The phytochemical content and anti-diabetic properties of *Aloe ferox* and *Aloe greatheadii* var. *davyana*

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Co-Promotor: Prof. M. Pieters

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2009
To my husband Schalk Botes for your love, patience, understanding, support, and unwavering faith in me throughout this study and the completion of my thesis. Life would be so much less without you.
Rev. 3 v 8

"See, I have placed before you an open door that no one can shut."
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ABSTRACT

Motivation: Diabetes mellitus is a non-communicable disease considered to be one of the five leading causes of death worldwide, characterized by hyperglycaemia and hyperlipidaemia as a result of altered glucose and lipid metabolism. Recently the search for suitable antidiabetic agents has focused on plants used in traditional medicine. Various Aloe species have been used for centuries in the management of various diseases, including diabetes. The majority of the scientifically based research on this topic was done on Aloe vera (or Aloe barbadensis) and Aloe arborescens. However, in the rural communities, the type of Aloe which is chosen as a traditional medicine would depend on its immediate availability to the specific community. Hence, various communities in different parts of the world would use the species of Aloe indigenous to their immediate surroundings. Aloe ferox (indigenous to the Western provinces of South Africa) and Aloe greatheadii var. davyana (indigenous to the Northern provinces of South Africa) are the most frequently used among the rural communities of South Africa to treat diabetes, even though very little scientific evidence, if any, exists to substantiate its use in diabetes. Different Aloe species would have varying phytochemical contents, health benefits and possible toxicities. Hence, it is of relevance for scientists, industry, and rural communities to not only investigate the relevant medicinal uses of their indigenous Aloe species, but also to determine the active components and their individual or combined mechanisms of biological function.

Objectives: The main objective of this study was to determine and compare the anti-diabetic effects of A. ferox and A. greatheadii ethanol leaf gel extracts using a streptozotocin (STZ)-induced diabetic rat model. In order to provide a foundational body of evidence for the aforementioned, a secondary objective included the characterization and comparison of the phytochemical content of A. ferox and A. greatheadii leaf gel extract (LGE) and 95% ethanol
leaf gel extract (ELGE) using gas chromatography mass spectrometry (GC-MS) and spectrophotometry prior to this, in order to confirm the presence of phytochemicals with health related benefits and to determine the most optimal extraction conditions for these.

Methods: The phytochemical content of both *A. ferox* and *A. greatheadii* var davynana LGE and ELGE were analyzed and compared via standard extraction methods and analysis on GC-MS (Agilent, USA) and spectrophotometrically (Shimadzu UV-1601 spectrophotometer). The extract obtained from the extraction method providing the most phytochemicals with previously proposed antidiabetic action, was chosen for the intervention study that followed. The intervention study was done using a STZ diabetic rat model in order to confirm the predicted antidiabetic effects based on the phytochemical characterization.

In order to accomplish this, fifty male Wistar rats were divided into five groups: Group 1 consisted of normal control rats (NC), group 2 of diabetic control rats (DC), group 3 of diabetic rats receiving 300 mg/kg *A. greatheadii* (DAG), group 4 of diabetic rats receiving 300 mg/kg *A. ferox* (DAF), and group 5 of diabetic rats receiving glibenclamide (DGL). After a 16 hour fast, the rats in the DC, DAG, DAF and DGL groups were injected (intraperitoneally) with 40mg/kg STZ dissolved in 0.1M cold sodium citrate buffer (pH 4.5) and left for one week, in order for diabetes to develop. Diabetes was confirmed after a 12 hour fast (blood glucose > 13.875mmol/L or 250mg/dL) by measuring blood glucose from a cut to the tail. The *A. ferox* ELGE, *A. greatheadii* ELGE and glibenclamide were given with an intragastric tube once daily for 5 weeks during which the rats had unlimited access to food and water. At the end of the intervention period, the rats were sacrificed and tissue and blood samples were collected. The effects of these interventions on the STZ induced diabetic state was monitored by measurement of various biochemical diabetes markers which included: serum
glucose, insulin, insulin resistance, fructosamine, triacylglycerol (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), very low-density lipoprotein cholesterol (VLDL-C), alanine transaminase (ALT), alkaline phophatase (ALP), ferric reducing antioxidant power (FRAP), and diacron reactive metabolites (dROMs).

**Results:** GC-MS and spectrophotometric analyses revealed a wide range of compounds with potential health benefits in both *A. ferox* and *A. greatheadii* LGE and ELGE. GC-MS analysis revealed that separate ethyl acetate/diethyl ether and hexane extractions of the LGE, is better suited to general phytochemical characterization purposes, whereas 95% aqueous ethanol extraction effectively concentrated selective groups of health related compounds, hence justifying its application to biological *in vivo* efficacy studies. Apart from these health related phytochemicals, sugar determinations revealed that *A. ferox* ELGE consisted of 96.9% sugar and *A. greatheadii* ELGE consisted of 83.75% sugar.

In the animal study, diabetes was confirmed one week after the injection of 40 mg/kg STZ by measuring fasting glucose concentrations via a cut to the tail. Compared to the NC group, STZ resulted in increased relative liver and kidney mass, end-point plasma glucose, fructosamine, oxidative stress, liver enzymes, total cholesterol, triglycerides, VLDL-C, and TC:HDL-C values, and reduced serum insulin levels. The majority of these diabetes markers, including fasting end-point glucose concentrations, fasting serum insulin levels, insulin resistance, and lipid levels, returned to near normal levels with glibenclamide supplementation, confirming that STZ injections resulted in an insulin independent diabetes that closely resembles type 2 diabetes biochemical abnormalities in human subjects.
Treatment with *A. greatheadii* moderately increased serum insulin accompanied by a modest decreased end-point plasma glucose and decreased liver enzyme ALP, in addition to moderately increased HDL-C and decreased TC:HDL-C values. *A. ferox* supplementation resulted in moderately increased serum insulin, accompanied by slight corrections in ALP and HDL-C, however, without a decrease in end-point plasma glucose. Little effect was seen on other diabetes markers.

**Conclusion:** Oral administration of the *Aloe* extracts, *A. greatheadii* in particular, resulted in moderate improvements in the STZ induced diabetic state, especially when considering the changes observed in the end-point plasma glucose and serum insulin levels, hence, justifying further investigations into the use of these traditional remedies for the treatment of diabetes. However, considering the phytochemical contents and previous literature using other *Aloe* species, more significant results were expected. Consequently, it is proposed that these effects should be studied using higher dosages, longer intervention periods, alternative extracts and perhaps larger sample groups for future antidiabetic investigations using these indigenous plants.

**Key words:** *Aloe*; GC-MS; Phytochemical characterization; Type 2 diabetes; Streptozotocin; Ethanol extracts
Motivering: Diabetes mellitus is ’n nie-oordraagbare siekte wat gesien word as een van die vyf hoof oorsake van streftes wêreld wyd, en word gekenmerk deur hiperglukemie en hiperlipidemie as gevolg van veranderde glukose- en lipiedmetabolisme. Die soeke na meer gepaste anti-diabetiese middels het onlangs begin fokus op plante wat gebruik word in traditionele medikasie. Aalwyn spesies word al vir eeue gebruik in die behandeling van verskeie siektes, insluitend diabetes. Die oorgrote meerderheid van die wetenskaplik-gebasseerde navorsing op hierdie onderwerp is op *Aloe vera* (of *Aloe barbadensis*) en *Aloe arborescens* gedoen. Die tipe aalwyn wat as tradisionele medisyne in landelike gemeenskappe gebruik word, hang van die onmiddellijke beskikbaarheid in die spesifieke gemeenskap af. Daarom sal gemeenskappe in verskillende dele van die wêreld die aalwyn spesies gebruik wat inheems is tot die onmiddelige omgewing. *Aloe ferox* (inheems tot die Westelike provinsies van Suid-Afrika) en *Aloe greatheadii* var. davyana (inheems tot die Noordelike provinsies van Suid-Afrika) word tans geredelik deur die landelike gemeenskappe van Suid-Afrika vir die behandeling van diabetes gebruik, al is daar baie min wetenskaplike bewyse om die gebruik daarvan te staaf. Verskillende aalwyn spesies sal verskillende fitochemikalie-inhoude, gesondheidsvoordele, en moontlike toksisiteite hê. Daarom is dit besonder relevant vir wetenskaplikes, die industrie, en landelike gemeenskappe om die relevante medisinale gebruikte van die inheemse alwyn spesies, asook die aktiewe komponente en die individuele of gekombineerde meganismes van biologiese funksie, te ondersoek.

Doelwitte: Die hoof doelwit van die studie was om die antidiabetiese effekte van *A. ferox* en *A. greatheadii* etanol blaar jel ekstrakte te bepaal en te vergelyk deur gebruik te maak van ’n streptozotocin (STZ)-geïnduseerde diabetiese rotmodel. Om ’n funksionele liggaam van bewyse vir die bogenoemde te verskaf, sluit ’n sekondêre doelwit die karakterisering en vergelyking van die fitochemikalie-inhoud van *A. ferox* en *A.
**greatheadii**blaar jel ekstrak en 95% etanol blaar jel-ekstrak in, deur gebruik te maak van
gas chromatografie-massa-spektrometrie (GC-MS) en spektrofotometrie.

**Metodes:** Die fitochemiekaal-inhoud van 'n watersuspensie van die blaar jel-ekstrak en
etanol blaar jel-ekstrak is geanalyseer via standaard ekstraksie-metodes deur van GC-MS
(Agilent, VSA) en spektrofotometrie (Shimadzu UV-1601 spektrofotometer) gebruik te
maak. Die ekstrak met die meeste antidiabetiese komponente, op grond van die
fitochemiekaal-inhoud, is gekies om in die intervensie te gebruik. Die intervensie is
gedoen deur gebruik te maak van 'n STZ diabetiese rot-model om die moontlike
antidiabetiese effekte te bevestig.

Vir hierdie doel is vyftig mannetjies Wistar rotte in vyf groepe verdeel: Groep 1 het
bestaan uit normaal kontrole rotte (NK), groep 2 uit diabetiese kontrole rotte (DK), groep
3 uit diabetiese rotte wat 300 mg/kg *A. greatheadii* ekstrak ontvang het (DAG), groep 4
uit diabetiese rotte wat 300 mg/kg *A. ferox* ekstrak ontvang het (DAF), en groep 5 het
bestaan uit diabetiese rotte wat glibenklamied ontvang het (DGL). Na 'n 16 uur vas is
die rotte in die DK, DAG, DAF en DGL groepe met 40mg/kg STZ opgelos in 0.1M koue
natriumsitraat-buffer (pH 4.5) ingespuight (intraperitoneaal) en gelos vir een week vir
diabetes om te ontwikkels. Diabeties is bevestig na 'n 12 uur vas (bloedglukose > 13.875
mmol/L of 250mg/dL) deur bloedglukose te meet via 'n sny aan die stert. *A. ferox* en *A.
greatheadii*blaar jel-ekstrakte en glibenklamied is een keer per dag vir vyf weke met 'n
intragastriese buis toegedien, waartydens die rotte onbeperkte toegang tot kos en water
gehad het. Aan die einde van die intervensieperiode is die rotte dood gemaak en weefsel-
en bloedmonster is versamel. Die effekte van die intervensies op STZ-geïnduseerde
diabetiese toestand is ondersoek deur die bepaling van verskeie biochemiese
diabetesmkerkers insluitend: serumglukose, insulien, insulien weerstandigheid,
fruktosamien, triasielgliserol (TG), totale cholesterol (TC) hoë-digtheidslipoproteïen-
cholesterol (HDL-C), lae-digtheidslipoproteïen-cholesterol (LDL-C), baie lae-
digtheidslipoproteïen-cholesterol (VLDL-C), alanien transaminase (ALT), alkalien
fosfate (ALP), "ferrie reduising antioxidant power (FRAP)", en "diacron reactive
metabolites (dROMs).
Resultate: GC-MS- en spektrofotometriese- analises het 'n groot verskeidenheid komponente met potensiële gesondheidsvoordele in *A. ferox* en *A. greatheadii* blaar jet- ekstrak en etanol blaar jellekstrak, getoon. GC-MS analises het getoon dat aparte etiel asetatadi-etiel ete en heksaan-ekstraksies van die blaar jet-ekstrak meer geskik vir algemene fitochemikalie-karakteriseringsdoeleindes is, en dat die 95% water etanol ekstraksie sekere komponente met gesondheidsvoordele meer effektief gekonsentreer het om die gebruik daarvan in biologiese *in vivo* studies te regverdig. Suikerbepalings het getoon dat *A. ferox* etanol blaar jet-ekstrak uit 96.9% suiker bestaan het en *A. greatheadii* blaar jet-ekstrak uit 83.75% suiker bestaan het.

