Senna alexandrina</em> Mill.</td>
<td>Leaves and bark</td>
<td>Displayed inhibitory activity against α-amylase and α-glucosidase&lt;sup&gt;177&lt;/sup&gt;</td>
</tr>
<tr>
<td>Fabaceae</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Sutherlandia frutescens</em> (L.) R.Br.</td>
<td>Leaves and shoots Pinosundia&lt;sup&gt;60&lt;/sup&gt;</td>
<td>Prevented insulin resistance in Chang liver cells&lt;sup&gt;113&lt;/sup&gt;, increased glucose utilization in C2C12&lt;sup&gt;114&lt;/sup&gt; and 3T3-L1 cells&lt;sup&gt;115&lt;/sup&gt;, and increased insulin secretion from INS-1 cells&lt;sup&gt;114&lt;/sup&gt;</td>
</tr>
<tr>
<td>Fabaceae</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Tarchonanthus camphorates</em> L.</td>
<td>Leaves and twigs</td>
<td>Increased glucose utilization in Chang liver and C2C12 cells&lt;sup&gt;108&lt;/sup&gt;</td>
</tr>
<tr>
<td>Asteraceae</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Terminalia sericea</em> Burch. ex. DC.</td>
<td>Stem-bark</td>
<td>Displayed inhibitory activity against α-amylase and α-glucosidase&lt;sup&gt;177&lt;/sup&gt;</td>
</tr>
<tr>
<td>Combretaceae</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Tulbaghia violacea</em> Harv.</td>
<td>Whole plant</td>
<td>Increased glucose utilization in Chang liver and C2C12 cells&lt;sup&gt;188&lt;/sup&gt;</td>
</tr>
<tr>
<td>Alliaceae</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Vernonia amygdalina</em> Deile</td>
<td>Leaves</td>
<td>Increased glucose utilization in C2C12 and 3T3-L1 cells&lt;sup&gt;175&lt;/sup&gt;, and displayed inhibitory activity against α-amylase and α-glucosidase&lt;sup&gt;187&lt;/sup&gt;</td>
</tr>
<tr>
<td>Asteraceae</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Vinca major</em> (L.)</td>
<td>Leaves, roots and stems</td>
<td>Increased glucose utilization in Chang liver, C2C12 and 3T3-L1 cells&lt;sup&gt;90&lt;/sup&gt;</td>
</tr>
<tr>
<td>Apocynaceae</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Warburgia salutaris</em> (Bertol.f.) Chiou. Canellaceae</td>
<td>Leaves</td>
<td>Displayed inhibitory activity against α-amylase and α-glucosidase&lt;sup&gt;177&lt;/sup&gt;</td>
</tr>
</tbody>
</table>
i. *Sclerocarya birrea*

*Sclerocarya birrea* (A. Rich.) Hochst. subsp. *caffra* (Sond.) Kokwaro (Anacardiaceae) is commonly known as the marula tree (Figure 9). The tree is widespread in Africa, from Ethiopia to South Africa. In South Africa, it is more dominant in the Ba-Phalaborwa area, in Limpopo\textsuperscript{118}.

This is a medium to large deciduous tree (7 - 19 m in height), with an erect trunk and rounded and spreading crown\textsuperscript{118–121}. The leaves consist of ten or more pairs of leaflets (about 60 mm long)\textsuperscript{120,121}. The bark is rough and grey, and usually peels off in round, flat disks\textsuperscript{119}. It has a mottled appearance due to contrasting grey and pale brown patches\textsuperscript{120,121}. Male and female flowers are usually found on separate trees\textsuperscript{120}. Male flowers are dark red when young and later turn pink or white\textsuperscript{119}. Female flowers are blood red, but change colour from purple to white, after opening\textsuperscript{119}. The fruit is a fleshy round or oval drupe, falling while green and turning yellow on the ground (February - June)\textsuperscript{121}.

The bark contains alkaloids, procyanidins and tannins, while flavonoids and tannins are present in the leaves\textsuperscript{122}. The fruit has a high ascorbic acid content and the seeds contain myristic, oleic, stearic and amino acids\textsuperscript{122}.

The bark of *S. birrea* is traditionally used to treat malaria, diarrhoea, constipation, dysentery, proctitis, stomach ailments, headaches, ulcers, toothache, rheumatism, haemorrhoids, backache, leprosy, infertility and to regulate the gender of the child according to the gender of the tree\textsuperscript{118–122}. In South Africa, different parts of the plant are traditionally used to treat DM\textsuperscript{120,122,123}. 

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Figure 9: Picture of the (A) tree and (B) bark of *Sclerocarya birrea* (A. Rich.) Hochst. subsp. *caffra* (Sond.) Kokwaro. 

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ii. *Ziziphus mucronata*

*Ziziphus mucronata* Willd. subsp. *mucronata* (Rhamnaceae) is commonly known as the buffalo-thorn tree (Figure 10). It is distributed throughout the summer rainfall areas of sub-Saharan Africa, extending from South Africa northwards to Ethiopia and Arabia\(^{124}\).

This tree is small to medium-sized (3 - 20 m high), with a spreading canopy\(^{124}\). The leaves are simple, alternate, ovate or broadly ovate and vary in size from tree to tree\(^{124}\). The leaves are bright green and shiny above, slightly paler beneath, with three main veins arising from the base and with the margins toothed in the upper half\(^{125}\). Young stems are reddish brown\(^{126}\), while the bark is dark grey-brown and smooth when young, becoming darker brown and fissured with age\(^ {127}\). Small yellowish green flowers are borne in clusters above each leaf\(^{125}\). The fruit is a reddish brown smooth shiny spherical drupe, 12 - 20 mm in diameter.

There is a limited amount of literature on the phytochemistry of *Z. mucronata*. Alkaloids commonly referred to as cyclopeptide alkaloids, are known from the *Ziziphus* species\(^{125}\).

The bark of *Z. mucronata* is used to treat chronic coughs, respiratory and stomach ailments, rheumatism and used as an emetic\(^ {128}\). Decoctions of the leaves and roots are applied externally on boils, glandular swellings and sores to promote healing and pain relief\(^{125}\). In South Africa, a tea is prepared from the leaves of the plant combined with powdered material from the *Viscum* species, to treat DM\(^ {129}\).
Figure 10: Picture of the (A) tree\textsuperscript{125} and (B) bark\textsuperscript{124} of *Ziziphus mucronata* Willd. subsp. *mucronata*. 

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1.8 *In vitro* models used to assess anti-diabetic activity

*In vitro* models provide valuable tools during early drug development, as these models are designed to simulate specific events occurring at molecular, sub-cellular and cellular levels\textsuperscript{130}. These models are extremely useful during the screening phase of drug discovery, where a large number of compounds are tested for selected pharmacological activity\textsuperscript{130}. Furthermore, *in vitro* testing is faster, more cost-effective and requires less test material, compared to *in vivo* testing\textsuperscript{131}. These advantages show the vital and growing role *in vitro* testing is playing during early drug development, especially in light of the three R's of animal testing: replacement, reduction and refinement\textsuperscript{131}. The aim of present study was to evaluate the anti-diabetic activity of crude extracts of *S. birrea* and *Z. mucronata* using *in vitro* models. Commonly used models include: inhibition of carbohydrate-digesting enzymes, glucose uptake in liver cells, adipocytes and muscle cells and insulin secretion from pancreatic β-cells.

### i. Inhibition of carbohydrate-digesting enzymes

*In vitro* assays to assess the effects of compounds on the inhibition of the two key enzymes involved in carbohydrate metabolism, namely, α-amylase and α-glucosidase, has been described by several authors\textsuperscript{64,78}. Inhibition of these enzymes results in a delay in the degradation of starch and oligosaccharides, which decreases the absorption of glucose and consequently inhibits post-prandial hyperglycaemia\textsuperscript{132}. Most of these enzymatic assays are colorimetric. Currently, the search for compounds that possess mild α-amylase and strong α-glucosidase inhibitory activity, is regarded as one of the therapeutic approaches for developing novel anti-diabetic agents\textsuperscript{133–135}. 
ii. Glucose uptake in liver cells, adipocytes and muscle cells

Adipose tissue, skeletal muscle and the liver, are the three major target tissues of insulin. T2DM is characterized by insulin resistance and an insulin secretion deficiency\(^\text{11,21,22}\). The latter results in a reduction of glucose uptake in adipose tissue and skeletal muscle, as well as increased gluconeogenesis in the liver. Cell lines commonly used to assess glucose uptake are: L6 (rat myoblasts), C2C12 (mouse myoblast), 3T3-L1 (mouse fibroblast), HepG2 (human hepatocarcinoma) and Chang liver cells (human liver). Glucose uptake assays assess the ability of the experimental compounds to enhance glucose uptake in the major target tissues of insulin, represented by the cell lines. If a compound exerts enhanced glucose uptake in these cell lines, it suggests that the compound has insulin-mimetic properties and could be a potential anti-diabetic drug, which could alleviate hyperglycaemia by enhancing glucose uptake.

Initially the methodology to assess glucose uptake entailed the measurement of labelled radioactive glucose\(^{133}\). Methods employing flow cytometry and fluorescence to measure glucose uptake have been developed and optimized, and are currently in use\(^{136}\). A glucose utilization method was developed in South Africa, at the Nelson Mandela Metropolitan University, by van de Venter et al.\(^{30}\). It entails a scoring system devised to consider both the anti-diabetic activity and toxicity of experimental compounds on three cell lines, namely: Chang liver cells, C2C12 myoblasts and 3T3-L1 adipocytes\(^{30}\). The scoring system is applied to all three cell lines together. For the glucose utilization assay, the scoring criteria was established using the responses observed in the positive controls used in each line, as well as the range of responses obtained with the different crude extracts\(^{30}\). A response < 120% of control is allocated a score of 0; a response between 120% and the mean response of the positive control is scored +1 and any response greater than that of the positive control, +2\(^{30}\). The sum of the three activity scores yields a maximum achievable activity of +6, indicating very strong potential to increase glucose utilization\(^{30}\).
iii. Insulin secretion from pancreatic β-cells

Insulin secretion assays assess the ability of experimental compounds to stimulate insulin secretion from insulin-secreting cell lines. The secreted insulin is measured by radioimmunoassay or enzyme-linked immunosorbent assay (ELISA). The most widely used insulin-secreting cell lines are: RIN (rat pancreatic β-cells), HIT (hamster pancreatic β-cells), β-TC (transgenic mouse pancreatic β-cells), MIN6 (mouse pancreatic β-cells) and INS-1 (rat pancreatic β-cells) cells\textsuperscript{133,137–139}. These cell lines provide valuable tools for the study of molecular events underlying β-cell function\textsuperscript{137} and can be used to identify experimental compounds with insulin-stimulating properties. Insulin secretion is defective in T2DM, resulting in hyperglycaemia. If a compound is capable of significantly increasing insulin secretion from these cell lines, it suggests that the compound could exhibit hypoglycaemic effects by enhancing insulin secretion. Furthermore, it suggests that the compound has the potential to be developed into an anti-diabetic drug.
1.9 Aim

The aim of the study was to assess the in vitro anti-diabetic activity of aqueous and methanol extracts of *Sclerocarya birrea* and *Ziziphus mucronata*.

1.10 Objectives

The objectives of the study were to determine:

1. Total polyphenolic content in terms of phenolic compounds and flavonoids using the Folin-Ciocalteau and aluminium trichloride methods, respectively.
2. Antioxidant activity using the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS\(^{**}\)) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assays.
3. α-Amylase inhibitory activity using an enzymatic assay.
4. α-Glucosidase inhibitory activity using an enzymatic assay.
5. Effect on cell viability using the Sulforhodamine B assay.
7. Effect on insulin secretion in RIN-m5F β-islet cells, using ELISA.
1.11 Project overview

T2DM is a known multi-factorial disorder\textsuperscript{140}. Therefore, a broad understanding of how the different parameters contribute to the development of T2DM is essential in order to better understand the disorder and aid in the development of new targeted therapies. The effect of the various parameters involved in the pathogenesis of T2DM is represented in Figure 11. A high density of the key enzymes involved in carbohydrate metabolism, α-amylase and α-glucosidase, in the jejunum and a diet rich in refined carbohydrates results in rapid metabolism causing hyperglycaemia\textsuperscript{63}, which attenuates oxidative stress\textsuperscript{71,74,79}. Oxidative stress and other factors cause damage to the β-cells, resulting in a reduction of insulin secretion\textsuperscript{61}. This results in decreased glucose uptake in adipose tissue and skeletal muscle, and increased gluconeogenesis in the liver. The end result of the various parameters is hyperglycaemia, which contributes to the development of secondary complications associated with T2DM. The effects of aqueous and methanol extracts of \textit{S. birrea} and \textit{Z. mucronata} were evaluated on each of these different parameters, in order to assess the anti-diabetic activity of these two plants.

The annual global morbidity and mortality figures attributable to DM are on rise, despite the availability of various treatment options\textsuperscript{9}. Since DM is one of the most prevalent NCD’s worldwide, it has become a threat to public health. Moreover, the commonly available drugs have undesirable side-effects. Therefore, new treatment strategies based on medicinal plants are the subject of current focus, in the hope of finding safer alternative treatments for T2DM.
Figure 11: The various parameters involved in the pathogenesis of T2DM, all leading to hyperglycaemia. (images taken from National Aids Treatment Advocacy Project\textsuperscript{91}). T2DM = type 2 diabetes mellitus.
Chapter 2: Materials and Methods

A list of reagents that were used in the study and the suppliers purchased from is provided in Annexure A. A list of all the equipment used and their manufacturers is provided in Annexure B.

2.1 Plant material

Bark from *Sclerocarya birrea* (A. Rich.) Hochst. subsp. *caffra* (Sond.) Kokwaro (Anacardiaceae) (voucher no.: NH 1910) and *Ziziphus mucronata* Wild. subsp. *mucronata* (Rhamnaceae) (voucher no.: NH 1809) were collected from the Limpopo province of the Republic of South Africa. Plant identities were confirmed by Dr N. Hahn, botanist and adjunct research professor at the University of Venda. Voucher specimens are lodged at the Soutpansbergensis herbarium (Makhado, South Africa).

2.2 Preparation of crude extracts

2.2.1 Aqueous extracts

The dried bark of *S. birrea* and *Z. mucronata* were ground to a fine powder. Of this powder, 1.5 g was added to 15 ml of distilled water. The mixture was brewed in boiling water for 10 min. The extracts were allowed to cool and then centrifuged at 1000 g for 10 min, after which the supernatant was removed and filtered through a 0.22 µm filter. The extracts were lyophilized and yields were determined gravimetrically. Prior to *in vitro* use, the extracts were dissolved in culture medium using 0.5% dimethyl sulfoxide (DMSO) and filter sterilized (0.22 µm).
2.2.2 Methanol extracts

A mass of 1.5 g of powdered plant material was added to 15 ml of methanol. The mixture was sonicated for 30 min, put on a shaker for 2 h and then incubated overnight at 4°C. Thereafter, the extracts were centrifuged at 1000 g for 10 min and the supernatant was removed and filtered through a 0.22 µm filter. The solvent was evaporated using a rotavapor at 40°C under reduced pressure, before the crude extracts were resuspended in DMSO. Extracts were diluted with phosphate buffered saline (PBS) solution to a concentration of 1 mg/ml and stored at -70°C.

2.3 Phytochemical analyses

2.3.1 Total phenolic content

Total phenolic content was determined using the Folin-Ciocalteu method as described by Zhang et al.\textsuperscript{141}, with slight modifications. Into a 96-well microplate was pipetted: 20 µl of the extract and 60 µl of Folin-Ciocalteu reagent (2 N). Following incubation of the resultant mixture for 8 min, 100 µl of sodium carbonate (3% w/v) was added. Gallic acid (final concentration 7.8 - 500 µg/ml) was used to establish a calibration curve. Distilled water served as a blank to account for background signal. Plates were incubated for 2 h in the absence of light. Absorbance was measured at 765 nm and results were expressed as milligrams of gallic acid equivalents per gram of plant extract, as calculated by the following equation:

$$Gallic\ acid\ equivalents\ (GAE) = c \times v \times \frac{df}{m}$$

Where, \(c\) = concentration obtained from the calibration curve (mg/ml); \(v\) = volume obtained from initial extraction of plant material (ml); \(df\) = dilution factor of sample; and \(m\) = total weight of extract (g).
2.3.2 Total flavonoid content

Total flavonoid content was determined using the aluminium trichloride assay method of Dewanto et al.\textsuperscript{142}, with minor modifications. Into a 96-well microplate was pipetted: 20 μl of the extract, 20 μl of sodium nitrate (3% w/v), 20 μl of aluminium trichloride (1% w/v) and 100 μl of sodium hydroxide (2 M). Rutin hydrate (15.6 - 1000 μg/ml) was used to establish a calibration curve. Distilled water served as a blank to account for background signal. Absorbance was measured at 570 nm and results were expressed as milligrams of rutin equivalents per gram of plant extract, as calculated by the following equation:

\[
\text{Rutin equivalents (RE)} = c \times v \times \frac{df}{m}
\]

Where, \( c \) = concentration obtained from the calibration curve (mg/ml); \( v \) = volume obtained from initial extraction of plant material (ml); \( df \) = dilution factor of sample; and \( m \) = total weight of extract (g).

2.4 Antioxidant activity

2.4.1 ABTS\(^{•+}\) radical scavenging activity

Antioxidant activity in terms of the ABTS\(^{•+}\) free radical was determined following the methods of Awika et al.\textsuperscript{143} and Erel\textsuperscript{144}, with slight modifications. The ABTS\(^{•+}\) free radical was freshly generated by addition of 3 mM of potassium peroxodisulfate solution to 8 mM ABTS\(^{•+}\). The mixture was left to react in the dark for \( > 12 \) h at room temperature. The working solution was prepared by diluting ABTS\(^{•+}\) stock solution with phosphate buffer (0.2 M, pH 7.4). A preliminary experiment was carried out with the crude extracts (half serial dilutions from 1 mg/ml) to give an indication of the concentrations between which the IC\(_{50}\) value would lie.
From there, a concentration range was selected for further experimentation. Into a 96-well microplate was pipetted: 15 µl of the extract (final concentration 0.1 - 100 µg/ml) and 200 µl of ABTS⁺ solution. Trolox was used as positive control. Plates were incubated for 15 min in the absence of light. Absorbance was measured at 630 nm.

2.4.2 DPPH radical scavenging activity

DPPH radical scavenging activity of the extracts was determined following the method as described by Gyamfi et al., with minor modifications. A preliminary experiment was carried out with the crude extracts (half serial dilutions from 1 mg/ml) to give an indication of the concentrations between which the IC₅₀ value would lie. From there, a concentration range was selected for further experimentation. Into a 96-well microplate was pipetted: 15 µl of the extract (final concentration 0.1 - 300 µg/ml) and 185 µl of DPPH (0.24 mM). Trolox was used as positive control. Plates were incubated for 15 min in the absence of light. Absorbance was measured at 570 nm.

2.5 In vitro enzymatic inhibition assays

2.5.1 α-Amylase inhibition assay

The bioassay method was adopted from Conforti et al., with slight modifications. The principle of this assay entails α-amylase breaking down starch and water into maltose. A colour reagent is then added, which binds to maltose and to produce a colorimetric product that can be measured spectrophotometrically.

A 0.5% (w/v) starch solution was prepared by mixing 0.125 g of potato starch in 25 ml of 20 mM sodium phosphate buffer with 6.7 mM sodium chloride (pH 6.9), for 15 min at 65°C.
The colorimetric reagent was prepared by mixing sodium potassium tartrate solution (12 g of sodium potassium tartrate tetrahydrate in 8 ml of 2 M sodium hydroxide) and 96 mM of 3,5-dinitrosalicylic acid solution (0.88 g of 3,5-dinitrosalicylic acid in 46 ml of distilled water). Into a tube was pipetted: 50 μl of the extract (final concentration 15.6 - 62.5 μg/ml) and 50 μl of porcine pancreatic α-amylase (2 U/ml). After incubation of the mixture at 25°C for 5 min, 100 μl of starch solution was added. After a further incubation of 3 min, 100 μl of colorimetric reagent was added and the mixture was incubated at 85°C for 15 min. The final mixture was diluted with 800 μl distilled water and 100 μl was pipetted into a 96-well microplate. Acarbose was used as positive control (62.5 μg/ml). The absorbance was measured at 540 nm and the α-amylase inhibitory activity was determined using the following equations:

\[
Reaction\ Rate\ (%) = \frac{[\text{starch}]_{\text{test}}}{[\text{starch}]_{\text{control}}} \times 100
\]

\[
Inhibition\ (%) = 100 - \text{reaction rate} \pm SEM
\]

2.5.2 α-Glucosidase inhibition assay

The α-glucosidase inhibition assay was done according to the methods described by Kim et al. and Sancheti et al., with minor modifications. In this assay, α-glucosidase breaks down the substrate 4-nitrophenyl α-D-glucopyranoside into two products namely, α-D-glucose and 4-nitrophenol. The colorimetric product 4-nitrophenol can be measured spectrophotometrically.

Rat intestinal acetone powder (0.5 g) was suspended in 10 ml of 0.9% saline and the suspension was sonicated for 5 min. After centrifugation (5000 g, 30 min, 4°C), the resulting supernatant was used. Into a 96-well microplate was pipetted: 15 μl of rat intestinal α-glucosidase solution (0.1 M, pH 7), 10 μl of the extract (final concentration 15.6 - 62.5 μg/ml) and 50 μl of phosphate buffer (0.1 M, pH 7).
After incubation of the mixture at 37°C for 10 min, 30 µl of substrate (0.5 mM 4-nitrophenyl α-D-glucopyranoside) was added and the plates were incubated at 37°C for 30 min. Acarbose was used as positive control. The absorbance was read at 405 nm and the α-glucosidase activity was calculated using the following equations:

\[
Reaction\ Rate\ (%) = \frac{[4 - \text{nitrophenol}]_{\text{test}}}{[4 - \text{nitrophenol}]_{\text{control}}} \times 100
\]

\[
Inhibition\ (%) = 100 - \text{reaction rate\ (%)} \pm SEM
\]

2.6 Cells and cell maintenance

Ethical approval for conducting experiments on the commercially available cell lines was obtained from the Faculty of Health Sciences Research Ethics Committee of the University of Pretoria (Annexure C). All the cell lines were purchased from the American Tissue Culture Collection® (ATCC).

i. C2C12 cells

The C2C12 mouse myoblast cell line (ATCC® CRL-1772™) is a subclone established from normal adult C3H mouse leg muscle. C2C12 myoblasts undergo in vitro myogenesis to form myotubes. Myogenesis can be induced by changing pre-confluent C2C12 cultures from high serum (10% foetal calf serum (FCS)) to low serum conditions (2 - 5% horse serum), which induces cell cycle exit, commitment to myogenic differentiation and fusion between myoblasts to form myotubes. Myotubes are elongated, multi-nucleated and protein-rich cells, which express myosin, α-actin, troponin, GLUT-4 and other components of the muscle-contractile machinery.
C2C12 cells were cultured in 75 cm$^2$ culture flasks at 37°C under an atmosphere of 5% CO$_2$ and humidified air. The cells were maintained in Dulbecco's Modified Eagle’s Medium (DMEM), containing non-essential amino acids, supplemented with 10% FCS and 1% streptomycin/penicillin. The medium was replaced every 2 - 3 days, as required. The differentiation of C2C12 myoblasts into myotubes was done according to the method of Su and Zhu$^{152}$. The myoblasts were transferred into differentiation medium consisting of DMEM with 5% horse serum for 4 days. The medium was changed every 24 h. Differentiation was confirmed using phase contrast microscopy. In all experiments, C2C12 myotubes were used at day 4.