In die dierestudie is diabetes een week na die inspuiting van 40 mg/kg STZ bevestig deur vastende glukosekonsentrasies met 'n sny aan die stert te bepaal. Al die diabetes merkers, insluitend vastende eind-punt glukosekonsentrasies, vastende seruminsulinvlakke, insulienweerstand, en lipiedvlakke, het terug gekeer na so te sê normale vlakke met gliibenklamied-supplementasie, wat bevestig dat die STZ inspuitings wel diabetes wat vergelykbaar is met type 2 diabetes in mense, veroorsaak het.


Gevolgtrekking: Orale toediening van die aalwyn ekstrakte, spesifiek *A. greatheadii*, het gelei tot matige verbeteringe in die STZ geëinduseerde diabetiese toestand, veral wanneer
die verandering in die eindpunt plasmaglukose en seruminsulienwaardes oorweeg word. Dus regverdig dit verdere navorsing in die gebruik van hierdie tradisionele medikasie vir die behandeling van diabetes. Wanneer die fitochemikalie-inhoud en vorige literatuur wat ander aalwyn spesies gebruik het, egter oorweeg word, is meer betekenisvolle resultate verwag. As gevolg hiervan word die studie van hierdie effekte, deur gebruik te maak van hoër dosisse, langer intervensi periodes, alternatiewe ekstrakte en moontlik groter groepgroottes vir toekomstige anti-diabetiese navorsing van hierdie inheemse plantes, voorgestel.

Sleutelwoorde
Aalwyn; GC-MS; Fitochemikalie-karakterisering; Tipe 2 diabetes; Streptozotocin; Etanol ekstrak
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<td>A. arborescens</td>
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<tr>
<td>ACAT</td>
<td>Acyl CoA: cholesterol acyltransferase</td>
</tr>
<tr>
<td>ACE/AACE</td>
<td>American College of Endocrinologists/American Association of Clinical Endocrinologists</td>
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<td>ADA</td>
<td>American Diabetic Association</td>
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<td>ADP</td>
<td>Adenosine diphosphate</td>
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<td>Aloe greatheadii</td>
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<td>AGE</td>
<td>Advanced glycation endproducts</td>
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<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>BSTFA</td>
<td>bis (trimethylsilyl) trifloroacetamide</td>
</tr>
<tr>
<td>CAT</td>
<td>Catalase</td>
</tr>
<tr>
<td>CE</td>
<td>Catechin equivalents</td>
</tr>
<tr>
<td>CHD</td>
<td>Coronary heart disease</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular disease</td>
</tr>
<tr>
<td>DAF</td>
<td>Diabetic rats receiving A. ferox</td>
</tr>
<tr>
<td>DAG</td>
<td>Diabetic rats receiving A. greatheadii</td>
</tr>
<tr>
<td>DC</td>
<td>Diabetic control</td>
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<tr>
<td>DCCT</td>
<td>Diabetic Control and Complications Trial</td>
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<tr>
<td>DK</td>
<td>Diabetic controle</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxiribonucleic acid</td>
</tr>
<tr>
<td>dROM</td>
<td>Diacron reactive metabolites</td>
</tr>
<tr>
<td>ELGE</td>
<td>Ethanol leaf gel extract</td>
</tr>
<tr>
<td>ET-1</td>
<td>Endothelin-1</td>
</tr>
<tr>
<td>FADH$_2$</td>
<td>Flavine adenine dinucleotide</td>
</tr>
<tr>
<td>ABBREVIATION</td>
<td>DESCRIPTION</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>FFA</td>
<td>Free fatty acid</td>
</tr>
<tr>
<td>FRAP</td>
<td>Ferric reducing antioxidant power</td>
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<tr>
<td>GAPDH</td>
<td>Glyceraldehyde phosphate dehydrogenase</td>
</tr>
<tr>
<td>GAE</td>
<td>Gallic acid equivalents</td>
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<tr>
<td>GC-MS</td>
<td>Gas chromatography mass spectrometry</td>
</tr>
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<td>GLUT4</td>
<td>Glucose transporter 4</td>
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<tr>
<td>GPx</td>
<td>Glutathion peroxidase</td>
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<tr>
<td>GSH</td>
<td>Reduced glutathione</td>
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<tr>
<td>GST</td>
<td>Glutathione-s-transferase</td>
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<tr>
<td>HbA1c</td>
<td>Haemoglobin A1c</td>
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<td>High-density lipoprotein cholesterol</td>
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<td>HNF</td>
<td>Hepatocyte nuclear factor</td>
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<tr>
<td>HOMA</td>
<td>Homeostasis assessment model</td>
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<tr>
<td>HPO</td>
<td>Horseradish peroxidase</td>
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<td>HSL</td>
<td>Hormone sensitive lipase</td>
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<td>Intermediate density lipoproteins</td>
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<tr>
<td>LDL-C</td>
<td>Low-density lipoprotein cholesterol</td>
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<tr>
<td>LGE</td>
<td>Leaf gel extract</td>
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<tr>
<td>MSG</td>
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<td>NADH</td>
<td>Nicotinamide adenine dinucleotide</td>
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<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
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<td>Nuclear factor</td>
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<td>Normaal controle</td>
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<td>Nitric oxide</td>
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<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
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<tr>
<td>OGTT</td>
<td>Oral glucose tolerance test</td>
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<tr>
<td>ORAC</td>
<td>Oxygen radical absorbance capacity</td>
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<tr>
<td>PAI-1</td>
<td>Plasminogen activator inhibitor-1</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
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<tr>
<td>PPAR</td>
<td>Peroxisome proliferators activated receptors</td>
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<tr>
<td>PVD</td>
<td>Peripheral vascular disease</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
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<td>SOD</td>
<td>Superoxide dismutase</td>
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<td>STZ</td>
<td>Streptozotocin</td>
</tr>
<tr>
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<td>DESCRIPTION</td>
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<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>STZ</td>
<td>Streptozotocin</td>
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<tr>
<td>TC</td>
<td>Total cholesterol</td>
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<td>TCA</td>
<td>Tricarboxylic acid</td>
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<td>Trolox equivalents</td>
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<tr>
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<td>Triglycerides</td>
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<td>TGF</td>
<td>Tumour growth factor</td>
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<tr>
<td>TMCS</td>
<td>Trimethylchlorosilane</td>
</tr>
<tr>
<td>UDP</td>
<td>Uridine diphosphate</td>
</tr>
<tr>
<td>UKPDS</td>
<td>United Kingdom Prospective Diabetes Study</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VLDL-C</td>
<td>Very low-density lipoprotein cholesterol</td>
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<tr>
<td>WHO</td>
<td>World Health Organization</td>
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<td>α</td>
<td>Alpha</td>
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<td>β</td>
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Chapter 1

Preface
1. BACKGROUND AND MOTIVATION

According to the World Health Organization (WHO), diabetes can be defined as persistent hyperglycaemia as a result of decreased insulin secretion (WHO Department of Non Communicable Disease Surveillance, 1999). Symptoms include excessive thirst, weight loss, increased urine volumes, recurrent infections, unexplained weight loss, drowsiness, coma, and high levels of glucosuria, and these may prompt further tests in order to delineate a positive or negative diagnosis of diabetes (The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 2002). Two types of diabetes can be diagnosed as a result of either; 1) complete β-cell destruction (type 1 diabetes) with severely diminished insulin secretion and the dependency on exogenous insulin, or 2) diminished tissue sensitivity to insulin together with impaired β-cell function (type 2 diabetes), which is worsened by obesity, often treatable with diet and exercise without medical intervention (WHO Department of Non Communicable Disease Surveillance, 1999), however, if left untreated, may worsen into an insulin dependent diabetic state.

For the purpose of this thesis, the remaining literature and discussion will focus mainly on type 2 diabetes.

Diabetes mellitus is considered to be one of the main threats to human health (Zimmet, 2001) and is classified as one of the 5 leading causes of death in developed countries (Amos et al., 1987). Figures reported by the International Diabetes Federation in 2006 paint a grim picture with an estimation of 333 million people expected to be diagnosed with diabetes by 2025 (International Diabetes Federation, 2006). The amount of diagnosed cases in Africa is set to rise from 7 million reported in 2003, to 15 million by the year 2025 (International Diabetes Federation 2006).
The ultimate aim of diabetes management is strict glucose control. Literature suggests that this can be accomplished through lifestyle interventions including weight management, the correct diet, physical activity, and medical management (The Diabetes Control and Complications Trial Research Group, 1993; Nathan et al., 2009). With reference to the above, hypoglycaemic agents (Luna & Feinglos, 2001), antioxidant therapy (Wohaieb & Godin, 1987; Koya et al., 1997; Studer et al., 1997; Bursell et al., 1999; Cameron & Cotter, 1999; Kowrulu & Kennedy, 2001) and poly (ADP-ribose) polymerase (PARP) inhibitors (Brownlee, 2005) can be used to achieve this. The American Diabetes Association recommends that fasting blood glucose levels should be maintained at, or corrected to 4.44 - 6.10 mmol/L (70 - 100 mg/dL) (American Diabetes Association, 2002).

The populations of developing countries worldwide continue to rely heavily on the use of traditional medicine as their primary source of healthcare (Cunningham, 1993). Our interest lies in finding plants indigenous to South Africa, with possible medicinal applications with regards to diabetes, hence the interest in Aloe ferox and Aloe greatheadii var. davyana. Since the preparation of these plants to treat diabetes varies from various tea extracts to dried leaf preparations, it was very difficult to compare the different preparations to each other and the literature. Hence our decision to investigate the anti-diabetic effects of Aloe ferox and Aloe greatheadii var. davyana using extracts similar to extracts previously described in literature investigating its anti-diabetic properties. A. ferox and A. greatheadii var. davyana (indigenous to Western Cape and the Northern Provinces of South Africa) are used among rural South African communities for the treatment of diabetes (personal communication with traditional healers). These treatments are based on anecdotal evidence or research findings done almost exclusively on Aloe vera. Different Aloe species would have varying phytochemical contents, health benefits and possible toxicities. Hence, the investigation of research of the relevant medicinal uses of indigenous Aloe species, as well as the determination of the active components and their individual or combined mechanisms of biological function may be of relevance for scientists, industry and rural communities.
2. AIMS AND OBJECTIVES OF THE STUDY

2.1. Aim

The aim of the study was to determine whether *A. ferox* and *A. greatheadii* var. *davyana* contained certain substances with antidiabetic activity, justifying their use as traditional antidiabetic medication.

2.2. Objectives

The above-mentioned aim will be accomplished by completion of the following objectives:

1. To characterize and compare the phytochemical composition of *A. ferox* and *A. greatheadii* leaf gel extract (LGE) and 95% ethanol leaf gel extract (ELGE) using gas chromatography mass spectrometry (GC-MS) and spectrophotometry, in order to substantiate possible antidiabetic activity and optimal extraction conditions based on the analysed phytochemical contents of these two extracts.

2. To determine the antidiabetic action of a suitable extract (identified above) using a STZ-induced diabetic rat model, by the measurement of various biochemical diabetes markers related to diabetes induced abnormalities in blood glucose, lipid, insulin, and liver enzyme levels, and correlate this biological activity to the phytochemical composition analysed.

3. STRUCTURE OF THESIS

Ethical approval for the study was obtained from the Ethical Committee of the North West University. The reference number for the study is: 06D06. This thesis is a
Chapter 1: Preface

compilation of chapters written specifically to comply with the requirements of the North-West University, Potchefstroom Campus and the journals to which manuscripts were submitted for publication. In particular, directives in terms of English language usage, formatting and bibliography styles were adhered to. All chapters will have their own reference index.

Following this chapter, Chapter 2 provides background information necessary for the interpretation of the data in the articles that follow. An overview of the pathogenesis and management of diabetes is given. Furthermore, possible anti-diabetic effects of different Aloe species using mainly STZ-induced diabetic animal models will be discussed.

Chapter 3 comprises a published manuscript (Loots et al., 2007 - Journal of Agricultural Food Chemistry) describing the phytochemicals in A. ferox LGE and ELGE characterized using GC-MS and spectrophotometric methods. In this chapter the phytochemical contents of the two extracts of A. ferox are discussed and compared in order to determine if these contain phytochemicals with health related benefits and which of these extraction procedures function best in extracting these compounds.

Chapter 4 comprises a published manuscript (Botes et al., 2008 - Molecules) describing the phytochemicals in A. greatheadii var davyana LGE and ELGE, also characterized using GC-MS and spectrophotometric methods. In this chapter the phytochemical contents of the two extracts of A. greatheadii are discussed and compared in order to determine if these contain phytochemicals with health related benefits and which of these extraction procedures function best in extracting these compounds, and how these compare to that of A. ferox as described in the publication of Chapter 3.

Chapter 5 consists of the manuscript describing the anti-diabetic action of the above-mentioned A. ferox ethanol leaf gel extract (ELGE) and A. greatheadii ELGE.
comparatively, in a STZ-induced diabetic rat model. This was done in order to determine whether these extracts truly show antidiabetic action, as was predicted by their phytochemical contents in Chapters 3 and 4. The manuscript was submitted for publication to The Journal of Agricultural Food Chemistry and is currently in review.

Chapter 6 is an integrated discussion and conclusion of the results of Chapters 3, 4, and 5. Recommendations regarding further research and practical applications are additionally made in this chapter. Attached as an addendum are the Instructions for Authors concerning the requirements of the specific journals for the 3 manuscripts as requested by the North-West University.
4. Authors contributions

The principal author of this thesis is Ms L Botes. The contribution of the co-authors and co-workers made towards this is given in Table 1.

The following is a statement from the co-authors confirming their individual roles in the study and giving their permission that the publications generated may form part of this thesis.

I declare that I have approved the above-mentioned publications and that my role in the study as indicated above is representative of my actual contribution and that I hereby give my consent that these may be published as part of the Ph.D. thesis of Lisa Botes.

Prof Du Toit Loots

Prof Marlien Pieters

Prof Francois van der Westhuizen

Dr Shahidul Islam
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Prof Du Toit Loots

Prof Marlien Pieters

Prof Francois van der Westhuizen

Dr Shahidul Islam
<table>
<thead>
<tr>
<th>Co-author</th>
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<th>Contribution</th>
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<tr>
<td>L. Botes (M.Sc. Dietetics)</td>
<td>-</td>
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</tr>
<tr>
<td>Prof. Du T. Loots (Ph.D. Biochemistry)</td>
<td>-</td>
<td>Promoter: Guidance all aspects of the study: designing, planning, execution, writing of all publications and documentation of the study.</td>
</tr>
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<td>Prof. M. Pieters (Ph.D. Nutrition)</td>
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<td>Co-promoter: Guidance in designing, planning, execution, statistical analyses, publication of chapter 3 and documentation of the study.</td>
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</tr>
<tr>
<td>Dr. S. Islam (Ph.D. Biochemistry)</td>
<td>-</td>
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<td>-</td>
<td>Mnr. C. Bester (Experimental Animal Centre)</td>
<td>Guidance and collaboration in the care and handling of the experimental animals.</td>
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<tr>
<td>-</td>
<td>Mrs. A. Fick (Experimental Animal Centre)</td>
<td>Guidance and collaboration in the care and handling of the experimental animals.</td>
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5. LITERATURE CITED


BOTES L., PIETERS M., ISLAM M.D.S., LOOTS DU T. Antidiabetic effects of *Aloe ferox* and *Aloe greatheadii* var. *davyana* leaf gel extracts in a streptozotocin diabetes rat model. *Journal of agricultural and food chemistry*. In review


Chapter 2

Literature Review
1. INTRODUCTION

Diabetes mellitus, long considered a disease of minor significance to world health, is now taking its place as one of the main threats to human health in the 21st century (Zimmet et al., 2001). It is the most common non-communicable disease worldwide and one of the top five leading causes of death in developed countries (Amos et al., 1987). The global figure of people with diabetes is set to rise from the estimated of 194 million in 2003, to 333 million in 2025 (International Diabetes Federation 2006). In Africa alone, approximately 7 million people between the ages of 20 and 79 were diagnosed with diabetes in 2003 and this figure is expected to rise to approximately 15 million in 2025 (International Diabetes Federation 2006).

*Aloe* species have been used for centuries for their laxative, anti-inflammatory, immuno-stimulant, antiseptic (Capasso et al., 1998), wound and burn healing (Chithra et al., 1998), anti-ulcer (Koo, 1994), anti-tumor (Saito, 1993) and especially anti-diabetic (Bunyaphraphatsara et al., 1996) properties. Many of these applications have been attributed to *Aloe*’s antioxidant phytochemicals (Reynolds & Dweck, 1999). However, these plants have been reported to contain various other compounds which function via a variety of alternative mechanisms such as antibacterial agents, antimicrobial agents (De Oliveira et al., 2008), and cathartic agents (Kametani et al., 2007). Although *Aloe vera* is the species most extensively described in the literature, the possibility of discovering useful properties among the more than 300 other *Aloe* species used as traditional medicines and as ingredients to commercial tonics is enough to excite curiosity.

This literature review will discuss diabetes and the underlying biochemical mechanisms associated with this disease, the traditional use of *Aloe* as a diabetes treatment and the mechanisms involved in their action (relating this
to their phytochemical content), in addition to the use of diabetes animal models as a tool in diabetes research.

2. DIABETES MELLITUS

2.1. Introduction

The first accepted definitions of diabetes were published by the National Diabetes Data Group in 1979 (National Diabetes Data Group, 1979) followed by the World Health Organization (WHO) in 1980. Currently, diabetes mellitus can be defined as "a chronic disease", which occurs when the pancreas does not produce enough insulin, or when the body cannot effectively use the insulin it produces. This leads to an increased concentration of glucose in the blood (hyperglycaemia) (WHO Department of Non Communicable Disease Surveillance, 1999). This may subsequently lead to further liver, kidney and pancreatic β-cell damage, as well as abnormal carbohydrate, protein and fat metabolism (Baynes, 1991; The Diabetes Control and Complications Trial research group, 1993; UK Prospective Diabetes Study research group, 1998; Brownlee, 2003). Diabetes can additionally be characterized by excessive thirst, weight loss, and in some cases progressive destruction of small blood vessels leading to such complications as infections and gangrene of the limbs or blindness (The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 2002). Type 1 diabetes (previously referred to as insulin-dependent or childhood-onset diabetes), is a more severe form of diabetes mellitus in which insulin production by the β-cells of the pancreas is impaired, usually resulting in dependence on externally administered insulin. Type 2 diabetes (formerly called non-insulin-dependent or adult-onset diabetes) is the milder, sometimes asymptomatic form, characterized by diminished tissue sensitivity to insulin.
and sometimes by impaired β-cell function, exacerbated by obesity and often treatable through diet and exercise (WHO, 1999).

In the sections to follow, the pathophysiology, complications and the diagnosis and management of diabetes mellitus will be discussed.

### 2.2. Pathophysiology of diabetes mellitus

The clinical diagnosis of diabetes is often prompted by symptoms of increased thirst and urine volumes, recurrent infections, unexplained weight loss, and in severe cases, drowsiness and coma where high levels of glucosuria are usually present. Due to the impact of diabetes on the lifestyle of an affected individual, the criteria used to make a diagnosis must be highly robust in order to omit as few people as possible who may have diabetes, while preventing any false positive diagnoses in others. Even though an individual’s diurnal blood glucose levels vary continuously, dependent on food intake as well as the body’s homeostatic responses, it is still used as the main criteria for diagnosing diabetes (Kernohan et al., 2003). The diagnostic criteria for diabetes mellitus have been modified from those previously recommended by the National Diabetes Data Group (National Diabetes Data Group, 1979) or the World Health Organization (WHO, 1985). According to the Expert Committee on the diagnosis and classification of diabetes mellitus, revised criteria for the diagnosis of diabetes include three possible ways to diagnose this disease (Table 1).
Table 1
Criteria for the diagnosis of diabetes mellitus (The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 2002).

1. Symptoms of diabetes plus a casual plasma glucose concentration of 11.1 mmol/L (200 mg/dL), where casual is defined as any time of day without regard to time since the last meal. The classic symptoms of diabetes include polyuria, polydipsia and unexplained weight loss.

   Or

2. Fasting plasma glucose ≥ 7.0 mmol/L (126 mg/dL). Fasting is defined as no caloric intake for at least 8 hours.

   Or

3. 2 hour plasma glucose ≥ 11.1 mmol/L (200 mg/dL) during an oral glucose tolerance test (OGTT). The test should be performed as described by the WHO, using a glucose load containing the equivalent of 75 g anhydrous glucose dissolved in water (WHO, 1985).

   In the absence of unequivocal hyperglycaemia with acute metabolic decompensation, these criteria should be confirmed by repeat testing on a different day. The OGTT is not recommended for routine clinical use.

In addition to the above-mentioned criteria for the diagnosis of diabetes, the Expert Committee for the Diagnosis and Classification of Diabetes Mellitus recommends the testing for diabetes in asymptomatic individuals who may be at risk also (The Expert Committee on the Diagnosis and Classification of
Diabetes Mellitus, 2002). This includes individuals who are 45 years of age and older. Testing should also be considered at a younger age or be carried out more frequently in individuals who are overweight (BMI ≥ 25kg/m²), have a first-degree relative with diabetes, are a member of a high-risk ethnic group (African-American, Hispanic American, Native American, Asian American, or Pacific Islander), have delivered a baby weighing 4.08kg (> 9lb) or have been diagnosed with gestational diabetes mellitus, are hypertensive (≥ 140/90mmHg), have an HDL-C level ≤ 0.90mmol/L (35mg/dL) and/or a triglyceride level ≥ 2.82mmol/L (250mg/dL), or had impaired glucose tolerance or impaired fasting glucose on previous testing (The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 2002).

As previously mentioned, diabetes mellitus is characterized by chronic hyperglycaemia, leading to disorders in carbohydrate, protein and fat metabolism (Kim et al., 2006). Diabetes can be characterized as type 1 or type 2 diabetes. In type 1 diabetes the β-cells are gradually destroyed, resulting in reduced insulin production (Nair, 2007). In type 2 diabetes, the body produces enough insulin, but due to insulin resistance, glucose does not move into the cells and thus cannot be utilized to produce energy (Nair, 2007). The pancreas attempts to correct this by secreting more insulin, which ultimately results in β-cell burnout, decreased insulin production, and finally complete insulin deficiency. A person with initial type 2 diabetes may, therefore, later develop an insulin dependence due to β-cell destruction, if the condition is left untreated. In healthy individuals euglycaemia is regulated by a negative feedback system: the rise in blood glucose after carbohydrate intake stimulates insulin secretion by the β-cells of the islets of Langerhans in the pancreas, resulting in glucose uptake by the cells, and the consequent lowering of blood glucose levels. This in turn lowers insulin secretions. However, this negative feedback system may become impaired in diabetic individuals (Figure 1).
Chapter 2: Literature review

Figure 1:
Insulin control and the influence of diabetes on the negative feedback mechanisms

Under normal conditions, elevated serum glucose levels due to increased carbohydrate intake are normalised by insulin secreted by the β-cells. In type 1 diabetes, no insulin is produced due to total β-cell destruction, resulting in chronic hyperglycaemia. In type 2 diabetes, insulin resistance causes cells to be less responsive to insulin and serum glucose levels remain elevated.
Several pathogenic processes are involved in the destruction of pancreatic β-cells and ultimately the development of either type 1 and type 2 diabetes. These processes include auto-immune destruction of β-cells, insulin resistance, genetic β-cell defects, genetic defects in insulin secretion, diseases of the exocrine pancreas, endocrinopathies, and infections (The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 2002). These pathogenic processes will be discussed briefly in the following section.