**ii. HepG2 cells**

The HepG2 human hepatocarcinoma cell line (ATCC® HB-8065™) has been widely used for many years in *in vitro* studies. These cells possess most of the genotypic and phenotypic features, and functions of normal human liver cells$^{153}$. These cells express insulin-like growth factor II and the insulin receptor$^{154}$.

The cells were cultured in 75 cm$^2$ culture flasks at 37°C under an atmosphere of 5% CO$_2$ and humidified air. The cells were maintained in Eagle’s Minimal Essential Medium (EMEM) supplemented with non-essential amino acids and fortified with 10% FCS and 1% streptomycin/penicillin. The medium was changed every 2 - 3 days, as required.

**iii. 3T3-L1 cells**

The 3T3-L1 mouse fibroblast cell line (ATCC® CL-173™) was developed from murine Swiss 3T3 cells$^{155}$. The cell line expresses the insulin receptor$^{155}$. The cell line is commonly used in adipogenesis studies because of its potential to differentiate from fibroblasts to adipocytes, which is achieved by treating the cells with selected pro-differentiative agents. Insulin$^{156,157}$, dexamethasone$^{157}$ and 3-isobutyl-1-methylxanthine (IBMX)$^{158}$, are the most widely used agents to stimulate differentiation into adipocytes that accumulate lipids in the form of lipid droplets.
3T3-L1 cells were cultured in 75 cm² culture flasks at 37°C under an atmosphere of 5% CO₂ and humidified air. The cells were maintained in DMEM supplemented with non-essential amino acids, 10% FCS and 1% streptomycin/penicillin. The medium was replaced 2 - 3 days, as required. Differentiation of 3T3-L1 fibroblasts to adipocytes was done according to a Life Science™ protocol. Briefly, the 3T3-L1 fibroblasts were treated with adipocyte differentiation medium (DMEM, 10% FCS, 1 µM dexamethasone, 0.5 mM IBMX and 10 µg/ml insulin) for 3 days. On day 4, the cells were then treated with adipocyte maturation medium (DMEM, 10% FCS and 10 µg/ml insulin) for a further 4 days. Differentiation was confirmed by phase contrast microscopy. Differentiated 3T3-L1 cells were used at day 8, in all experiments.

iv. RIN-m5F cells

The RIN-m5F rat islet β-cell line (ATCC® CRL-11605™) is a clone derived from the RIN-m rat islet β-cell line. These cells produce and secrete insulin, and produce L-dopa-decarboxylase.

The cells were cultured in 75 cm² culture flasks at 37°C under an atmosphere of 5% CO₂ and humidified air. The cells were maintained in Roswell Park Memorial Institute Medium-1640 (RPMI-1640), supplemented with non-essential amino acids and fortified with 10% FCS and 1% streptomycin/penicillin. The medium was replaced every 2 - 3 days, as required.

2.6.1 Cell harvest and counting

The propagation medium was discarded and the cell culture flask was rinsed twice with sterile PBS, to wash off excess FCS or horse serum. A volume of 2 ml of 0.25% trypsin/versene (w/v) was added to the flask and incubated for 3 - 5 min at 37°C. The cell suspension was transferred to a sterile 15 ml tube, filled with cell culture medium containing 10% FCS to inactivate the trypsin/versene and then centrifuged at 200 g for 5 min.
The supernatant was decanted and the cell pellet resuspended in 1 ml of the appropriate cell culture medium supplemented with 2% FCS. The suspension was mixed thoroughly with an autopipette to obtain a homogenous single-cell suspension. The cell suspension was diluted 1:9 into trypan blue counting solution (0.04% w/v in PBS). The mixture was loaded onto a haemocytometer and the cells were counted using a microscope. The cell suspension was diluted to a density of $1.5 \times 10^5$ cells/ml, $2.5 \times 10^5$ cells/ml, $1.5 \times 10^5$ cells/ml and $2.5 \times 10^5$ cells/ml for C2C12, HepG2, 3T3-L1 and RIN-m5F cells, respectively, in the appropriate medium supplemented with 2% FCS.

2.6.2 Cytotoxicity

In vitro cytotoxicity testing remains one of the crucial assays employed for safety evaluation. The objective of in vitro cytotoxicity testing is to evaluate the intrinsic ability of a compound to kill cells\textsuperscript{161}. Factors that feature in the toxicology of an entity include: the dosage of the compound, the duration of exposure to a compound and the mechanism of toxicity of the compound\textsuperscript{162}. In vitro toxicity can present itself at a cellular level in the following ways: diminished cellular adhesion, decrease in the replication rate, reduction in overall viability and dramatic morphological changes\textsuperscript{163}.

Many different assays have been developed to determine in vitro cytotoxicity. Commonly used assays include: the neutral red uptake assay, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and the sulforhodamine B (SRB) assay. SRB is one of the most widely used assays for in vitro cytotoxicity testing\textsuperscript{164}.

SRB is a bright pink dye that binds to the protein components of fixed cells\textsuperscript{165}. Since SRB binds in a stoichiometric fashion, the amount of dye extracted from the stained cells is directly proportional to the cell mass\textsuperscript{165}.

The SRB assay has several advantages over other assays\textsuperscript{165}:

- The assay is very sensitive.
It allows for a large number of samples to be tested and only requires simple equipment and inexpensive reagents, making it a highly cost-effective method.

It does not rely on a reduction reaction to determine viability, which excludes the possibility of making type I errors (false positives) in the presence of interfering compounds.

Into a 96-well plate was added: 80 μl of the appropriate medium supplemented with 2% FCS. A volume of 100 μl of the cell suspension was added to every well using an autopipette, and incubated for 24 h at 37°C and 5% CO₂ to allow for attachment, in preparation for further experimentation. A volume of 20 μl of the aqueous and methanol extracts (final concentration 0.78 μg/ml - 100 μg/ml) was added to seeded 96-well plates. The plates were further incubated for 24 h. Wells containing 200 μl of medium served as medium control, while wells containing 100 μl medium and 100 μl of the cell suspension served as untreated control.

After the incubation period, the SRB cell enumeration assay was performed according to Vichai and Kirtikara, with slight modifications. A wide concentration range (final concentration 0.78 μg/ml - 100 μg/ml) was selected in order to determine sub-toxic concentrations, which were then used to evaluate the anti-diabetic activity of crude extracts of *S. birrea* and *Z. mucronata* in subsequent cellular assays. Following exposure, a volume of 100 μl of the supernatant was removed from each well. Trichloroacetic acid (100 μl of 30% (v/v)) was then added to all wells, after which the plates were incubated overnight at 4°C. The plates were then washed four times with distilled water, left to dry and 100 μl of SRB (0.057% (w/v)) added to all wells. After a 30 min incubation period, plates were rinsed twice with 1% acetic acid to remove unbound dye. Plates were left to dry, after which 200 μl of tris-base solution (10 mM, pH 10.5) was added to the wells to dissociate bound dye. Plates were incubated for 30 min at room temperature and the absorbance was measured spectrophotometrically at 540 nm (reference wavelength 630 nm) using a microplate reader. Cell viability was calculated using the equation:
% Cell viability = \[
\frac{\text{average sample absorbance} - \text{average blank absorbance}}{\text{average control absorbance} - \text{average blank absorbance}} \times 100
\]

### 2.7 Glucose uptake assay

2-[[N-(7-nitrobenz-2-oxa-1,3-diaxol-4-y]amino]-2-deoxyglucose (2-NBDG) is a fluorescent analogue of 2-deoxyglucose and can be used for directly monitoring glucose uptake in normal, malignant and non-malignant cells, ranging from bacteria, to yeast, to mammalian cells. If a compound possesses insulin-mimetic effects, an enhanced rate of 2-NBDG uptake into the cells will be observed.

The glucose uptake assay was carried out according to Zou et al., with minor modifications. C2C12, HepG2 and 3T3-L1 cells were plated at the appropriate densities in 96-well plates and used at subconfluence after 24 h incubation. Cells were exposed to 50 µl of culture medium in the presence of fluorescent 2-NBDG together with 3 sub-toxic concentrations (final concentration 1.56 - 6.25 µg/ml) of the plant extracts. Insulin was used as positive control (6.25 µg/ml). Medium controls were included to account for any background signal, whereas wells containing medium and cells served as negative control. All control wells received culture medium in the presence of fluorescent 2-NBDG. Plates were incubated at 37°C with 5% CO₂ for 1 h. The 2-NBDG uptake reaction was stopped by removing the incubation medium and washing the cells once with cold PBS. Cells in each well were resuspended in 100 µl cold PBS and the plates were read at an excitation wavelength of 460 nm and emission wavelength of 544 nm. The activity was evaluated by comparing the fluorescence intensities of the experimental wells with the negative and positive controls.
2.8 Insulin secretion assay

ELISA kits were used to quantify the insulin secreted by RIN-m5F cells in the insulin secretion assay. The kit is a solid phase two-site enzyme immunoassay, based on the direct sandwich technique where two monoclonal antibodies are directed against separate antigenic determinants on the insulin molecule. During the incubation step, insulin in the sample reacts with peroxidase-conjugated anti-insulin antibodies and anti-insulin antibodies bound to the microplate well. The washing step removes unbound, enzyme-labelled antibody and the bound conjugate is detected by reaction with 3,3',5,5'-tetramethylbenzidine. The reaction is terminated by the addition of acid to give a colorimetric endpoint that is read spectrophotometrically.\textsuperscript{177}

The insulin secretory activity was carried out according to Nomura et al.\textsuperscript{178}. The RIN-m5F cells were plated into 96-well plates and used at subconfluence after 24 h incubation. Cells were exposed to 100 µl of 3 sub-toxic concentrations (final concentration 1.56 - 6.25 µg/ml) of the plant extracts and incubated at 37°C with 5% CO\textsubscript{2} for 3 h. Medium controls were included to account for any background signal. Wells containing medium and cells served as negative control. Following treatment, plates were centrifuged at 1000 g for 10 min. The insulin concentration of each supernatant was determined according to the manufacturer’s instructions. Absorbance was measured at 450 nm and the activity was evaluated by comparing the absorbance of the experimental wells with the negative control.
2.9 Statistical analyses

All experiments were carried out in triplicate on three separate occasions and results were expressed as mean ± standard error of the mean (SEM). GraphPad Prism 5 was used for all data manipulation and analyses.

For the enzymatic assays, significantly higher differences from the positive control (acarbose) were detected by performing an unpaired \( t \)-Test. Significance was set at \( p < 0.05 \) and is indicated by \( \bullet \).

For antioxidant activity and cell viability, the inhibitory concentration that results in inhibition of 50% of biological activity (IC\(_{50}\)) was determined through the use of non-linear regression (variable slope).

For the glucose uptake assay, significantly higher differences from the negative and positive controls (insulin) were detected by performing an unpaired \( t \)-Test. Significance was set at \( p < 0.05 \) and is indicated by \( \star \) for significantly higher differences than the negative control and \( \bullet \) for significantly higher differences than the positive control.

For the insulin secretion assay, significantly higher differences from the negative control were detected by performing an unpaired \( t \)-Test. Significance was set at \( p < 0.05 \) and is indicated by \( \bullet \).
Chapter 3: Results

3.1 Extraction

The extraction yields of the plants investigated are presented in Table 4. Both crude extracts of *S. birrea* had higher yields than *Z. mucronata*. Furthermore, the methanol extracts of both plants had higher yields than their aqueous counterparts.