2.2.1. Immune mediated disease:
Cellular-mediated auto-immune destruction of β-cells results in total destruction of pancreatic β-cells as seen in type 1 diabetes (Maclaren et al., 1999; Abel & Krokowski, 2001). Markers of immune destruction of the β-cells include islet cell autoantibodies (Marker & Maclaren, 2001), autoantibodies to insulin (Maclaren et al., 1999), autoantibodies to glutamic acid decarboxylase (Taplan & Barker, 2008), and autoantibodies to the tyrosine phosphatases IA-2 and IA-2β (Myers et al., 1995). The rate of β-cell destruction varies and individuals may present with ketoacidosis as the first manifestation of the disease (reviewed by The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 2002). In the presence of stress or infection, some individuals may also present with modest fasting hyperglycaemia that can rapidly change to severe fasting hyperglycaemia (reviewed by The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 2002).

2.2.2. Insulin resistance:
In type 2 diabetes, insulin resistance is usually associated with relative, rather than absolute insulin deficiency (Turner & Clapham, 1998), and treatment with exogenous insulin is usually not required (reviewed by The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 2002). Although the causes for insulin resistance are not well defined, it can be accepted that autoimmune destruction of β-cells is not involved (reviewed by
The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 2002). Obesity is present in most individuals that present with insulin resistance. Moreover, obesity itself may cause some degree of insulin resistance (Pietiläinen et al., 2005; Ingelsson et al., 2009). Due to the gradual development of hyperglycaemia, insulin resistance and associated type 2 diabetes may go largely undiagnosed and are usually only diagnosed in relation to stress or infection (The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 2002). The risk of the development of diabetes associated with insulin resistance increases with age, obesity and physical inactivity (Zimmet, 1992; Ferrannini et al., 1997).

2.2.3. Genetic β-cell defects:
Several forms of diabetes are associated with defects in β-cell function and are associated with the early onset of hyperglycaemia (The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 2002). This type of diabetes is characterized by impaired insulin secretion with minimal or no defects in insulin action (Bell & Polonsky, 2001). To date, abnormalities at three genetic loci on different chromosomes have been identified: 1) mutations on chromosome 12 in a hepatic transcription factor referred to as hepatocyte nuclear factor (HNF)-1α; 2) mutations in the glucokinase gene on chromosome 7p resulting in a defective glucokinase molecule and; 3) mutations in the HNF-4α gene on chromosome 20q (reviewed by The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 2002).

2.2.4. Genetic defects in insulin action:
Formerly known as type A insulin resistance, genetic abnormalities in insulin action as a result of mutations of the insulin receptor may range from hyperinsulinaemia with modest hyperglycaemia, to severe diabetes (Maclaren et al., 1999; reviewed by The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 2002). It is assumed that these result from
lesions residing in the postreceptor signal transduction pathways of these genes.

2.2.5. Diseases of the exocrine pancreas:
Any injury to the pancreas may result in the development of diabetes (The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 2002), and may additionally be associated with cancer, pancreatitis, trauma, infection, pancreatectomy, and pancreas carcinoma (The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 2002). Even though only extensive damage to the pancreas will result in diabetes, adenocarcinomas that involve only a small portion of the pancreas have also been associated with this disease (The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 2002). This implies that additional mechanisms, apart from a simple reduction of ß-cell mass, may also result in the development of this disease (The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 2002).

2.2.6. Endocrinopathies:
Excessive amounts of insulin antagonizing hormones (growth hormone, cortisol, glucagon, epinephrine) may cause diabetes (The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 2002; Resmini et al., 2009). This generally occurs in individuals with pre-existing defects in insulin secretion. Hyperglycaemia usually returns to normal when the excess hormone is removed. Diabetes, as a result of hypokalaemia induced by somatostatinoma and aldosteronoma, can generally be resolved after successful removal of the tumour (Conn, 1965, reviewed by The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 2002).


2.2.7. Infections:
Certain viruses such as congenital rubella (Jun & Woon, 2001; Hyöty & Taylor, 2002), koksaki-virus B, cytomegalovirus, adenovirus, and mumps, have been implicated in β-cell destruction and diabetes (Jun & Woon 2001).

A variety of complications may result from hyperglycaemia and these are broadly classified as micro and macro vascular complications. Micro vascular complications include retinopathy, nephropathy, and neuropathy. Macro vascular complications include cardiovascular disease (CVD), peripheral vascular disease (PVD) and cerebrovascular disease. These will be described in section 5.

The mechanisms by which hyperglycaemia induces the above-mentioned micro and macro vascular complications are due to an abnormal lipid profile as well as a variety of other hyperglycaemia-induced mechanisms including the polyol pathway, advanced glycation end product (AGE) formation, the protein kinase C (PKC) pathway and the hexosamine pathway, which are all thought to be induced through oxidative stress mechanisms. These will be discussed in detail in section below. For the purpose of this thesis, the discussion will focus on type 2 diabetes as the empirical work was done using a type 2 diabetes animal model.

3. HYPERGLYCAEMIA INDUCED OXIDATIVE STRESS MECHANISMS

Diabetes mellitus related hyperglycaemia is associated with, amongst other factors, oxidative stress (Wright et al., 2006). Both diabetic humans and animal models reportedly exhibit high oxidative stress due to persistent and chronic hyperglycaemia (Singh et al., 2005). Chronic postprandial hyperglycaemia results in multiple biochemical reactions, of which oxidative stress, as a result of free radical production, is the best described for its role in
diabetes induction and its associated complications (Martín-Gallán et al., 2002). The majority of glucose entering the cell is metabolized through glycolysis via a number of steps to acetyl Coenzyme A, which then enters the tricarboxylic (TCA) cycle. The metabolism of glucose in the TCA cycle generates 2 electron donors: NADH, which donates electrons to complex I of the electron transport chain and flavin adenine dinucleotide (FADH$_2$), which donates electrons to complex II (Brownlee, 2005). In healthy cells, electrons from both these complexes are passed to coenzyme Q, complex III, cytochrome C, complex IV and finally to molecular oxygen, which they reduce to water (Brownlee, 2005). As the electrons pass through the electron transport chain, energy is generated in the form of adenosine tri-phosphate (ATP) (Brownlee, 2005). However, in diabetes, high amounts of glucose are being oxidized in the TCA cycle, resulting in an over-influx of reduced NADH and reduced FADH$_2$ into the electron transport chain (Korshunov et al., 1997, Brownlee, 2005). This causes the electrons to back-up at coenzyme Q, which diverts the electrons to molecular oxygen, thereby generating superoxides (Brownlee, 2005), consequently causing oxidative stress. Super oxides, in turn, are responsible for the formation of other free radicals such as hydroperoxides and peroxides, which also result in mitochondrial damage and cell apoptosis (Brownlee, 2005). In effect, this results in a decreased production of ATP, and in turn a reduction in the ATP/ADP ratio (Brownlee, 2005), leading to $\beta$-cell destruction and ultimately decreased insulin secretion by the $\beta$-cells (Ceriello & Motz, 2004). The free radicals, in particular superoxide production, exert their damaging effects through various mechanisms. It is thought to inhibit the rate limiting enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Du et al., 2000) by modifying the enzyme with polymers of ADP-ribose (Brownlee, 2005), resulting in increased levels of the upstream metabolites such as advanced glycation end products (AGE) and protein kinase-C (PKC). The inhibition of GADPH, as well as the overproduction of superoxides, activate four major pathways of hyperglycaemic damage: The polyol pathway (Allen et al., 2005), non-
enzymatic protein glycation, PKC activation (Rolo & Palmeira, 2006), and the hexosamine pathway (Du et al., 2000; Brownlee, 2001).

Hyperglycaemia-induced oxidative stress, as well as the pathways of hyperglycaemic damage will be discussed in greater detail below.

### 3.1 Polyol pathway

As indicated in Figure 2, high amounts of glucose inside the cell are reduced to sorbitol by aldose reductase in a process that consumes nicotinamide adenine dinucleotide phosphate (NADPH). Since the affinity of aldose reductase for glucose is low, maximal rates of aldose reductase-catalyzed formation of sorbitol can be attained only with high intracellular concentrations of glucose, such as in the early stages of type 2 diabetes (Kawanishi et al., 2003). The conversion of sorbitol to fructose impairs the NADPH-dependent generation of reduced glutathione (GSH), an intracellular antioxidant (Allen et al., 2005), which in turn may lead to hyperglycaemia-induced cell apoptosis and oxidative stress.
Some of the high glucose inside the cell is reduced to sorbitol by aldose reductase in a process that consumes NADPH. The conversion of sorbitol to fructose impairs the NADPH-dependent generation of reduced GSH leading to hyperglycaemia-induced cell apoptosis and oxidative stress.

3.2 Advanced glycation end products (AGEs) pathway

Non-enzymatic glycation occurs through the covalent binding of aldehyde or ketone groups of reducing sugars to free amino groups of proteins to form labile Schiff’s base (Singh et al., 2001, Basta et al., 2004). The initial Schiff’s base undergoes rearrangement to form Amadori’s products, which is responsible for some of the biological consequences in glycation (Basta et al., 2004). Additionally, Amadori’s products can be degraded into a variety of highly active carbonyl groups, such as 3-deoxy-glucosone, which can react again with free amino groups to form intermediate glycation products (Basta et al., 2004). These intermediate glycation products (including 3-deoxy-glucosone, glyoxal, and methyl-glyoxal) sporadically undergo a series of chemical rearrangements to yield irreversible advanced glycation end products.
(AGEs). Glyoxal and methyl-glyoxal products can also be formed by glucose auto-oxidation or produced from glycolipids (Thornalley et al., 1999) (Figure 3). This process is also known as the Maillard reaction (Singh et al., 2001). Glycation is concentration-dependent in the early stages of the Maillard reaction (Furth, 1997) and is thus enhanced in diabetic patients. AGEs accumulate in most sites of diabetes complications including the kidney, retina and atherosclerotic plaques (Makita et al., 1994; Bucala & Vlassara, 1995; Hammes et al., 1999). The formation of AGEs is, however, catalyzed by transition metals and can duly be inhibited by reducing compounds such as antioxidants (Chappey et al., 1997). Tissue AGE concentration correlates to the degree of atherosclerotic lesions (Basta et al., 2004) by the following mechanisms:

1) Mechanical cross-bridge dysfunction among the vessel wall macromolecules (Sell and Monnier, 1989).
2) Circulating blood cells adhering to the blood vessel walls (Basta et al., 2004),
3) Perturbation of cellular function through binding to a variety of receptors on macrophages, endothelial cells, smooth muscle cells, renal cells, and neuronal cells (Hori et al., 1995; Yan et al., 1996).
Reducing sugars such as glucose, react non-enzymatically with amino groups in proteins, lipids and nucleic acids through a series of reactions to form AGEs. This causes cell damage via the modification of proteins, the modification of extracellular matrix molecules by AGE precursors, and the modification of circulating proteins by AGE precursors.

3.3 Protein kinase C (PKC) pathway

In diabetes, the activity of sorbitol dehydrogenase is increased, resulting in an increased reduction of fructose to sorbitol (polyol pathway). This reduction of fructose to sorbitol causes an increase in the nicotinamide adenine dinucleotide(reduced)/nicotinamide adenine dinucleotide(oxidised) (NADH/NAD\(^+\)) ratio resulting in the increase synthesis of diacylglycerol. Increased diacylglycerol serves as a PKC activator (Rolo & Palmeira, 2006) (Figure 4). PKC activation has many biochemical consequences that relate to diabetes complications including the following: increased tumour growth factor (TGF)-\(\beta\), increased vascular endothelial growth factor, increased
endothelin-1, increased NAD(P)H oxidase, increased nuclear factor (NF)-κB and increased ROS production (Inoguchi et al., 1991; Ishii et al., 1996; Brownlee, 2001). The activation of the PKC pathway may result in the development of microvascular complications of diabetes such as retinopathy and nephropathy (Brownlee, 2005).

Figure 4
Hyperglycaemia-induced PKC activation (Brownlee, 2005)

Inside the cell hyperglycaemia indirectly acts as an activating co-factor for PKC isoforms β, δ, and α. This affects gene expression and results in increased endothelin-1 (ET-1), increased vascular endothelial growth factor (VEGF), increased tumour growth factor (TGF)-β, increased plasminogen activator inhibitor-1 (PAI-1), increased nucleic factor (NF)-κB and increased NAD(P)H oxidase and ROS production and consequently blood-flow abnormalities, compromised vascular permeability, capillary and vascular occlusion, pro-inflammatory gene expression, and oxidative stress.
3.4 Hexosamine pathway

The hexosamine pathway (Figure 5), is an additional pathway of glucose metabolism that may mediate some of the toxic effects of high blood glucose (Du et al., 2000; Brownlee, 2001). Under usual metabolic conditions, 2-5% of the glucose entering the cells is directed to the hexosamine pathway and is metabolised through glycolysis, resulting in the conversion of fructose-6-phosphate to glucosamine 6-phosphate (Brownlee, 2001; James et al., 2002). However, during hyperglycaemia much of the excess glucose is shunted into the hexosamine pathway, resulting in the overproduction of uridine diphosphate (UDP)-N-acetylglucosamine, the substrate for the glycosylation of important intracellular factors such as growth factor-β1 and plasminogen activator inhibitor-1 (PAI-1). Both of these are deleterious to blood vessels (McClain and Crock, 1996; Du et al., 2000). PAI-1 is the primary physiological inhibitor of plasminogen activation in vivo (Loskutoff & Samat, 1998). PAI-1 also regulates fibrinolysis (Alessi & Juhan-Vague, 2006). Elevations in plasma PAI-1 levels result in abnormal fibrin clearance mechanisms and the formation of atherosclerotic plaques (Loskutoff & Samat, 1998; Alessi & Juhan-Vague, 2006). Clinical evidence suggests that increased PAI- levels are associated with atherothrombosis (Kohler & Grant, 2000; Sobel et al., 2003) and it is also a predictor for myocardial infarction (Hamsten et al., 1987; Juhan-Vague et al., 1996; Smith et al., 2005). This over production is linked to insulin resistance (Robertson, 2001) and leads to the development of diabetic micro-vascular complications (Gabriely et al., 2002; Goldberg et al., 2002).
Figure 5

Hexosamine pathway (Brownlee, 2001).

During hyperglycaemia in early stages of type 2 diabetes, excess glucose is shunted into the hexosamine pathway resulting in the overproduction of uridine diphosphate (UDP)-N-acetylglucosamine, leading to glycosylation of growth factor-β1 (TGF-β1) and plasminogen activator inhibitor-1 (PAI-1). This ultimately leads to altered fibrinolysis, insulin resistance, cardiovascular events, and micro vascular complications.

High postprandial glucose through the activation of the polyol pathway, the hexosamine pathway, the PKC pathway and the AGE pathway, in turn results in a number of diabetic complications. These will broadly be discussed in sections 4 and 5.
4. HYPERGLYCAEMIA INDUCED LIPID PROFILE ABNORMALITIES

Altered insulin secretion induced by mechanisms explained above, in turn, results in dyslipidaemia characterized by increased levels of total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), very low-density lipoprotein cholesterol (VLDL-C) and triglycerides (TG) and decreased levels of high-density lipoprotein cholesterol (HDL-C) (Krauss, 2004). Abnormalities in lipid profiles are one of the most common complications in diabetes. Hyperglycaemia and insulin resistance both play a pivotal role in the metabolism of TG-rich lipoproteins, which ultimately leads to diabetic dyslipidaemia (Figure 6). Krauss summarizes this as follows: Firstly, increased hepatic secretion and impaired clearance of very low-density lipoproteins (VLDL) results in prolonged plasma retention of this as partially lipolyzed remnants, or cholesterol-enriched intermediate-density lipoproteins (IDL). Secondly, it also results in increased production of small, dense LDL particles, which are inversely related to plasma HDL particles. The production of small, dense LDL particles arises from the TG enrichment of the lipolytic products through the action of cholesteryl ester transfer protein as well as the hydrolysis of TG and phospholipids by hepatic lipase. A major factor involved in the reduction in HDL particles associated with diabetes, appears to be the increased transfer of cholesterol from HDL particles to TG-rich lipoproteins, with the mutual transfer of TG to HDL. TG-rich HDL particles are hydrolyzed by hepatic lipase and are rapidly catabolized and cleared from the plasma, resulting in low levels of plasma HDL (Krauss, 2004). Apart from the over production of VLDL by the liver due to hyperglycaemia, insulin resistance also increases the action of hormone sensitive lipase, thereby increasing lipolysis in adipose tissue, which ultimately results in increased free fatty acids (FFA) which are then transported to the liver (Izkhakov et al., 2003), and once again contributing to VLDL overproduction. Additionally, FFA in the form of TG is deposited in
the muscle, liver, heart, and pancreas, resulting in decreased insulin sensitivity in this tissue (Krauss, 2004). Furthermore, insulin resistance also increases hepatic lipase activity (Watson et al., 1994; Tan et al., 1995).

**Figure 6** (Krauss, 2004)

**Altered lipid metabolism in diabetes**

Insulin resistance and accompanied increased free fatty acids (FFA) and hyperglycaemia cause the production of increased small, dense low-density lipoprotein (LDL) particles and decreased high-density lipoprotein (HDL) particles. Hyperglycaemia-induced very low-density lipoprotein (VLDL) production ultimately leads to the increased deposition of FFA (in the form of triglycerides (TG)) in the liver, muscle and heart and pancreas resulting in reduced insulin sensitivity in this tissue.
5. MACRO AND MICRO VASCULAR DIABETIC COMPLICATIONS

In the light of this study and the mechanisms described above, the focus will be on the link between abnormalities in lipid metabolism and oxidative stress and their role in the development of micro and macro vascular complications. Due to the fact that the diabetes associated micro and macro vascular complications are not the focus of this thesis, these complications will only briefly be discussed in the light of the underlying mechanisms associated with hyperglycaemia, dyslipidaemia and oxidative stress.

Hyperglycaemia, more specifically hyperglycaemia-induced oxidative stress, has been recognized as an independent risk factor for the development of both micro-vascular and macro-vascular complications (Giugliano et al., 1996; Capes et al., 2000; King & Loeken, 2004). At a cellular level, much is known about the deleterious effects of high glucose concentrations (Allen et al., 2005) with hyperglycaemia being widely recognized as the causal link between diabetes and diabetic complications (reviewed by Brownlee, 2003). The Diabetes Control and Complications Trial (DCCT) and the United Kingdom Prospective Diabetes Study (UKPDS) established that hyperglycaemia is the initiating cause of diabetic tissue damage. Additionally, scientific evidence confirms the association between diabetes related complications and oxidative stress (Baynes, 1991). The most likely explanation for hyperglycaemia-induced micro-vascular and macro-vascular complications during diabetes seems to entail the combined effects of increased levels of reactive oxygen species (ROS) and decreased capacity of the cellular antioxidant defence systems (Chung et al., 2003). Constant changes in the redox status of enzymes and transcription factors as well as changes in NAD⁺ cofactor ratios are important in normal cellular physiology (King & Loeken, 2004). However, normal cell physiology can adversely be affected by chronic or excess oxidant production (King & Loeken, 2004). For
example, while PKC and nitric oxide synthase (NOS) are necessary for normal cellular function, an overproduction may disrupt the normal function of affected tissue (King & Loeken, 2004). Similarly, the normal promotion of cell cycle progression in response to growth factors with the production of H$_2$O$_2$ may be overturned by excessive H$_2$O$_2$ production, resulting in cell cycle arrest (Savitsky & Finkel, 2002). Diabetic micro-vascular and macro-vascular complications may, therefore, transpire due to hyperglycaemia-induced oxidative stress through various pathways that may interfere with the normal function of affected tissue in the following ways: by increasing blood flow and disturbing hemodynamics in the retina (Kunisaki et al., 1995; Kowluru & Kennedy, 2001); by affecting the contractility of vascular smooth muscle cells (Sharpe et al., 1998), by damaging the mesangial cells in the renal glomerulus (Brownlee, 2005) and decreasing neural conductivity in peripheral nerves (Hounsom et al., 2001). Baynes and Thorpe (1999) suggested that the various pathways of hyperglycaemia-induced oxidative stress production overlap and intersect with one another, for example, AGE formation and polyol pathway activity may lead to oxidative stress. The increased AGE formation may additionally lead to the activation of the PKC pathway as well as increased growth factor expression, which, once again, leads to increased oxidative stress. Furthermore, this increase in oxidative stress may not be sufficient to induce total tissue destruction, however, it may cause enough damage to cause DNA strand breaks, thereby inducing cell death (Du et al., 2003) and consequently impair the integrity and function of the entire tissue. Apart from affecting the static function of the signalling pathways, hyperglycaemia-induced oxidative stress may also affect gene expression involved in cell survival or death, as well as cell function, ultimately causing compromised tissue function (King & Locken, 2004).

In addition to oxidative stress, lipid concentrations are also strongly related to the development of micro and macro vascular complications (Jenkins et al., 2003; Lyons et al., 2004; Tesfaye et al., 2005; Petitti et al., 2007).
Lipoprotein abnormalities, such as glycation and oxidation, are both important factors in the development of diabetic complications (Lyons et al., 1987). However, less known factors such as altered distribution in lipoprotein subclasses may also play a significant role in the development of diabetic complications (Austin et al., 1988; Havel, 1990; Fievet & Fruchart, 1991). Lipoproteins may, furthermore, exert their adverse effects through altered coagulation, fibrinolysis, vascular tone, or insulin resistance (Jenkins et al., 2003).

5.1. Micro vascular complications

5.1.1. Retinopathy:
Retinopathy is the most common complication of diabetes mellitus, affecting up to 90% of the diabetic population with loss of vision seen in about 5% patients diagnosed with diabetes (Yülek et al., 2007). Patients with poor, long-term glycaemic control, are more vulnerable to diabetic retinopathy than to other micro vascular complications of diabetes (Yülek et al., 2007). In addition to poor glycaemic control, the literature has confirmed the involvement of oxidative stress early in the course of the development of diabetic retinopathy (Kowluru, 2003). Unfortunately, these effects are not easily reversed, or the progression thereof slowed by the reinstatement of good glycaemic control (Kowluru, 2003).

5.1.2. Nephropathy:
Diabetic nephropathy is characterized by glomerular basement thickening, glomerular and tubular hypertrophy, mesangial expansion, glomerulosclerosis and tubulointerstitial fibrosis (Jacobsen, 2005), hypertension, a progressive increase in albuminuria, a high cardiovascular risk and an unrelenting decline in glomerular filtration rate (GFR). This in turn may lead to end stage renal disease (ESRD) (Rossing, 2007). Patients of 10 to 15 years of age with previously diagnosed type 1 diabetes are at risk of developing diabetic
nephropathy. The interval for type 2 diabetes is less clearly defined because onset of this type of diabetes is less defined (Rossing, 2007). Without specific intervention, 20% to 40% of diabetic patients will develop nephropathy with devastating consequences (Rossing, 2007). Contrary to the progressive and irreversible development of diabetic nephropathy in the past, recent advances in the management and treatment of diabetic nephropathy has led to the substantial improvement of this complication through early and aggressive blood pressure lowering (Parving et al., 2001).