3.2 Total polyphenolic content

Polyphenolic content was higher in the methanol extracts than the respective aqueous extracts. Both crude extracts of *S. birrea* and *Z. mucronata* were found to contain a greater concentration of flavonoids than phenols (Table 4). Furthermore, the methanol extracts of both plants contained higher concentrations of polyphenols, than the aqueous extracts. Crude extracts of *S. birrea* contained higher concentrations of polyphenols than *Z. mucronata*.

3.3 Antioxidant activity

The ability of the crude extracts of *S. birrea* and *Z. mucronata* to scavenge the ABTS\(^{**}\) and DPPH radicals is illustrated in Figures 12 and 13. Antioxidant activity was higher in methanol extracts than in aqueous extracts. Crude extracts of *S. birrea* displayed stronger antioxidant activity than those of *Z. mucronata*. Methanol extracts of *S. birrea* displayed the strongest antioxidant activity in both assays, which was stronger than that of the positive control, trolox, a known antioxidant\(^{179}\).
Table 4: Extraction yield, total flavonoid and phenol content of the crude extracts of *S. birrea* and *Z. mucronata*.

<table>
<thead>
<tr>
<th>SPECIES</th>
<th>EXTRACT</th>
<th>EXTRACTION YIELD (%)</th>
<th>TOTAL FLAVONOIDS (mg RE/g)</th>
<th>TOTAL PHENOLS (mg GAE/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. birrea</em></td>
<td>Aqueous</td>
<td>9.10</td>
<td>55.03 ± 0.62</td>
<td>12.30 ± 0.16</td>
</tr>
<tr>
<td></td>
<td>Methanol</td>
<td>15.00</td>
<td>574.50 ± 15.47</td>
<td>109.40 ± 7.18</td>
</tr>
<tr>
<td><em>Z. mucronata</em></td>
<td>Aqueous</td>
<td>4.70</td>
<td>7.70 ± 0.22</td>
<td>4.71 ± 0.20</td>
</tr>
<tr>
<td></td>
<td>Methanol</td>
<td>10.20</td>
<td>172.40 ± 2.91</td>
<td>89.08 ± 5.60</td>
</tr>
</tbody>
</table>

GAE = gallic acid equivalents; RE = rutin equivalents
Figure 12: ABTS$^{+}$ radical scavenging activity of the crude extracts of (A) *S. birrea* and (B) *Z. mucronata.*
Figure 13: DPPH radical scavenging activity of the crude extracts of (A) *S. birrea* and (B) *Z. mucronata*. 
Crude extracts of *S. birrea* displayed slight pro-oxidant effects at low concentrations (0.5 and 1.0 µg/ml) (Figures not shown). Only the methanol extract of *Z. mucronata* displayed slight pro-oxidant effects at low concentrations (0.1 and 0.5 µg/ml) (Figures not shown). The antioxidant capacity, described as IC$_{50}$ values, of the crude extracts of both plants are presented in Table 5.

### 3.4 α-Amylase and α-glucosidase inhibitory activity

The α-amylase and α-glucosidase inhibitory activity of the crude extracts *S. birrea* and *Z. mucronata* are illustrated in Figures 14 and 15. Acarbose belongs to the α-glucosidase inhibitor class of the oral hypoglycaemics and is known to inhibit both α-amylase and α-glucosidase$^{180}$. For this reason, acarbose was used as positive control in both assays.

Aqueous and methanol extracts of *S. birrea* and the methanol extract of *Z. mucronata* inhibited the activity of α-amylase in a dose-dependent manner. The aqueous extract of *Z. mucronata* stimulated α-amylase (Figure 14). At the highest concentration tested (62.5 µg/ml), the aqueous extract of *S. birrea* inhibited α-amylase by 90.8%, which was significantly ($p < 0.05$) higher than that of the positive control, acarbose (76.5%), at a similar concentration. Even though the aqueous extract of *S. birrea* (62.5 µg/ml) displayed the strongest inhibitory activity, the methanol extracts were found to be more potent at lower concentrations (15.6 - 31.2 µg/ml). At concentrations of 31.2 µg/ml and 62.5 µg/ml, the methanol extract of *S. birrea* also significantly ($p < 0.05$) inhibited the enzyme by ~ 80%, being significantly ($p < 0.05$) higher than acarbose.

Both crude extracts of *S. birrea* and *Z. mucronata* inhibited the activity of α-glucosidase in a dose-dependent manner (Figure 15). The methanol extract of *Z. mucronata* (62.5 µg/ml) displayed the strongest inhibitory activity (15.7%), followed by the methanol extract (62.5 µg/ml) of *S. birrea* (15.2%). However, at lower concentrations (15.6 - 31.2 µg/ml) the crude extracts of *S. birrea* displayed stronger inhibitory activity than crude extracts of *Z. mucronata*.
Table 5: Antioxidant capacity (IC\textsubscript{50}) of the crude extracts of *S. birrea* and *Z. mucronata*.

<table>
<thead>
<tr>
<th>SPECIES</th>
<th>EXTRACT</th>
<th>ABTS\textsuperscript{+} RADICAL INHIBITION (IC\textsubscript{50}) (µg/ml)</th>
<th>DPPH RADICAL INHIBITION (IC\textsubscript{50}) (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. birrea</em></td>
<td>Aqueous</td>
<td>5.60</td>
<td>19.60</td>
</tr>
<tr>
<td></td>
<td>Methanol</td>
<td>2.16</td>
<td>9.80</td>
</tr>
<tr>
<td><em>Z. mucronata</em></td>
<td>Aqueous</td>
<td>20.78</td>
<td>169.60</td>
</tr>
<tr>
<td></td>
<td>Methanol</td>
<td>11.18</td>
<td>89.81</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trolox</td>
<td></td>
<td>3.11</td>
<td>16.10</td>
</tr>
</tbody>
</table>
Figure 14: The effects of the crude extracts of *S. birrea* and *Z. mucronata* on the inhibition of α-amylase activity (%). Significantly higher differences from the positive control (PC), acarbose (62.5 µg/ml), are indicated by • representing $p < 0.05$. 

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Figure 15: The effects of the crude extracts of *S. birrea* and *Z. mucronata* on the inhibition of α-glucosidase activity (%). No significantly higher differences from the positive control (PC), acarbose (62.5 µg/ml), were noted.
The methanol extracts of *S. birrea* displayed stronger inhibitory activity than the aqueous extracts of *S. birrea* at all three concentrations tested. The aqueous extracts of *Z. mucronata* were found to be stronger inhibitors of α-glucosidase, than the methanol extracts at lower concentrations (15.6 - 31.2 µg/ml). Crude extracts displayed α-glucosidase inhibitory activity comparable to that of the positive control, acarbose.

### 3.5 Cell viability

The effects of the crude extracts of *S. birrea* and *Z. mucronata* on cell viability in the C2C12, HepG2, 3T3-L1 and RIN-m5F cell lines are presented in Figures 16 - 19.

The crude extracts of both plants displayed limited toxicity in the C2C12 cell line, where concentrations of 100 µg/ml of crude extracts of the plants did not induce 50% cell death (Figure 16). Less than 100% cell viability was observed in C2C12 cells treated with low concentrations (1.56 - 3.13 µg/ml) of the crude extracts, whereas 100% cell viability was observed in all the other cell lines at similar concentrations (Figure 16). A hormetic effect was observed in most survival curves of C2C12 cells, except for the survival curves where the cells were treated with the methanol extract of *S. birrea* and the aqueous extract of *Z. mucronata* (Figures 16A and 16D).

Aqueous and methanol extracts of *S. birrea*, and the aqueous extract of *Z. mucronata* displayed low toxicity in the HepG2 cell line (Figure 17). The methanol extract of *Z. mucronata* displayed significant toxicity (IC$_{50}$ = 85.32 µg/ml) in this cell line (Figure 17C). A hormetic effect was observed in all the HepG2 survival curves (Figure 17).

Similarly, crude extracts of *S. birrea* and *Z. mucronata* displayed low toxicity in the 3T3-L1 cell line (Figure 18). A hormetic effect was also observed in all the 3T3-L1 survival curves (Figure 18).

The majority of the crude extracts displayed low toxicity in the RIN-m5F cell line (Figure 19). Only the methanol extract of *Z. mucronata* displayed significant toxicity (IC$_{50}$ = 65.78 µg/ml) in this cell line (Figure 19C). A hormetic effect was observed in all the RIN-m5F survival curves (Figure 19).
Figure 16: Survival curves of C2C12 cells after 24 h exposure to (A) methanol extracts of *S. birrea*, (B) aqueous extracts of *S. birrea*, (C) methanol extracts of *Z. mucronata* and (D) aqueous extracts of *Z. mucronata*.
**Figure 17:** Survival curves of HepG2 cells after 24 h exposure to (A) methanol extracts of *S. birrea*, (B) aqueous extracts of *S. birrea*, (C) methanol extracts of *Z. mucronata* and (D) aqueous extracts of *Z. mucronata*. 
Figure 18: Survival curves of 3T3-L1 cells after 24 h exposure to (A) methanol extracts of *S. birrea*, (B) aqueous extracts of *S. birrea*, (C) methanol extracts of *Z. mucronata* and (D) aqueous extracts of *Z. mucronata*. 
Figure 19: Survival curves of RIN-m5F cells after 24 h exposure to (A) methanol extracts of *S. birrea*, (B) aqueous extracts of *S. birrea*, (C) methanol extracts of *Z. mucronata* and (D) aqueous extracts of *Z. mucronata*.
3.6 Glucose uptake and insulin secretion

In C2C12 cells, the crude extracts of both plants, caused a significantly ($p < 0.05$) higher increase in glucose uptake compared to the negative control, with the exception of the aqueous extract of *Z. mucronata* (1.56 µg/ml) (Figure 20). In the majority of cases, the crude extracts also caused greater increases in glucose uptake, than the positive control, insulin.

The effects of the crude extracts on glucose uptake were less marked in HepG2 cells and were only significantly ($p < 0.05$) higher than the negative control, for the aqueous extract of *S. birrea* and the methanol extract of *Z. mucronata* at a concentration of 6.25 µg/ml (Figure 21).

The crude extracts of both plants caused a significantly ($p < 0.05$) higher increase in glucose uptake at all concentrations tested in 3T3-L1 cells, in comparison to the negative control (Figure 22). Furthermore, the methanol (1.56 - 6.25 µg/ml) and aqueous (3.13 - 6.25 µg/ml) extracts of *S. birrea* and the methanol (3.13 - 6.35 µg/ml) extract of *Z. mucronata*, exerted significantly ($p < 0.05$) higher increases in glucose uptake, than that observed for insulin.

At all three concentrations tested, neither the aqueous nor the methanol extracts of *S. birrea* and *Z. mucronata*, caused a significant increase in insulin secretion from RIN-m5F cells (Figure 23).
Figure 20: The effects of the crude extracts of *S. birrea* and *Z. mucronata* on glucose uptake in C2C12 cells. Significantly higher fluorescence intensities from the positive control (PC), insulin (6.25 µg/ml), are indicated by * representing $p < 0.05$. Significantly higher fluorescence intensities from the negative control (NC) are indicated by ♦ representing $p < 0.05$. 

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**Figure 21:** The effects of the crude extracts of *S. birrea* and *Z. mucronata* on glucose uptake in HepG2 cells. Significantly higher fluorescence intensities from the negative control (NC) are indicated by * representing $p < 0.05$. No significantly higher differences from the positive control (PC), insulin (6.25 µg/ml), were noted.
Figure 22: The effects of the crude extracts of *S. birrea* and *Z. mucronata* on glucose uptake in 3T3-L1 cells. Significantly higher fluorescence intensities from the positive control (PC), insulin (6.25 µg/ml), are indicated by • representing $p < 0.05$. Significantly higher fluorescence intensities from the negative control (NC) are indicated by * representing $p < 0.05$. 