5.1.3. Neuropathy:
Diabetic neuropathy can be defined as signs and symptoms of peripheral nerve dysfunction in patients with diabetes mellitus in whom other causes of peripheral nerve dysfunction have been excluded (Bansal et al., 2006). Diabetic neuropathies affect both peripheral and autonomic nervous systems and cause considerable morbidity and mortality in diabetic patients (Vinik et al., 2008). Although the main causes of diabetic neuropathy are still largely unknown, ischaemic and metabolic components are implicated in the pathogenesis (Bansal et al., 2006). Hyperglycaemia induces rheological changes which in turn results in increased endothelial vascular resistance and reduced blood flow. Additionally, hyperglycaemia also causes the depletion of nerve myoinositol through a competitive uptake mechanism. Hyperglycaemia-induced oxidative stress, through mechanisms previously described, results in abnormal neuronal, axonal, and Schwann cell metabolism, further resulting in impaired axonal transport. Furthermore, increased vascular resistance results in hypoxia and additional nerve damage (Bansal et al., 2006). The treatment of diabetic neuropathy is aimed at decreased progression by ultimately controlling blood glucose levels, as well as symptomatic relief (Bansal et al., 2006).
5.2. Macro vascular complications

5.2.1. Cardiovascular disease (CVD):
CVD occurs with greater frequency in people with diabetes mellitus and the prevalence of CVD is even more prominent in diabetic women than diabetic men (Nathan, 1993; Huxley et al., 2006). Epidemiological studies have constantly shown that diabetes increases the risk for coronary heart disease (CHD), (Fuller et al., 2001; Almdal et al., 2004; Fox et al., 2004; Vaccaro et al., 2004) in part due to the strong association between the lipid profile and risk of CHD in adults with diabetes (Petitti et al., 2007). Wajchenberg (2007) and others suggested that CVD may be related to a poor control of hyperglycaemia. Increased LDL oxidation and endothelial dysfunction as a result of chronic hyperglycaemia directly affect most CVD risk factors (reviewed by Wajchenberg, 2007). Bloomgarden (2004) reviewed different factors that may have an effect on the development of CVD. Inflammation, as a result of hyperglycaemia, may contribute to the development of CVD through the hexosamine pathway (reviewed by Bloomgarden, 2004). Similarly, hyperglycaemia-induced oxidative stress results in increased nitric oxide (NO) production which ultimately leads to DNA damage (Wajchenberg, 2007). This DNA damage results in acute endothelial dysfunction contributing to the development of CVD (Wajchenberg, 2007). Furthermore, hyperglycaemia also results in the activation of the PKC pathway which has known effects on the development of diabetic complications (Brownlee, 2005).

5.2.2. Peripheral vascular disease (PVD):
PVD can be defined as lower extremity arterial atherosclerosis (Adler et al., 2002) and can present as painful aching, cramping, or tightness of muscles during exercise, due to insufficient blood flow to meet the metabolic demands (Falconer et al., 2008). PVD is more common in patients with diabetes and may increase the risk for lower extremity amputations (Barzilay et al., 1997).
The involvement of hyperglycaemia in the development of PVD is clearly defined, even though no prospective study has previously identified hyperglycaemia as an independent risk factor in type 2 diabetes (Adler et al., 2002). However, even in the absence of diabetes, insulin resistance increases the risk of PVD by nearly 50% (Muntner et al., 2005). Uncontrolled hyperglycaemia as well as increased HbA1c levels result in an increased risk for the development of PVD (Bartholomew & Olin, 2006) through various pathways including the polyol pathway, the AGE pathway, the PKC pathway, and the hexosamine pathway (Giugliano et al., 2008). These pathways are described in detail in section 3.

5.2.3. Cerebrovascular disease:
More than 40 years ago it was documented that cerebrovascular disease is present in twice as many diabetic patients beyond the age of 40 years, compared to non-diabetic individuals of the same age (Grunnet, 1963, Garcia et al., 1974). The pathogenesis of diabetes-associated stroke appears to be linked to excessive glycation and oxidation, endothelial dysfunction, increased platelet aggregation, impaired fibrinolysis and insulin resistance (Lukovits et al., 1999). As with most of the complications linked with diabetes, chronic hyperglycaemia and insulin resistance are, once again, being targeted as significant factors in the development and progression of diabetes-associated cerebrovascular disease (Ceriello et al., 1992; Folsom et al., 1999).

6. MANAGEMENT OF DIABETES MELLITUS AND DIABETIC COMPLICATIONS

The management of diabetes and diabetic complications are multi-factorial which can be addressed individually or in combination to manage diabetes and its associated complications (Nathan et al., 2009). These factors include lifestyle interventions including weight management, correct diet, physical activity, and medical management (The Diabetes Control and Complications
Trial Research Group, 1993; Nathan et al., 2009) using amongst others, hypoglycaemic agents, antioxidant therapy (Wohaib & Godin, 1987); and poly (ADP-ribose) polymerase (PARP) inhibitors (Brownlee, 2005). The main aim of these treatments being improved glucose control. These factors will be discussed in the following section.

6.1. Strict glucose control

Due to the fact that diabetes mellitus is associated with various abnormalities and complications (as previously discussed), there are different goals in the management of diabetes (Tables 2 & 3). The first consideration is glucose control, since all the diabetes-related complications are (directly or indirectly) caused by chronic high blood glucose levels. The American Diabetic Association (ADA) and the American College of Endocrinologists/American Association of Clinical Endocrinologists (ACE/AACE) suggest that a fasting blood glucose level of between 4.44 and 6.1 mmol/L (80 – 110 mg/dL) should be maintained, whereas HbA1c levels should be kept in a range between 6.5 and 7% (Collins, 2002) (Table 2). As hyperglycaemia and insulin resistance lead to the development of hypertension as well as a distinctive atherogenic lipid profile, the management of hyperglycaemia as well as the atherogenic lipid profile are important parts of diabetes management (Table 3). Lipid management of patients with diabetes should be directed at lowering LDL-C and TG levels, as well as raising HDL-C levels in order to reduce the development of macro vascular complications (American Diabetes Association, 2002).

6.2. Lifestyle interventions

Inactivity and overweight are major lifestyle factors contributing to the development of diabetes and may also result in poor management of diabetes.
and diabetic complications (Harris, 1991; American Diabetes Association, 2002). Modest weight loss of 5-10% of the total body weight translates into improved insulin sensitivity (McAuley et al., 2002) and decreased cardiovascular risk factors in patients with type 2 diabetes mellitus (Hollander et al., 1998; Kelley et al., 2002). Additionally, physical activity results in improved glycaemic control, blood pressure, and lipid levels (Wolf et al., 2004). It has been shown that weight loss in type 2 diabetes improves insulin resistance, increases insulin-stimulated nonoxidative-glucose metabolism, and enhances the effect of insulin to inhibit exogenous glucose production and suppress lipid oxidation (Henry et al., 1986; Kelley et al., 1993; Goodpaster et al., 1999; Kelley et al., 1999). Recently it has been shown that a diet consisting of 30% protein, 20% carbohydrate, and 50% fat over five weeks resulted in a 38% reduction in 24-hour integrated glucose area, a reduction in fasting glucose to near normal levels and a total glycated hemoglobin (HbA1c) reduction from 9.8% to 7.6% (Gannon & Nuttall, 2006). However, such diet may cause other complications over the long term due to its high fat:carbohydrate ratio. The level of HbA1c provides a measure of glycaemic control of diabetes patients during the previous two to three months (Jeffcoate, 2004). However, currently the American Diabetes Association recommends a carbohydrate intake of between 45% and 65% of the daily total energy intake (Delahanty et al., 2009).
Table 2  
Guidelines for glycaemic control in mmol/L (mg/dL) in diabetes (American Diabetes Association, 2002)

<table>
<thead>
<tr>
<th></th>
<th>Fasting BG</th>
<th>Pre-prandial BG</th>
<th>Post-prandial BG</th>
<th>Bedtime BG</th>
<th>HbA1c (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal range</td>
<td>3.88–5.60 (70–100)</td>
<td>&lt; 5.60 (&lt; 100)</td>
<td>&lt; 7.80 (&lt; 140)</td>
<td>&lt; 6.10 (&lt; 110)</td>
<td>&lt; 6.0</td>
</tr>
<tr>
<td>ADA</td>
<td>4.44–6.10 (80–110)</td>
<td>4.44–6.70 (80–120)</td>
<td>&lt; 10.1 (&lt; 180)</td>
<td>5.60–7.80 (100–140)</td>
<td>&lt; 7.0</td>
</tr>
<tr>
<td>ACE/AACE</td>
<td>&lt; 6.10 (&lt; 110)</td>
<td>&lt; 6.10 (&lt; 110)</td>
<td>&lt; 7.78 (&lt; 140)</td>
<td>-</td>
<td>&lt; 6.5</td>
</tr>
</tbody>
</table>

Table 3
Goals for blood pressure and lipid levels for adults with diabetes
(American Diabetes Association, 2002)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Goal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood pressure (mm Hg)</td>
<td></td>
</tr>
<tr>
<td>Systolic</td>
<td>&lt; 130</td>
</tr>
<tr>
<td>Diastolic</td>
<td>&lt; 80</td>
</tr>
<tr>
<td>Triglycerides in mmol/L (mg/dL)</td>
<td>&lt; 1.70 (&lt; 150)</td>
</tr>
<tr>
<td>HDL-C in mmol/L (mg/dL)</td>
<td></td>
</tr>
<tr>
<td>Men</td>
<td>&gt; 1.17 (&gt; 45)</td>
</tr>
<tr>
<td>Women</td>
<td>&gt; 1.43 (&gt; 55)</td>
</tr>
<tr>
<td>LDL-C in mmol/L (mg/dL)</td>
<td>&lt; 5.60 (&lt; 100)</td>
</tr>
</tbody>
</table>

HDL-C: High-density lipoprotein cholesterol; LDL-C: Low-density lipoprotein cholesterol

6.3. Medical management

The global mortality rate from diabetes has been largely reduced through the control of hyperglycaemia by the development of potent anti-diabetic substances (Joost, 1985), ultra pure recombinant human insulin (Heinemann et al., 1990), and new methods for insulin delivery (Mirouze, 1983). This has lead to a dramatic increase in life expectancy of people diagnosed with this disease. The various medicinal strategies that are currently being used will be discussed below.
6.3.1 Oral hypoglycaemic agents:
Current recommendations from the American Diabetes Association include a trial of diet and exercise as first line therapy for the treatment of patients with type 2 diabetes. If the desired level of glycaemia control is not achieved within three months, a pharmacological intervention is required (Luna & Feinflos, 2001). Different classes of pharmacological interventions include: Sulphonylureas, Meglitinides, Biguanides, Thiazolidinediones and Alpha(α)-glucosidase inhibitors. These will be discussed in the section below.

6.3.3.1 Sulfonylureas:
Sulfonylureas stimulate insulin release from the β-cells and may slightly improve insulin resistance in peripheral target tissue (Sturgess et al., 1985; Luna & Feinglos, 2001) by the closure of the ATP-dependent potassium channels in the target tissue (Gerich, 1989). This class of oral hypoglycaemic agents generally reduces HbA1c levels as well as fasting plasma glucose concentrations (Luna & Feinglos, 2001). Included in this class of treatment is tolbutamide, tolazamide, chlorpropamide, glibenclamide, glipizide and gliclazide (Luna & Feinglos, 2001).

6.3.3.2 Meglinitides:
The mechanism of action of meglinitides (repaglinide and nateglinide) closely resembles that of sulfonylureas (Luna & Feinglos, 2001). Meglinitides also stimulate insulin release from the β-cells, but are mediated through a different binding site of the β-cell. Unlike sulfonylureas, meglinitides have a very short onset of action and a short half-life (Luna & Feinglos, 2001).

6.3.3.3 Biguanides:
Metformin is currently the only agent in this class being used in America. It works by reducing hepatic glucose output and enhancing insulin sensitivity in hepatic and peripheral tissue (DeFronzo et al., 1995; Feinglos & Bethel, 1998; DeFronzo, 1999). Metformin has been shown to reduce HbA1c levels and
fasting plasma glucose concentrations. It also reduces plasma TG and LDL levels (Luna & Feinglos, 2001).

6.3.3.4 Thiazolidinediones:
Included in this class are pioglitazone and trozigitazone. These agents work by enhancing insulin sensitivity in muscle and adipose tissue. It also reduces hepatic glucose production to a lesser extent. These drugs noticeably improve insulin resistance, especially when used in combination with other drugs (Luna & Feinglos, 2001). However, it has no effect on insulin secretion (Luna & Feinglos, 2001). Monotherapy with these agents has been associated with reduced HbA1c levels as well as reduced fasting plasma glucose concentrations (Feinglos & Bethel, 1998; DeFronzo, 1999). Additionally, troglitazone is associated with decreased TG levels (Saltiel & Olefsky, 1996; Raskin et al., 2000). However, troglitazone has been removed from the market worldwide due to its negative side effects.

6.3.3.5 Alpha-glucosidase inhibitors:
Alpha-glucosidase inhibitors act by inhibiting the enzyme alpha-glucosidase which cleaves complex carbohydrates into sugars (Luna & Feinglos, 2001), thus inhibiting the absorption of sugars after a meal. The largest impact of the class is, therefore, on postprandial glycaemia (Luna & Feinglos, 2001), with a modest effect on fasting plasma glucose concentrations. Additionally, alpha-glucosidase inhibitors (acarbose and miglitol) have been associated with a reduction in HbA1c levels (Feinglos & Bethel, 1998; DeFronzo, 1999).