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Figure 23: The effects of the crude extracts of *S. birrea* and *Z. mucronata* on insulin secretion in RIN-m5F cells. No significantly higher differences from the negative control (NC) were noted.
Chapter 4: Discussion

4.1 Polyphenolic content and antioxidant activity

In the present study, polyphenolic content was higher in methanol extracts than aqueous extracts. Aqueous and methanol extracts of the bark of both *S. birrea* and *Z. mucronata* were found to contain more flavonoids than phenols. *S. birrea* contained ~ 5-fold more flavonoids than phenols, whereas *Z. mucronata* was shown to possess ~ 2-fold more flavonoids than phenols. Methanol extracts of the leaves of *S. birrea* have been reported to contain flavonol compounds equal to three times the amount of phenols\(^1\), which supports the findings of the present study. However, contradictory findings were reported in another study, where 50% aqueous-methanol extracts of young leaves and stems of *S. birrea* were found to contain more phenols than flavonoids\(^2\). As for *Z. mucronata*, ethanol and ethyl acetate extracts were reported to contain a higher phenolic content than the aqueous extracts, when different plant parts were investigated\(^3\). This finding supports the findings of the present study, in that methanol extracts of the plant were found to contain more phenols than the aqueous extracts. Also, aqueous, acetone and ethanol bark extracts of *Z. mucronata* were reported to contain more phenols than flavonoids\(^4\). These results contradict the findings of the present study. Extraction procedure, the season the plant was collected in, the part of the plant that was used and method used to determine polyphenolic content, could possibly explain the conflicting data. Season and plant part have been shown to cause variations in the assessment of polyphenolic content in plants\(^5\).

Oxidative stress plays a role in the pathogenesis of DM. It is increased during prolonged exposure to hyperglycaemia\(^6,7\), which co-exists with a reduction in antioxidant capacity\(^8\). Furthermore, oxidative stress is a known pathogenic mechanism in diabetic secondary complications like diabetic retinopathy, nephropathy and microangiopathy\(^9,10,11\). For these reasons, oxidative stress is a sensible target for preventing the development of both T2DM and its related secondary complications.
Antioxidant assays are commonly used, especially in plant and phytochemical research. However, determining the total antioxidant capacity (AOC) of a compound remains challenging because there are different sources of antioxidants, multiple sources of free radicals and oxidants, as well as multiple mechanisms and responses to different radicals\(^{188}\). It is therefore pertinent to include more than one antioxidant assay when determining AOC of a compound because no single assay will accurately reflect all of the radical sources or all antioxidants in a mixed or complex system.

The ABTS assay measures AOC by radical quenching via hydrogen atom transfer (HAT) reaction\(^{189}\). HAT-based assays measure the ability of an antioxidant to quench free radicals by hydrogen donation. The reaction occurs as follows\(^{188}\):

$$A^* + BH \rightarrow AH + B^*$$

Where \(A^*\) = free radical; \(BH\) = \(H^+\) donor (antioxidant); \(AH\) = neutralized radical; \(B^*\) = oxidized molecule.

The ABTS assay can be applied over a wide pH range and to test a variety of samples since it accommodates both lipophilic and hydrophilic compounds\(^{188}\). However, a disadvantage of this assay, is that it has a short endpoint time and AOC may differ when slow reactions are involved or reactions that have long lag phases, making it difficult to assess the antioxidant activity of a test sample\(^{188}\).

The DPPH assay measures AOC using a single-electron transfer (SET) reaction\(^{189}\). SET-based assays evaluate the ability of an antioxidant to transfer an electron to another molecule. The reaction occurs as follows\(^{188}\):

$$R^* + ST \rightarrow R^{*-} + ST^+$$

Where \(R^*\) = free radical; \(ST\) = electron donor (antioxidant); \(R^{*-}\) = neutralized radical; \(ST^+\) = oxidized molecule.
The DPPH assay is technically simple, however many antioxidants that react quickly with peroxyl radicals may react slowly or may even be inert to DPPH, due to steric inaccessibility\textsuperscript{190}. Also, DPPH is decolorized by reducing agents as well as H\textsuperscript{+} transfer, which also contributes to inaccurate interpretations of AOC\textsuperscript{190}.

In the present study, the IC\textsubscript{50} values obtained in the ABTS and DPPH assays correlated with each other in that methanol extracts always produced greater AOC’s than aqueous extracts, but AOC’s obtained for individual extracts differed substantially between the two assays. The discrepancy seen between the results of the two assays could be explained by the fact that the ABTS•\textsuperscript{+} and DPPH radicals are neutralized via different reaction mechanisms.

All extracts of \textit{S. birrea} were found to possess antioxidant activity. The methanol extract of \textit{S. birrea} displayed the lowest IC\textsubscript{50} value (2.16 µg/ml) in comparison to all other test samples, which implies that it has the strongest radical scavenging activity. Furthermore, the methanol extract also displayed stronger antioxidant activity than the positive control, trolox, in both antioxidant assays. The antioxidant activity of compounds isolated from methanol extracts of \textit{S. birrea} leaves have been reported, with TEAC values of 2.79 mM and 3.01 mM, respectively, which were stronger than the positive control, quercetin\textsuperscript{181}. Similar findings were reported for the same plant part of \textit{S. birrea} when using the cell free DPPH radical scavenging assay, where the methanol extracts displayed stronger DPPH radical scavenging activity (IC\textsubscript{50} = 5.02 µg/ml), than the positive control, ascorbic acid\textsuperscript{182}. Another study reported an IC\textsubscript{50} = 9.41 µg/ml, when acetone stem-bark extracts of the plant were investigated using the DPPH assay\textsuperscript{107}. The antioxidant activity of methanol extracts of \textit{S. birrea} using the DPPH assay as determined by Moyo \textit{et al.}\textsuperscript{182} (IC\textsubscript{50} = 5.02 µg/ml) and Nkobole\textsuperscript{107} (9.41 µg/ml), is comparable with the IC\textsubscript{50} value determined in the present study (IC\textsubscript{50} = 9.8 µg/ml). Furthermore, crude extracts of \textit{S. birrea} displayed stronger antioxidant activity than the positive control, which correlates with the findings of other authors\textsuperscript{181,182}. 
In the present study, all extracts of *Z. mucronata* displayed antioxidant activity, however, the positive control displayed stronger antioxidant activity. Antioxidant activity using the DPPH assay revealed that *Z. mucronata* bark possessed antioxidant activity, with IC$_{50}$ values of 64.6 µg/ml, 48.2 µg/ml and 42.2 µg/ml, for aqueous, acetone and ethanol extracts, respectively, being weaker than the positive control, vitamin C$^{184}$. The same authors also assessed antioxidant activity using the ABTS assay and their findings revealed that the plant possessed antioxidant activity, with IC$_{50}$ values of 42.9 µg/ml, 31.7 µg/ml and 30.6 µg/ml, for acetone, aqueous and ethanol extracts, respectively$^{184}$. Both the acetone and ethanol extracts of *Z. mucronata* displayed stronger antioxidant activity than the positive control, butylated hydroxytoluene$^{184}$. Antioxidant activity of aqueous, ethanol and ethyl acetate extracts from different parts of *Z. mucronata* indicated that the ethanol extracts displayed the strongest antioxidant activity, performing better than the positive controls, trolox and ascorbic acid$^{183}$. IC$_{50}$ values of 18.7 µg/ml and 29.1 µg/ml have been reported for methanol root extracts of *Z. mucronata*, analyzed using the ABTS and DPPH assays respectively$^{191}$. Moreover, the extract displayed stronger antioxidant activity than the positive control, trolox$^{191}$. The IC$_{50}$ value determined for the methanol extract of *Z. mucronata* (IC$_{50} =$ 11.2 µg/ml) in the present study using the ABTS assay, is comparable to the findings of Adewusi and Steenkamp$^{191}$ (IC$_{50} =$ 18.7 µg/ml). Furthermore, crude extracts of *Z. mucronata* also displayed weaker antioxidant activity than the positive control, which correlates with other findings in literature$^{184}$.

A noteworthy observation was that both extracts of *S. birrea* and the methanol extract of *Z. mucronata* also displayed pro-oxidant properties at low concentrations (0.1, 0.5 and 1.0 µg/ml). Evidence suggests that polyphenolics may exhibit pro-oxidant effects depending on factors such as: metal-reducing potential, chelating behaviour, pH and solubility characteristics$^{192}$. The crude extracts of both plants displayed antioxidant and pro-oxidant effects in the present study, indicating that under certain conditions these extracts could be more of an oxidative risk than benefit, warranting further study about the relationship of their bioactivity and their physiological antioxidant and pro-oxidant properties.
The established use of a *S. birrea* preparation, diabetisane, for the treatment of DM has been attributed to the antioxidant activity of compounds such as flavonol and phenolic derivatives\textsuperscript{181}. Flavonoids have been proposed as the most likely contributors to antioxidant activity of *Z. mucronata*\textsuperscript{184}. Flavonoids are known to possess strong radical scavenging abilities and antioxidant activity\textsuperscript{193}.

It is evident that crude extracts of both plants possess antioxidant activity and are abundant in flavonoids, which could possibly be responsible for the mode in which the plants exert their antioxidant effects. The findings of the present study, suggest that constituents contained in both plants could therefore be used to alleviate oxidative stress induced by chronic hyperglycaemia to prevent/stall diabetic secondary complications, improve insulin sensitivity, reduce damage to the pancreatic $\beta$-cells, and perhaps even delay the progression of DM itself. The findings of the present study are supported by other authors, where antioxidants have been shown to have beneficial effects in the treatment of DM\textsuperscript{9,87}.

### 4.2 $\alpha$-Amylase and $\alpha$-glucosidase inhibitory activity

One way of controlling T2DM is to limit intestinal carbohydrate digestion. Although the gastrointestinal tract does not play a major role in the pathogenesis of T2DM, modification of its physiological activities can be used to decrease post-prandial hyperglycaemia via inhibition of the carbohydrate hydrolyzing enzymes, $\alpha$-amylase and $\alpha$-glucosidase\textsuperscript{194,195}. Inhibition of these enzymes causes delayed carbohydrate digestion and prolonged overall carbohydrate digestion time, resulting in a reduction in the rate of glucose absorption and consequently blunting the post-prandial rise in blood glucose\textsuperscript{64,132}.

Acarbose and miglitol are examples of $\alpha$-glucosidase inhibitors, which are currently in clinical use. However, their prices are high and clinical side-effects such as abdominal distention, flatulence, meteorism and diarrhoea can occur\textsuperscript{195,196}. The search for new inhibitors of these enzymes from natural sources with less or no side-effects, is currently being investigated as an alternative to the synthetic drugs\textsuperscript{65}.
Both the aqueous and methanol extracts of 62.5 µg/ml *S. birrea* were more effective inhibitors of α-amylase than the positive control, acarbose, at the same concentration. Both extracts also displayed substantial α-glucosidase inhibitory activity (> 15%). The aqueous extracts were stronger inhibitors of α-amylase, whereas α-glucosidase was inhibited mostly by the methanol extracts. The present findings are supported by Mogale *et al.*\(^{197}\), where acetone, aqueous, hexane, and methanol stem-bark extracts of *S. birrea* were shown to have potent *in vitro* inhibitory activity against α-amylase (> 50%). Furthermore, both the acetone and methanol extracts inhibited the enzyme, more than the positive control, acarbose. These authors also reported that the acetone and hexane extracts displayed strong α-glucosidase inhibitory activity (> 80%). Acetone stem-bark extracts of *S. birrea* have also been reported to inhibit α-amylase and α-glucosidase activities (IC\(_{50} = 100\) µg/ml)\(^{107}\), which also supports the findings of the present study. Overall, crude extracts of *S. birrea* displayed the strongest α-amylase and α-glucosidase inhibitory activity. Compounds that possess very strong α-amylase inhibitory activity have been shown to cause similar side-effects to that of acarbose\(^{198}\). An ideal α-glucosidase inhibitor should possess mild α-amylase and strong α-glucosidase inhibitory activity\(^{135}\). It is possible that lower concentrations of the methanol extract would achieve mild α-amylase inhibitory activity, while still displaying strong α-glucosidase inhibitory activity. It has been suggested that α-amylase is inhibited mostly by polar metabolites, probably pseudosaccharides, and α-glucosidase by non-polar metabolites, contained within *S. birrea*\(^{197}\). Furthermore, the hypoglycaemic effects of *S. birrea* in *in vivo* models, have been postulated to be mediated through the inhibition of α-glucosidase\(^{197}\).

In the present study, both the aqueous and methanol extracts of the bark of *Z. mucronata* also displayed substantial inhibitory activity against α-glucosidase, whereas only its methanol extract displayed inhibitory activity against α-amylase. To date, this is the first study to investigate the effect of crude extracts of *Z. mucronata* on the activity of α-amylase and α-glucosidase, making it necessary to compare data with the genus *Ziziphus*. In the present study, the methanol extract of *Z. mucronata* displayed weak α-amylase inhibitory activity (< 30%), while the aqueous extract stimulated the enzyme.
Potent α-amylase inhibitory activity has been reported for ethanol extracts from the leaves of *Z. spina-christi* (IC$_{50}$ = 300 µg/ml), which supports the findings of the present study in that methanol extracts of *Z. mucronata* displayed α-amylase inhibitory activity, however it was weak.

The mechanism through which the crude extracts of both plants exert their inhibitory effects could be attributed to the presence of a high content of polyphenols, predominantly flavonoids. Plant polyphenols have been shown to inhibit α-amylase and α-glucosidase activities, and polyphenols such as anthocyanins, flavonoids and tannins are known to possess potent α-glucosidase inhibitory activities.

### 4.3 Cell viability

In the present study, low toxicity was evident in all four cell lines treated with crude extracts of *S. birrea*, with IC$_{50}$ values > 100 µg/ml. This low toxicity (IC$_{50}$ values > 100 µg/ml) observed in the present study is supported by findings from other *in vitro* studies. Dichloromethane/methanol extracts of the bark of *S. birrea* have been reported to display toxic effects to Chang liver cells and 3T3-L1 cells. Aqueous extracts of the roots of this plant were also toxic to 3T3-L1 cells. The aqueous and dichloromethane/methanol extracts of the stem, aqueous extract of the bark and dichloromethane/methanol extract of the roots were not toxic to Chang liver cells or 3T3-L1 cells. Neither was the aqueous extract of the root found to be toxic to Chang liver cells. Low toxicity (LC$_{50}$ (concentration of compound that kills 50% of the test animal) > 5 mg/ml) was reported for aqueous, hexane and methanol extracts of the bark of *S. birrea*, using the brine shrimp lethality assay. Contradictory results were found for aqueous extracts of the stem-bark of *S. birrea*, where significant death were noted in INS-1E (rat pancreatic) cells exposed to 50 µg/ml of extract for 24 h. The effect of ethanol extracts of the stem-bark of *S. birrea* on cell viability on LLC-PK1 (pig kidney) and MDBK (kidney from bovine) cells treated with various concentrations of *S. birrea* (100 - 1000 µg/ml) for different durations (24, 48 and 72 h), indicated a dose-dependent decrease in cell viability.
From literature, toxicity appears to be related to both the extraction solvent and plant part used. Furthermore, results of the present study are also supported by in vivo findings, where aqueous stem-bark extracts of *S. birrea* were found to be relatively non-toxic and safe in small mammals\(^\text{207,208}\).

As far as can be determined, this is the first study presenting toxicity data for crude extracts of the bark *Z. mucronata* in C2C12, HepG2, 3T3-L1 and RIN-m5F cells. Only the methanol extracts of *Z. mucronata* showed significant toxicity, specifically in HepG2 and RIN-m5F cells, suggesting potential selective toxicity for cells of a liver and pancreatic β-islet origin, however, this needs to be confirmed by other researchers. Similar findings of low toxicity (> 100 µg/ml) are reported by other in vitro studies, where the SH-SY5Y neuroblastoma cell line and the brine shrimp lethality test were used\(^\text{205,209}\). Methanol extracts of the roots of *Z. mucronata* have been reported to maintain cell viability at 75% in the HeLa (cervical carcinoma), HT29 (colon adenocarcinoma) and A431 (skin carcinoma) cell lines when exposed to 100 µg/ml\(^\text{210}\). Furthermore, results of the present study are also supported by in vivo studies, where no acute toxicity in small mammals treated with crude extracts of the *Ziziphus* genus was observed\(^\text{211,212}\).

Slight decreases in cell viability was observed at the highest concentration (100 µg/ml) tested of the crude extracts of *S. birrea* and *Z. mucronata*, while low concentrations (1.56 - 12.50 µg/ml) caused stimulation of growth in the different cell lines tested. This hormetic effect is a dose-response phenomenon, which is characterised by a counter intuitive switch over from high-dose inhibition to low-dose stimulation\(^\text{213}\). Molecular mechanisms that account for hormetic responses are numerous, being most thoroughly documented in pharmacological literature, with respect to receptor-based systems\(^\text{214}\).

Contrary to results obtained with the rest of the tested cell lines, cell viability was < 100% in C2C12 cells treated with low concentrations (1.56 - 3.13 µg/ml) of both aqueous and methanol extracts of the plants. Furthermore, the hormetic effect was not observed in the survival curves of C2C12 cells treated with the methanol extract of *S. birrea* and the aqueous extract of *Z. mucronata*.
This could be due to the fact that the C2C12 cell line is the fastest growing cell line, with a doubling time of 12 h\textsuperscript{215,216}, which explains why these observations were only noted in this specific cell line. It is possible that low-dose exposure to the crude extracts of the plants inhibited cellular proliferation, rather than causing cell death. This decrease in proliferation rate would manifest as a slight decrease in cell viability, compared to negative controls in which cell proliferation continued uninhibited.

### 4.4 Glucose uptake and insulin secretion

Various glucose uptake assays exist, employing different methodologies\textsuperscript{133,136}. In the present study, glucose uptake was monitored in C2C12, HepG2 and 3T3-L1 cells using 2-NBDG, a fluorescent analogue of 2-deoxyglucose\textsuperscript{166}. 2-NBDG has been shown to provide a good optical marker of glucose metabolism\textsuperscript{167}. An increase in fluorescence intensity corresponds to an enhanced uptake of 2-NBDG into the cells, which indicates that a compound possess insulin-mimetic properties.

Insulin secretion was assessed in the RIN-m5F cell line using ELISA. This cell line is a superior model cell line to use when assessing the ability of glucose-containing crude extracts to stimulate insulin secretion because, unlike other pancreatic β-cell lines, the RIN-m5F cell line is unresponsive to the insulin-secretory effects of glucose\textsuperscript{133}. This eliminates the possibility of making type I errors (false positives) due to contaminating glucose originating from the culture medium.

In the present study, crude extracts of \textit{S. birrea} caused significantly ($p < 0.05$) higher increases in glucose uptake in C2C12 and 3T3-L1 cells in comparison to the positive control, insulin. The latter may implicate that the crude extracts of \textit{S. birrea} may be potent therapies in preventing and alleviating hyperglycaemia. The effects of the crude extracts were less marked in HepG2 cells and were only significantly higher ($p < 0.05$) at a concentration of 6.25 µg/ml for the aqueous extract of \textit{S. birrea}. No significant increase in insulin secretion was observed in RIN-m5F cells treated with both crude extracts of the plant at concentrations of 1.56 - 6.25 µg/ml.
Aqueous and dichloromethane/methanol extracts of the bark, stem and roots of *S. birrea* were found to enhance glucose utilization in Chang liver, C2C12 and 3T3-L1 cells (12 and 50 µg/ml)\(^{30}\), findings that support the results of the present study. The glucose-lowering effects of *S. birrea* using *in vivo* models is known\(^{104,206,208,217}\). Aqueous stem-bark extracts have been reported to lower blood glucose levels in diabetic rats\(^{207,208}\). 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\(^{217}\). A similar hypoglycaemic effect with increased plasma insulin, was reported in diabetic rats treated with aqueous stem-bark extracts (300 mg/ml)\(^{206}\). Contradictory findings were reported in diabetic rats treated with ethanol stem-bark extracts (60, 120 and 240 mg/kg), where a reduction in blood glucose levels, without significantly affecting pancreatic insulin secretion was observed\(^{104}\).

This report suggests that the plant extracts exert their hypoglycaemic effects independently of insulin, which supports the findings of the present study.

In the present study, both the aqueous and methanol extracts of *Z. mucronata* caused a significantly (*p* < 0.05) higher increase in glucose uptake in C2C12 and 3T3-L1 cells, outperforming the positive control, insulin, warranting further investigation into its anti-diabetic potential. The crude extracts showed less marked effects on glucose uptake in HepG2 cells and was only significantly (*p* < 0.05) higher at a concentration of 6.25 µg/ml for the methanol extract of the plant. The crude extracts of the plant (1.56 - 6.25 µg/ml) failed to cause a significant increase in insulin secretion from RIN-m5F cells. Literature with regards to the effects of crude extracts of *Z. mucronata* on glucose uptake *in vitro* was not found. For this reason, observations from the present study were compared to *in vivo* studies that utilized plants from the *Ziziphus* species. Aqueous (3% and 6% decoctions) and hydro-alcoholic (100 mg/kg) extracts of the leaves of *Z. jujuba* have been reported to reduce blood glucose levels in diabetic rats\(^{218,219}\). Aqueous extracts of the leaves of *Z. mauritiana* (100 and 150 mg/kg) have been shown to decrease blood glucose levels in diabetic rabbits\(^{220}\). Alcoholic extracts of the leaves of *Z. sativa* (100 - 400 mg/kg) have also been reported to lower blood glucose levels in diabetic rats\(^{211}\). Butanol and ethanol extracts (200 mg/kg) of the leaves of *Z. spina-christi* have been shown to decrease plasma glucose levels by increasing plasma insulin levels in diabetic rats\(^{199,212}\).
The findings of these *in vivo* studies correspond to the results obtained in the present study in that the animals demonstrated increased glucose utilization following treatment with crude extracts of members from the *Ziziphus* species.

The findings of the present study confirm that the crude extracts of both plants possess hypoglycaemic effects. However, the crude extracts had no significant effect on insulin secretion from RIN-m5F cells. Possible reasons for this observation include: too low test concentrations of the crude extracts and too high passage number of the cell line. The latter have been shown to decrease insulin secretion in the RIN-m5F cell line\(^{221}\). Results from the present study suggest that the crude extracts of both plants exert their hypoglycaemic effects independently of insulin.

### 4.6 Possible mechanism(s) of action of the test compounds

T2DM is a multi-factorial disorder\(^{140}\) stemming from changes in many metabolic pathways in different tissues, many of which are potential targets for drug treatment. When screening for a potential anti-diabetic compound, it is important to include as many of these potential targets as possible to ensure that possible “hits” are not excluded\(^{30}\).

In the present study, crude extracts of *S. birrea* and *Z. mucronata* displayed inhibitory activity against the key enzymes in carbohydrate digestion, α-amylase and α-glucosidase, and significantly (*p* < 0.05) increased glucose uptake in C2C12, HepG2 and 3T3-L1 cells. The crude extracts displayed no significant increase in insulin secretion from RIN-m5F cells. Based on the findings of the present study, it can be hypothesized that the crude extracts of both plants exert their hypoglycaemic effects independently of insulin, via an extra-pancreatic mechanism.