6.3.2 Antioxidant therapy:
Even though many authors proved that oxidative stress markers can be normalized and early signs of micro vascular and macro vascular complications can be prevented with antioxidant therapy (Koya et al., 1997; Studer et al., 1997; Bursell et al., 1999; Cameron & Cotter, 1999; Kowrulu & Kennedy, 2001), the literature is still vague as to whether oxidative stress...
appears early in diabetes, preceding the development of complications, or whether it is merely a result of tissue damage (Baynes & Thorpe, 1999; Kuroki et al., 2003). Clinical and animal studies have, however, shown improvements in many parameters of oxidative stress such as in lipid peroxidation, increased isopropanes, plasma malondialdehyde and cellular markers of oxidative stress such as NF-kB (Koya et al., 1997; Studer et al., 1997; Bursell et al., 1999; Cameron et al., 1999; Beckman et al., 2001; Gaede et al., 2001; Kowrulu & Kennedy, 2001; Venugopal et al., 2002; Kuroki et al., 2003), as well as in early or functional markers of diabetic retinopathy, nephropathy, neuropathy and cardiovascular disease (Cameron et al., 1999) using antioxidant treatments such as vitamin C and E supplementation, either individually or in combination. Additionally, it has been reported that vitamin C and E supplementation resulted in improved blood flow, nerve conduction velocity, permeability, endothelial dysfunction, albuminuria and vascular contractility (Cameron et al., 1999).

6.3.3. PARP inhibitors:
Since the activation of PARP modifies and inhibits GAPDH, which in turn leads to the activation of the major pathways of hyperglycaemic damage, the inhibition thereof would block these pathways and ultimately prevent hyperglycaemia-induced oxidative stress. As shown by Du and co-workers in 2003, a specific PARP inhibitor prevented hyperglycaemia-induced activation of PKC, NF-kB, intracellular AGE formation, and the hexosamine pathway. In long-term experimental diabetes, treatment with a PARP inhibitor also completely prevented the major structural lesion of both human non-proliferative retinopathy and experimental diabetic retinopathy (Brownlee, 2005).

Apart from the above-mentioned medicines used in the Western world, the earliest recorded treatments for diabetes also involved the use of various plants. As early as 1550 BC high-fibre diets consisting of wheat grains and
ochre (a pigment made from the iron ores haematite (red), goethite (yellow) or limonite (brown), and often used for medicinal purposes), were recommended to normalise diabetes-associated hyperglycaemia (Bailey & Day, 1989). For this reason, orally active botanicals may serve as substitutes for oral hypoglycaemic agents. However, even though botanical therapies still remain the cornerstone of diabetes medicine in underdeveloped regions, the availability and advancement of insulin therapy resulted in the progressive disappearance of indigenous botanical treatments in Western societies (Bailey & Day, 1989). Due to renewed global attention to alternative medicine and natural therapies, renewed scientific interest in this field has evolved.

Many people in South Africa as well as the populations of developing countries still use plants for medicinal purposes (Cunningham, 1993; Thring & Weitz, 2005). Over 27 million people in South Africa use indigenous medicine and up to 60% of the population consult with one of 200 000 indigenous traditional healers (Reviewed by Thring & Weitz, 2005). Despite their extensive use, only few traditional plants have a scientific basis for their proposed actions (WHO 1999). However, through advances in human nutrition research, scientists have an increasing understanding of the relationship between the chemical composition of plants and the health status of those consuming them. In the next section, *Aloes* will be discussed as an indigenous plant widely used for the treatment of diabetes in South Africa.

7. ALOE

*Aloes* have been used therapeutically since ancient times (Morton, 1961, Crosswhite & Crosswhite, 1984) and popularity in the inner, colourless leaf gel has increased in the last two decades (Reynolds & Dweck, 1999). *Aloe* gel has been sold commercially in various parts of the world as part of a wide range of health care, cosmetic and therapeutic product ranges (Reynolds & Dweck, 1999). Apart from their extensive commercial use, various *Aloe*
species are also being used in rural communities throughout the globe to treat a variety of ailments. In South Africa for instance, the leaves of various *Aloe* species are used for their laxative, anti-inflammatory, immuno-stimulant, anticeptic (reviewed by Okyar et al., 2001), wound and burn healing (Chithra et al., 1998), anti-ulcer (Koo, 1994), and anti-tumor (Saito, 1993) activities, of which their anti-diabetic activity is the most common application (Bunyaphraphatsara et al., 1996; personal communications with traditional healers). In this study the focus is on the two *Aloe* species most widely distributed over South Africa. *Aloe ferox* is a tall single stemmed plant distributed over more than a thousand kilometres from the South Western Cape through to Southern Kwazulu-Natal, the south eastern corner of the Free State and Southern Lesotho, South Africa (Aubry, 2001). It prefers a cooler climate and creates a stunning winter display when in bloom. However, due to the large difference in the climate in the different parts of South Africa, these plants may differ physically from region to region (Aubry, 2001). *Aloe greatheadii* var. *davyana* on the other hand, occurs in all the northern provinces of South Africa (van Wyk & Smith, 1996). Both species occur in a broad range of habitats as a result of their wide distribution range and grow both in the open and in bushy areas (Figure 7).
Very little scientific data regarding the compounds occurring in these species, as well as their biological activities, exist. However, recent studies have investigated the mechanisms by which Aloe may improve diabetes and related complications (reviewed by Loots, 2008:459). Due to the fact that A. vera (Aloe barbadensis) and Aloe arborescens are the most extensively described in literature (Reynolds & Dweck, 1999), the use of Aloe in general for the treatment of diabetes is primarily based on research done on the two Aloe species indigenous to the Sudan and Middle East (Agarwal, 1985). Other Aloe species described for their possible antidiabetic activity include Aloe glibberellin, A. ferox Mill, Aloe perryi Baker, and Aloe africana (Reynolds & Dweck, 2009). As different Aloe species occur in a wide range of habitats worldwide, the use of specific Aloe species by rural communities will depend on the immediate availability of the Aloe species. Additionally, inter-species variation as well as variations in climate, soil etc, are all factors which may affect the phytochemical composition of these plants, and subsequently their biological activity. As a result, a direct correlation of biological activity from
A. vera, based on phytochemical content, to other Aloe species occurring in other parts of the world would be inaccurate. Hence, it is important for both commercial sectors and rural communities to better describe their local Aloe species' phytochemical contents and biological functions, and in so doing evaluate their applications to health and disease.

8. ANTIDIABETIC EFFECTS OF ALOE

8.1. Antioxidant properties

There is increasing interest in the antioxidant activities of various phytochemicals present in the diet. Antioxidants are believed to play a very important role in the body's defence system against ROS (Ou et al., 2002). It has been suggested that antioxidant action may be an important property of plant medicines associated with diabetes (Larson, 1988). Antioxidant polyphenols were recently isolated from A. vera and identified as aloersin derivatives (Lee et al., 2000; Yagi et al., 2002). After determining the antioxidant activity of crude and processed A. barbadensis, Lee and co-workers (2000) concluded that the extracts of the crude preparation exhibited appreciable antioxidant activity. Additionally, A. barbadensis has been shown to have hepatoprotective effects and this has mainly been attributed to its antioxidant capacity (Chandan et al., 2007).

Although there is very limited research confirming the antioxidant capacities of various Aloe extracts in vitro, a large amount of literature exists describing the in vivo antioxidant effects of various Aloe species. Beppu and co-workers describe the protective effects of A. arborescens boiled leaf skin against pancreatic β-cell oxidative damage caused by methyl (CH$_3$) radicals (Beppu et al., 2003, Beppu et al., 2006). Additionally, A. vera leaf pulp (500 mg/kg) and leaf gel (63 mg/kg) extracts resulted in increased liver GSH, and a reduction in non-enzymatic glycation and lipid peroxidation in streptozotocin.
(STZ)-diabetic rats (Can et al., 2004). Rajasekaran and co-workers (2005a & 2005b) also showed increased liver and kidney GSH levels and increased activities of the liver and kidney antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione-s-transferase (GST) with the administration of an A. vera leaf gel extract (300 mg/kg) to STZ-diabetic rats. These effects were largely attributed to the antioxidant polyphenols present in these Aloe species.

Apart from their antioxidant capacity, polyphenols may additionally result in decreased intestinal glucose absorption (Cao et al., 1997) which in turn may lead to reduced oxidative stress. Furthermore, the lowering of blood glucose levels by Aloe supplementation can, additionally be expected to ultimately reduce polyol, PKC, and hexoamine pathway activities, as well AGE production (Loots, 2008:459), further protecting tissue from hyperglycaemia-induced damage. Possible interventions to decrease diabetes induced oxidative stress, may therefore include improved glycaemic control in addition to antioxidant therapy (Sharma et al., 2000) and hence may very well include plants/plant extracts that have both hypoglycaemic and anti-oxidative properties.

8.2. Glucose lowering effects

The management of diabetes without any side effects is a challenge to the medical systems, thus increasing the demand for natural products with anti-diabetic activity (Can et al., 2004). As reviewed by Loots, (2008), the glucose lowering effects of Aloe in human and animal studies have been demonstrated by various authors (Reynolds & Dweck, 1999; Volger & Ernst, 1999). Different Aloe extracts (gel, juice, dried exudates, polysaccharide fractions, bitter crystals, leaf skin pulps, and acetone precipitates) of various Aloe species in both humans as well as Alloxan (ALX) and STZ-induced diabetic animals, have been used to investigate its possible glucose lowering effects.
The reported hypoglycaemic effects have mainly been attributed to two glycans (isolated from \textit{A. aborescense}) (Hiniko \textit{et al.}, 1986), acemannan (carbohydrate fraction of the \textit{Aloe} gel), glycoproteins and various polysaccharides (as identified in \textit{A. barbadensis} Miller (\textit{A. vera} Linne) and \textit{Kidachi Aloe}) and various phenols (Beppu \textit{et al.}, 2006).

One of the first reports on the positive effects of \textit{A. vera} appeared in 1985 when Agarwal investigated the lipid and glucose lowering effects of a diet containing "Husk of Isabgol" and \textit{A. vera}, in the absence of anti-diabetic drugs, in patients with diabetes and atheromatous heart disease over a period of five years (Agarwal, 1985). He reported a marked decrease in blood sugar, serum TC and TG levels in 3167 patients who followed the diet consisting of \textit{A. vera} leaves (Agarwal, 1985) and speculated that these positive effects may have been due to the high soluble fibre content of \textit{A. vera} (Agarwal, 1985). Since then, various authors investigated the glucose lowering effects of different \textit{Aloe} species (Ghannam \textit{et al.}, 1986; Ajabnoor, 1990; Beppu \textit{et al.}, 1993; Rajasekaran \textit{et al.}, 2004, Gundidza \textit{et al.}, 2005; Beppu \textit{et al.}, 2006; Rajasekaran \textit{et al.}, 2006; Kim \textit{et al.}, 2009). In 1990, the acute glucose lowering effects of the exudates of \textit{A. barbadensis} (500 mg/kg body weight) was reported after oral supplementation over four days in ALX-induced diabetic mice (Ajabnoor, 1990). The proposed positive effects were attributed to the stimulation of insulin release from the \(\beta\)-cells, resulting in reduced glucose levels. Abuelgasim reported slight, but not significant reductions in glucose levels with acute \textit{A. vera} supplementation (gastric dosing of 100 and 500 mg/kg ethanol \textit{A. vera} extract) over a six day period in 18 hour fasted normal rats after the intraperitoneal administration of 50% glucose at a dose of 2 mg/kg body weight (Abuelgasim \textit{et al.}, 2008).