When insulin levels are low, GLUT-4 is stored in vesicles in adipose and skeletal muscle. Once insulin binds to its cell-surface receptor, it initiates intracellular signalling cascades. The MAPK and PI3K pathways are commonly activated through this process. The activation of PI3K involves a linkage to receptor activation of IRS’s (Figure 24).
Figure 24: Pathway of GLUT-4 translocation in adipose tissue and skeletal muscle.\(^{222}\)

IR = insulin receptor; IRS = insulin receptor substrates; PI3K = phosphatidylinositol-3-kinase; PKB = protein kinase B; PKC = protein kinase C.
Activated PI3K phosphorylates membrane phospholipids, the major product being phosphatidylinositol-3,4,5-trisphosphate (PIP$_3$). PIP$_3$, in turn, activates the enzyme protein kinase B (PKB), also known as Akt. Akt1, Akt2, and Akt3 are the three members of the PKB/Akt family of serine/threonine kinases. Akt2 is important in insulin-mediated glucose homeostasis. Additional enzymes activated by insulin receptor signalling are PIP$_3$-dependent kinase (PDK), some isoforms of protein kinase C (PKC) and small ribosomal subunit protein 6 (p70) kinase (p70S6K). Activation of PKB and PKC leads to translocation of GLUT-4 to the plasma membrane, resulting in increased glucose uptake. Once glucose enters the cell, it is phosphorylated by hexokinase to form glucose-6-phosphate, which can be converted into glycogen and stored, or it can enter glycolysis where it is broken down into pyruvate.

In the present study, C2C12 myoblasts were differentiated into myotubes before execution of the glucose uptake assay. Myotubes are elongated cells, that express GLUT-4 and other components of the muscle-contractile machinery. 3T3-L1 adipocytes also express high densities of GLUT-4, as well as the insulin receptor. As described earlier, GLUT-4 is the transporter responsible for insulin-mediated uptake of glucose. The findings of the present study suggest that the manner in which the crude extracts exert their hypoglycaemic effects in C2C12 and 3T3-L1 cells could possibly be mediated via interactions with the insulin receptor, resulting in the translocation of GLUT-4 to the plasma membrane.

PKB/Akt causes activation of mammalian target of rapamycin (mTOR) (Figure 25). The concerted actions of mTOR and p70S6K are responsible for insulin’s increased protein synthesis effect, including GLUT-1 synthesis. GLUT-1 causes basal glucose uptake. Once glucose enters the liver cells, it is converted by glucokinase into glucose-6-phosphate, which can enter glycolysis or be converted to glycogen. Furthermore, insulin also exerts its mitogenic, growth promoting, and differentiation effects via the MAPK pathway, which leads to the synthesis of GLUT-3. Activation of the MAPK pathway could be a possible reason for the observed hormetic effect in the cell viability assay, in the present study.
Figure 25: Pathway of GLUT-1 and GLUT-3 biosynthesis in the liver.<sup>224</sup>

IRS = insulin receptor substrates; MAPK = mitogen-activated protein kinase; mTOR = mammalian target of rapamycin; PI3K = phosphatidylinositol-3-kinase; PKB = protein kinase B.
The HepG2 cell line expresses insulin-like growth factor II\textsuperscript{154}, the insulin receptor\textsuperscript{154} and GLUT-1\textsuperscript{225}. GLUT-1 is the major contributor to glucose influx in HepG2 cells\textsuperscript{225}. The findings of the present study suggest, that the manner in which the crude extracts of both plants exert their hypoglycaemic effects in HepG2 cells, could possibly be via interactions with the insulin receptor, resulting in GLUT-1 synthesis. Moreover, crude extracts of both plants could possibly have an effect on the MAPK and PI3K pathways.

Metformin belongs to the biguanide class of oral hypoglycaemics. It exerts its hypoglycaemic effect by activation of the AMPK pathway, via mechanisms that are currently still not fully understood\textsuperscript{9,22,90}. Various other factors can also cause AMPK activation, including stimulation of the leptin and α-adrenergic receptors, and conditions such as, hypoxia, hypoglycaemia, heat shock and ischaemia, which cause increased levels of intracellular cAMP\textsuperscript{226}. Adiponectin is also known to activate this pathway\textsuperscript{226}. Adiponectin is mainly synthesized and secreted by adipose tissue, however, studies indicated that skeletal muscle and the liver also synthesize and secrete adiponectin. This hormone is known to increase insulin sensitivity and improve glucose tolerance\textsuperscript{92}. Adiponectin binds to the adiponectin receptor 1 (AdipoR1), which recruits adaptor protein containing pleckstrin homology domain, phosphotyrosine-binding domain and leucine zipper motif (APPL1), to the intracellular NH\textsubscript{2} terminus of AdipoR1\textsuperscript{227} (Figure 26). This results in activation the AMPK and MAPK\textsuperscript{227} pathways, which causes increased glucose uptake in adipose tissue and skeletal muscle via GLUT-4 translocation, and inhibition of gluconeogenesis in the liver\textsuperscript{227,228}.

C2C12\textsuperscript{229}, HepG2\textsuperscript{230} and 3T3-L1\textsuperscript{229,231} cells express AdipoR1. Furthermore, the activation of the AMPK and MAPK pathways via interactions with AdipoR1 by adiponectin have been shown in these cell lines\textsuperscript{229,231,232}. The crude extracts of both plants could possibly also mediate their hypoglycaemic effects by activation of the AMPK and MAPK pathways. This would result in increased glucose uptake in adipose tissue and skeletal muscle via GLUT-4 translocation, and inhibition of gluconeogenesis in the liver\textsuperscript{227,228}.
Figure 26: Model of APPL-mediated adiponectin GLUT-4 translocation in adipose tissue and skeletal muscle. Ad = adiponectin; AdipoR1 = adiponectin receptor 1; AMPK = 5’ adenosine monophosphate activated protein kinase; APPL1 = adaptor protein containing pleckstrin homology domain, phosphotyrosine-binding domain and leucine zipper motif; MAPK = mitogen activated protein kinase.
Furthermore, the AMPK pathway has also been shown to regulate glucose uptake via GLUT-1\(^{233}\), which is responsible for glucose influx in HepG2 cells\(^{225}\). The precise mechanism of activation of these two pathways by the crude extracts of *S. birrea* and *Z. mucronata* could perhaps be via interactions with AdipoR1.

A further possible mechanism of action for the crude extracts of both plants, could be that they are mediated by similar mechanisms as thiazolidinediones, via activation of PPAR-\(\gamma\), a nuclear receptor found in adipose tissue, skeletal muscle and the liver, but predominantly in adipose tissue. It modulates the expression of the genes involved glucose metabolism, is involved in insulin signal transduction, and regulates adipocyte differentiation\(^9,21,90\). PPAR-\(\gamma\) links with retinoid X receptor (RXR), forming the PPAR-\(\gamma\)-RXR heterodimer\(^{234}\). In the presence of a PPAR-\(\gamma\) ligand, this heterodimer binds to peroxisome proliferator response element (PPRE-\(\gamma\)), which promotes transcription of certain insulin-sensitive genes. Glucose uptake also occurs via increased GLUT-4 translocation to the cell membrane, in adipocytes\(^{234,235}\). C2C12, HepG2 and 3T3-L1 cells all express PPAR-\(\gamma\) and hypoglycaemic effects in these cells via PPAR-\(\gamma\) have also been shown\(^{236–238}\). It has been hypothesized that PPAR-\(\gamma\) agonists cause their hypoglycaemic effects mainly in adipose tissue, with secondary effects in skeletal muscle and the liver\(^{239}\).

Two main mechanisms have been proposed as to how *S. birrea* exerts its hypoglycaemic effects. One proposal postulates that the hypoglycaemic effects of *S. birrea* appears to be mediated via a mechanism that is similar to that of chlorpropamide, a sulphonylurea anti-diabetic drug, or is related to insulin secretion from pancreatic \(\beta\)-cells\(^{207,208}\). The second hypothesises that the hypoglycaemic effects of *S. birrea* may be associated with renoprotective and hypotensive effects, via a mechanism similar to that of metformin\(^{104}\). Furthermore, the authors proposed that *S. birrea* exerts its hypoglycaemic effect via an extra-pancreatic mechanism\(^{104}\). The latter mechanism of action has also been proposed based on *in vitro* findings\(^{30}\).

The hypoglycaemic of *Z. sativa* has been attributed to the presence of tannins\(^{211}\), whereas the hypoglycaemic effect of *Z. spina-christi* has been attributed to the presence of one of its major saponin glycosides, christinin-A\(^{212}\).
More than 100 cyclopeptide alkaloids have been isolated from the genus *Ziziphus* and the family Rhamnaceae is known to be a rich source of cyclopeptide alkaloids. Cyclopeptide alkaloids have been shown to possess hypoglycaemic effects, implicating that they might be responsible for the anti-diabetic effects of the *Ziziphus* species. The findings of the present study supports the latter of the two above-mentioned proposals, an extra-pancreatic mechanism of action for the hypoglycaemic effects of crude extracts of both *S. birrea* and *Z. mucronata*, possibly acting as insulin-mimetic agents. The plant extracts also exert additive extra-pancreatic hypoglycaemic effects by specifically inhibiting α-amylase and α-glucosidase, likely due to their polyphenolic contents, which have been shown to inhibit α-amylase and α-glucosidase activities, possess anti-hyperglycaemic effects and inhibit the development of DM. Plant polyphenols have also been shown to enhance glucose uptake via the MAPK and PI3K pathways, and GLUT-4 translocation. In addition to enzyme inhibition, the crude extracts also displayed strong antioxidant activity, implying that the extracts could alleviate oxidative stress, improve insulin sensitivity, prevent damage to the β-cells and prevent/delay the development of the secondary complications associated with T2DM. The proposed mechanism of the anti-diabetic activity of *S. birrea* and *Z. mucronata* is presented in Figure 27. The extra-pancreatic mechanism of action proposed in this study is supported by *in vitro* and *in vivo* findings. Results from the present study provide evidence for the hypoglycaemic effects of *S. birrea* and *Z. mucronata*, which correlates with findings in literature.
Crude extracts of *S. birrea* and *Z. mucronata*

Inhibition of α-amylase and α-glucosidase

- Complications of DM and damage to the pancreas
- Oxidative Stress
- Insulin sensitivity

Alleviation of Hyperglycaemia

Interactions with the insulin receptor and/or AdipoR1 and/or PPAR-γ

Activation of the AMPK, MAPK and PI3K pathways, resulting in GLUT-4 translocation and GLUT-1 synthesis

Liver
Adipose tissue
Skeletal muscle

Glucose uptake

**Figure 27:** Proposed mechanism of the anti-diabetic and hypoglycaemic effects of *S. birrea* and *Z. mucronata* (images taken from National Aids Treatment Advocacy Project\textsuperscript{91} and Medical Biochemistry Page\textsuperscript{223}).
Chapter 5: Conclusion

DM is the world’s fastest growing metabolic disease and as knowledge of the heterogeneity of this disorder increases, so does the need for more multi-factorial therapies\textsuperscript{97}. Herbal remedies could provide a cost-effective alternative to the current anti-diabetic drugs, devoid of the side-effects associated with commercially available drugs. The aim of this study was to assess the \textit{in vitro} anti-diabetic activity of aqueous and methanol extracts of two plants, \textit{S. birrea} and \textit{Z. mucronata}.

Crude extracts of \textit{S. birrea} and \textit{Z. mucronata} were found to possess potent \textit{in vitro} anti-diabetic activity. Most of the crude extracts displayed \(\alpha\)-amylase and \(\alpha\)-glucosidase inhibitory activities and caused significant \((p < 0.05)\) increases in glucose uptake in cells originating from the three target tissues of insulin, with effects comparable to that of insulin. Crude extracts of both plants had no significant effect on insulin secretion from RIN-m5F cells. Based on the findings of the present study an extra-pancreatic mechanism of action is proposed.

In conclusion, the \textit{in vitro} anti-diabetic properties of both \textit{S. birrea} and \textit{Z. mucronata} were confirmed in the present study. Results support previous proposals of an extra-pancreatic mechanism of action for both plants. Investigations to elucidate the mechanism(s) of action requires assessment of the crude extracts of the plants on targeted \textit{in vitro} models of the AMPK, MAPK, PI3K and PPAR-\(\gamma\) biochemical pathways, and GLUT-1 and GLUT-4 expression. Furthermore, synergistic effects between the different pathways should also be assessed. The effects of the crude extracts on glycogen formation in the different cell lines should also be determined. Additionally the inhibitory effects of the crude extracts on the activity of angiotensin-1 converting enzyme, which is an enzyme greatly involved in the pathophysiology of CVD, also needs to be investigated, which would be an additional benefit in the treatment of DM, as CVD is a common complication arising from DM. Moreover, isolation and identification of the active constituent(s) within the plant extracts responsible for their hypoglycaemic effects, also needs to be carried out.
DM, a globally prevalent NCD, is a growing threat to human health. The current pharmacological agents for the treatment of DM cause undesirable side-effects. Herbal remedies offer the potential for alternative treatment strategies, which may be cost-effective and devoid of the undesirable side-effects. The aim of this study was to evaluate the *in vitro* anti-diabetic activity of aqueous and methanol extracts of *Sclerocarya birrea* and *Ziziphus mucronata*, which are traditionally used in the treatment of diabetes mellitus in southern Africa.

Polyphenolic contents of crude extracts were quantified using the aluminium trichloride and Folin-Ciocalteau methods. Antioxidant activity was carried out by determining the ability of the extracts to scavenge the ABTS•+ and DPPH free radicals. The inhibitory effects of the crude extracts of both plants on the activities of the key enzymes in carbohydrate metabolism, α-amylase and α-glucosidase, were determined using colorimetric enzymatic assays. The effects of the crude extracts on cell viability was assessed on C2C12 myotubes, HepG2 hepatocarcinoma cells, 3T3-L1 adipocytes and RIN-m5F pancreatic β-islet cells, using the SRB assay. Fluorescence detection was used to investigate the effects of the crude extracts on glucose uptake on C2C12, HepG2 and 3T3-L1 cells, whereas, ELISA was used to assess insulin secretion from RIN-m5F cells.

Crude extracts of *S. birrea* and *Z. mucronata* were found to contain flavonoids and phenols, however flavonoid content was predominantly higher. All the crude extracts displayed antioxidant activity, with the methanol extract of *S. birrea* displaying the strongest antioxidant capacity (IC$_{50}$ = 2.16 µg/ml). The majority of the crude extracts of both plants displayed α-amylase and α-glucosidase inhibitory activities. Most of the crude extracts displayed low toxicity, where concentrations of 100 µg/ml did not induce 50% cell death.
Crude extracts of both plants caused significant \( (p < 0.05) \) increases in glucose uptake in C2C12, HepG2 and 3T3-L1 cells, with effects comparable to the positive control, insulin. However, the plant extracts did not cause a significant increase in insulin secretion from RIN-m5F cells.

The findings of the present study, suggest that the plant extracts exert their hypoglycaemic effects via an extra-pancreatic mechanism, independently of insulin. This possibly involves the inhibition of \( \alpha \)-amylase and \( \alpha \)-glucosidase, and interactions with the insulin receptor and/or AdipoR1 and/or PPAR-\( \gamma \), leading to the activation of the AMPK, MAPK and PI3K pathways, which results in the translocation and synthesis of different glucose transporters. The findings of the present study provide evidence that \( S. \) birrea and \( Z. \) mucronata possess \textit{in vitro} anti-diabetic activity. Further investigations are required to elucidate the mechanism(s) of action of the crude extracts of both plants using more targeted \textit{in vitro} models.
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Annexure A: Reagents

2-NBDG

2-NBDG was procured in powder form from Invitrogen (USA).

3,5-dinitrosalicylic acid

3,5-dinitrosalicylic acid was purchased in powder form from Sigma-Aldrich (St. Louis, USA).

4-nitropheryl α-D-glucopyranoside

4-nitropheryl α-D-glucopyranoside was obtained from Sigma-Aldrich (St. Louis, USA), in powder form.

ABTS•+

ABTS•+ radical was obtained from Sigma-Aldrich (St. Louis, USA).

Acarbose

Acarbose was purchased from Sigma-Aldrich (St. Louis, USA), in powder form.

Aluminium trichloride

Aluminium trichloride was supplied by Sigma-Aldrich (St. Louis, USA), in powder form.
Culturing consumables

Sterile 96-well white plates suitable for fluorescence was procured from Nunc (Denmark). Cellstar tissue culture flasks (25 cm² and 75 cm²) were supplied by Greiner BioOne (Austria).

Dexamethasone

Dexamethasone was purchased from Sigma-Aldrich (St. Louis, USA), in powder form.

DMSO

DMSO was obtained from Sigma-Aldrich (St Louis, USA) and used undiluted.

DMEM

DMEM powdered medium was procured from Sigma-Aldrich (St Louis, USA). A mass of 67.35 g of medium powder was dissolved in 5 L sterile, deionised water. Sodium bicarbonate (11 g) was added to adjust the pH. The solution was filter sterilized twice using 0.22 µm cellulose acetate filters, dispensed into sterile 500 ml bottles, fortified with 1% penicillin/streptomycin and stored at 4°C. Medium was fortified with 10% (v/v) FCS prior to use.

DPPH

DPPH was obtained in powder form from Sigma-Aldrich (St. Louis, USA).
EMEM

EMEM powdered medium was supplied by Sigma-Aldrich (St Louis, USA). A mass of 48 g of EMEM powdered medium was dissolved in 5 L sterile deionised water. Sodium bicarbonate (11 g) was added to adjust the pH. The solution was filter sterilized twice using 0.22 µm cellulose acetate filters, dispensed into sterile 500 ml bottles, fortified with 1% penicillin/streptomycin and stored at 4°C. Medium was fortified with 10% (v/v) FCS prior to use.

FCS

FCS was purchased from PAA (Pasching, Austria) and was inactivated prior to use by incubation at 56°C for 45 min.

Folin-Ciocalteau reagent

Folin-Ciocalteau reagent was supplied by Sigma-Aldrich (St. Louis, USA), in liquid form and used undiluted.

Horse serum

Horse serum was obtained from PAA (Pasching, Austria) and was inactivated prior to use by incubation at 56°C for 45 min.

IBMX

IBMX powder was procured from Sigma-Aldrich (St. Louis, USA).

Insulin

Insulin was obtained in powder form from Sigma-Aldrich (St. Louis, USA).
**Insulin (rat) ultrasensitive ELISA kits**

Insulin (rat) ultrasensitive ELISA kits were purchased from DRG International (USA).

**Methanol**

Methanol was supplied by Merck Chemicals (Darmstadt, Germany).

**Penicillin/streptomycin**

A solution containing 10 000 U of penicillin and 10 000 µg streptomycin was obtained from BioWhittaker (Walkersville, USA).

**PBS**

PBS powder was procured from BD Biosciences (Sparks, USA). A PBS solution was prepared by dissolving 9.23 g of PBS powder in deionised water and stored at 4°C until use. The solution was filter-sterilised through a 0.22 µm pore size filter, prior to use.

**Potato starch**

Potato starch powder was purchased from Sigma-Aldrich (St. Louis, USA).

**Potassium peroxodisulfate**

Potassium peroxodisulfate was obtained in powder form from Sigma-Aldrich (St. Louis, USA).
Porcine pancreatic α-amylase

Porcine pancreatic α-amylase was procured from Sigma-Aldrich (St. Louis, USA), in powder form.

Rat intestinal acetone powders

Rat intestinal acetone powders was purchased from Sigma-Aldrich (St. Louis, USA), in powder form.

RPMI-1640

RPMI-1640 powdered medium was supplied by Sigma-Aldrich (St Louis, USA). A mass of 48 g powdered medium was dissolved in 5 L sterile deionised water. Sodium bicarbonate (11 g) was added to adjust the pH. The solution was filter sterilized twice using 0.22 µm cellulose acetate filters, dispensed into sterile 500 ml bottles, fortified with 1% penicillin/streptomycin and stored at 4°C. Medium was fortified with 10% (v/v) FCS prior to use.

SRB

SRB dye was procured in powder form, from Sigma-Aldrich (St. Louis, USA).

Sodium hydroxide

Sodium hydroxide was purchased from Merck Chemicals (Darmstadt, Germany) in powder form.

Sodium potassium tartrate tetrahydrate

Sodium potassium tartrate tetrahydrate was obtained in powder form, from Sigma-Aldrich (St. Louis, USA).
**Sodium nitrate**

Sodium nitrate was obtained in powder form, from Merck Chemicals (Darmstadt, Germany).

**Trichloroacetic acid**

Trichloroacetic acid was supplied by Merck Chemicals (Darmstadt, Germany), in crystalline form.

**Trolox**

Trolox was obtained from Sigma-Aldrich (St. Louis, USA) in powder form.

**Trypan blue counting solution**

Powdered trypan blue was supplied by BDH Laboratories Supplies (UK). A mass of 200 mg of trypan blue powder, was dissolved in 50 ml PBS to obtain a 0.04% (w/v) solution, which was then filtered through a 0.45 µm syringe filter to remove any insoluble particles.

**Trypsin/versene**

A trypsin/versene solution was purchased from Highveld Biological (Johannesburg, RSA) and stored at 4°C.
Annexure B: Equipment

Plant material was ground using a YellowLine Grinder. A Branson 52 sonicator was used during extraction of the plants and to dissolve powdered reagents. Aqueous extracts were concentrated using a Freezone® 6 Freeze Dry System lyophilizer. Methanol extracts were concentrated using a Büchi Rotovapor R-200 Rotary-Evaporator. Cell culture plates and the extracts were shaken on a VRN-200 shaker. A Beckman-Coulter Allegra X22 centrifuge was used for cellular and acellular work. A Reichert-Jung Microscope was used for cellular work. A Biotek ELX 800 universal plate reader was used for spectrophotometrical readings and a BMG FluoStar Optima Fluorescent plate reader was used for fluorometric readings.
Annexure C: Ethical Approval

| NUMBER | 58/2012 |
| TITLE OF THE PROTOCOL | In vitro assessment of the anti-diabetic activity of Sclerocarya birrea and Ziziphus mucronata |
| PRINCIPAL INVESTIGATOR | Student Name & Surname: Mr N M H da Costa Mousinho  
Dept: Pharmacology; Steve Biko Academic Hospital; University of Pretoria.  
E-Mail: vanessa.steenkamp@uwp.ac.za |
| SUB INVESTIGATOR | None |
| STUDY COORDINATOR | None |
| STUDY DUREE | MSc |
| SPONSOR COMPANY | Not applicable |
| MEETING DATE | 28/03/2012 |

The Protocol is acceptable as an in vitro study, on 28 / 03 /2012 by a properly constituted meeting of the Ethics Committee subject to the following conditions:
1. The approval is valid for 5 years period (till the end of December 2016), and
2. The approval is conditional on the receipt of 6 monthly written Progress Reports, and
3. The approval is conditional on the research being conducted as stipulated by the details of the documents submitted to and approved by the Committee. In the event that a need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

Members of the Research Ethics Committee:
- Prof M J Bester
- Prof R Delport
- Prof. J A Ker
- Dr K. L. M. H. M.
- Dr MP Muthuba
- Prof. A. N. M. N.
- Mrs MC Nzeku
- Profs L. M. Nithye
- Snr S St John Phatoli
- Dr R. Reynolds
- Dr T. Rossouw
- Prof L. E. Schoeman
- Mr Y. S. S. S.
- Dr R. Sommers
- Prof T. P. Swart
- Prof C. W. van Staden

Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

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Annexure D: Research Outputs

National conferences:


Publication:

N.M.H. Da Costa Mousinho, J.J. van Tonder, V. Steenkamp. *In vitro* anti-diabetic activity of *Sclerocarya birrea* and *Ziziphus mucronata*. Submitted to Natural Product Communications.