The glucose lowering effects following a longer duration of \textit{Aloe} supplementation have also been investigated. Beppu and co-workers (1993) confirmed the blood glucose lowering effects of \textit{Aloe} in 1993 by the oral
administration of *Kidachi Aloe* leaf pulp (*A. arborescens* var. *natalensis*) in STZ-induced diabetic rats. In 2006, Beppu and co-workers investigated the anti-diabetic effects of dietary supplements of whole leaf, leaf pulp and dried powder of *Kidachi Aloe* and *A. vera* in low dose STZ treated diabetic mice with less decisive results. Significant reductions in fasting blood glucose levels were seen after 19 days of STZ treatment with oral administration of *Kidachi Aloe* whole leaf (245 ± 69.8 mg/dL) as well as with leaf pulp administration (216 ± 42.1 mg/dL) compared to the basal diet control group (258 ± 67.5 mg/dL). However, 73 days after STZ injection, *Kidachi Aloe* and *A. vera* leaf pulp showed rather high fasting blood glucose levels (269 ± 94.9 mg/dL) after 19 days, but not after 73 days (334 ± 75.7 mg/dL) following the STZ injections, compared to STZ-induced diabetic rats receiving the basal diet (258 ± 67.5 mg/dL and 374 ± mg/dL respectively) (Beppu et al., 2006). Similar confounding results on blood glucose were also seen by other groups. Okyar showed similar hyperglycaemic effects using *A. vera* leaf gel (as opposed to of leaf pulp) in type 2 diabetic rats (Okyar et al., 2001). These findings are supported by previous experiments showing an elevation of blood glucose levels in alloxan-diabetic mice treated with a health product containing *A. vera* leaf gel (Koo, 1994). Okyar concluded that the hyperglycaemic agent of *A. vera* probably resides in the leaf gel, and that leaf pulp, devoid of the gel, may be useful in the treatment of diabetes mellitus (Okyar et al., 2001). However, Beppu and co-workers speculated that the lack in glucose lowering effects seen in the two leaf pulps may be due to the high polysaccharide and disaccharide content of these, putting extra strain on the pancreatic β-cells during the digestion and absorption of these sugars. Considering previous results showing glucose lowering effects with the intraperitoneal administration of *Kidachi Aloe* leaf (inclusive of the pulp) (Beppu et al., 1993), Beppu and co-workers (2006) speculated that the method of dosing may be an important consideration when using these *Aloes* for their anti-diabetic action.
8.3. Lipid lowering effects

The lipid lowering effects of *A. vera* reported by Agarwal in 1985 was repeated in 1996 and 2006 (Bunyanpraphatsara *et al.*, 1996; Yongchaiyudha *et al.*, 1996; Rajasekaran *et al.*, 2006). Bunyanpraphatsara and co-workers (1996) as well as Yongchaiyudha and co-workers (1996) reported reduced serum TG levels in 2 human parallel studies with the supplementation of a tablespoon of *A. vera* gel over a period of 42 days. In the same way, Rajasekaran and co-workers (2006) showed marked reductions in plasma, liver and kidney cholesterol, TC, TG, LDL-C, VLDL-C, free fatty acids and phospholipids, as well as augmentations in HDL-C to near normal levels with oral dosing for 21 days of 300mg/kg/day *A. vera* leaf gel extract in STZ-induced diabetic rats.

Considering the literature, not only does the type of *Aloe* used influence the outcome of a diabetes intervention, but so does the part of the *Aloe* used (e.g. leaf, inner gel, pulp, skin), the way in which these parts are prepared/extracted prior to administration (e.g. ethanol extracts, lyophilization etc.), as well as the mode of administration (orally, gastric gavage, intraperitoneal administration, etc).

9. INDUCTION OF EXPERIMENTAL DIABETES IN EXPERIMENTAL ANIMALS

Diabetic animal models are increasingly being used for investigating the management of diabetes and its long term complications. These models can be divided into 2 broad categories: 1) genetically induced spontaneous diabetes models and 2), experimentally/chemically induced non-spontaneous diabetes models (Islam & Loots, 2009). For the purpose of this thesis, the focus will be only on the chemically-induced diabetic animal models.
A review written by Islam and Loots (2009) will be used primarily as the source of information for this section due to its comprehensive discussion of the topic.

Due to the fact that very little is known about the possible toxicities of new medications, initial drug development is done using diabetic animal models, which can be induced using a variety of methods. Chemically-induced diabetic animal models are convenient and simple to use due to their lower cost, wider availability, and ease of diabetes induction (Islam & Loots, 2009). As summarized by Islam and Loots (2009), the most frequently used substances to induce diabetes are ALX and STZ (Szkudelski, 2001) via intraperitoneal or intravenous injections, even though various other methods to produce diabetes animal models are also being used. The first chemical described to induce diabetes was ALX (Goldner & Gomori, 1943), followed by STZ in 1963 (Rakieten et al., 1963). Both these chemicals induce diabetes by destroying the pancreatic β-cells, mainly by inducing oxidative stress. Although both STZ and ALX can be used to induce type 1 or type 2 diabetes (depending on the dosage administered), the literature seems to favor the use of STZ due to its stability, mechanism of action and the diabetogenic dose needed.

In the 1950s, the first report of a diabetic animal model induced via partial pancreatectomy was described (Pauls & Bancroft, 1950) and was characterized by hyperglycaemia, reduced β-cell mass and associated reduced pancreatic weight, reduced insulin content, and reduced insulin response. Further development of this model involved varying the degree of pancreatectomy (Leahy et al., 1988; Jonas et al., 1999; Kurup & Bhonde, 2000), with and without the aid of diabetes inducing chemicals, alone or in combination, depending on the animal used and the degree of pancreatectomy (Islam & Loots, 2009). Even although most of these models developed
hyperglycaemia and insulin resistance, other characteristics typical of type 2 diabetes such as dyslipidaemia and impaired liver function were absent.

Apart from the development of diabetic animal models using adult animals, diabetes can also be induced during neonatal stages. Bonner-Weir and co-workers (1981) introduced a neonatal animal model with the intraperitoneal injection of STZ using 2-day old rats. With further refinement of this model it became clear that there are varying inter-species sensitivities to this drug. The need for different doses of STZ to induce diabetes, resulting in diabetes animal models with different diabetes characteristics, have been reported by various investigators (Hemmings & Spafford, 2000; Shinde & Goyal, 2003; Emonnot et al., 2007). The use of ALX to induce diabetes in neonatal animals has, to our knowledge, only been attempted by one group of investigators (Kodama et al., 1993) and resulted in chronic high postprandial blood glucose levels.

In addition to the use of a low to moderate dose of STZ or ALX, dietary modification has also been described as a possible way to induce diabetes in rats and mice (Hutton et al., 1976; Luo et al., 1998; Reed et al., 2000; Zhang et al., 2003; Srinivasan et al., 2005; Islam & Choi, 2007). In particular, a high-fat diet in combination with STZ injections of various doses have been used to induce a type 2 diabetes rat or mouse model that presented with characteristics similar to that found in human type 2 diabetic patients. These include insulin resistance, significant increases in body weight, increased fasting blood glucose levels, increased serum insulin, increased free fatty acids, increased triglycerides (Lewis et al., 1991; Reed et al., 2000), and hyperlipidaemia (Lewis et al., 1991).

Various authors have noted that intra-uterine growth retardation (IUGR) can also be used to develop a type 2 diabetes rat model (Dacou-Voutetakis et al., 1975; Hales et al., 1991; Simmons et al., 2001; Vuguin et al., 2004) and is
characterized by hyperglycaemia, impaired glucose tolerance, and insulin resistance. Mono-sodium glutamate (MSG), usually used in food preparation, has been shown to have necrotic effects when injected intraperitoneally. When injected for a pre-determined number of days, MSG resulted in type 2 diabetes related characteristics such as obesity, impaired glucose tolerance, hyperinsulinaemia, increased triglyceride levels (Iwase et al., 1998; Nagata et al., 2006), abnormal lipid profiles, and abnormal liver function (Nagata et al., 2006).

For the purpose of this review, only the mechanism of STZ action will be discussed, as this was the method that was used in this study.

9.1. Mechanism of streptozotocin (STZ) action

As mentioned earlier, STZ was first used to produce diabetes in experimental animal models in 1963 (Rakieten et al., 1963). Since the initial discovery of the use of STZ for this purpose, various groups have manipulated the process to develop experimental animal models that closely resemble type 1 diabetes (Like and Rossini, 1976; Ganda et al., 1976; Rossini et al., 1977; Like et al., 1978). However, since the first separate classification of type 1 and type 2 diabetes by the WHO in 1980, the process of inducing diabetes using STZ was modified to attempt the development of an experimental animal model that resembles type 2 diabetes (WHO, 1980).

STZ (2-deoxy-2-(3-(methyl-3-nitosoureido)-D-glucopyranose) is used to induce diabetes (Szkudelski, 2001) via destruction of the pancreatic β-cells. This is accomplished by the intraperitoneal or intravenous injection of STZ into various species of male rats and mice (Junod et al., 1967; Rerup, 1970; Ozturk et al., 1996; Rajasekaran et al., 2006; Srinivasan & Ramarao, 2007; Jafarnejad et al., 2007). Depending on the dosage used, a model more closely resembling that of type 1 diabetes, or that of type 2 diabetes can be induced.
As STZ causes β-cell destruction, the diabetes model that results from higher doses of STZ may be thought to be closer to a type I diabetic state. However, at lower STZ dosages, only partial destruction of β-cells occurs, hence resulting in a diabetes animal model with enough insulin producing β-cells remaining for this model to be classified as non-insulin dependent responding to conventional type II diabetes medication. Judging from the literature, a dose of 50 mg/kg is generally used for inducing a type 2 state, and doses of greater than 65 mg/kg for a type 1 diabetic state (Abdel-Zaher et al., 2005; Guerrero-Analco et al., 2007). Most, if not all the literature uses the lower dose STZ model for investigating the anti-diabetic effects of various plant extracts, and additionally use glibenclamide (an oral hypoglycemic sulfonylurea commercially used in type 2 diabetes to control blood glucose levels) as a positive control. Apart from the intended induction of type 2 diabetes with a single intraperitoneal injection of STZ in mature rats, multiple low dose injections are also used in either infant or mature mice or rats to induce a type 2 diabetic state (Szkudelski, 2001).

An important consideration when determining the dose of STZ to induce diabetes, is the age and species of animals selected. As early as the 1970s the literature reported an age-depended effect of STZ in rats or mice (Masiello et al., 1979; Riley et al., 1981; Reddy & Sandler, 1995; Ranhotra & Sharma, 2000), concluding that older animals were less sensitive to the effects of STZ than their younger counterparts. The weight of the animal was deemed less important as the dosage of STZ is calculated per kg body weight. Mice are also more tolerant to the toxic effects of STZ as compared to rats, hence lower doses are used in the latter to induce a similar diabetic state.

The diabetogenic effects of STZ can be observed within 2 hours after injection with a rise in blood glucose levels accompanied by a drop in insulin. After 6 hours, hypoglycaemia and hyperinsulinaemia occurs followed by a final rise in glucose levels accompanied by reduced insulin (West et al., 1996).
impairs glucose oxidation and decreases insulin biosynthesis and secretion through various processes: STZ causes oxidative stress by inhibiting the TCA cycle (Turk et al., 1993) which limits mitochondrial oxygen consumption (Bedoya et al., 1996). This results in the overproduction of ROS in the mitochondria. Furthermore, the STZ action in the mitochondria limits mitochondrial ATP production causing ATP depletion (Szkudelski, 2001). The restriction of ATP generation is further augmented by NO production (Szkudelski, 2001) as well as the depletion of NAD$^+$ by the activation of poly ADP-ribosylation (Sandler & Swenne, 1983). Low ATP levels which ultimately result in β-cell destruction is therefore responsible for reduced insulin secretion. These processes finally result in β-cell DNA damage and ultimately β-cell death (Elsner et al., 2000; Szkudelski, 2001).

10. CONCLUSION

As previously mentioned, diabetes is characterized by hyperglycaemia, due to reduced insulin secretion and/or insulin resistance. Pathological processes involved in β-cell destruction (and ultimately hyperglycaemia) include autoimmune destruction of β-cells, insulin resistance, genetic β-cells defects, genetic defects in insulin secretion, diseases of the exocrine pancreas, endocrinopathies, and infections. Chronic hyperglycaemia, as in diabetes, leads to a variety of complications via various pathways. From the literature it is clear that oxidative stress typically accompanies the development of diabetes and its complications. Most notably, hyperglycaemia is one of the leading causes of oxidative stress. The mechanisms inducing this state includes glucose auto-oxidation, the non-enzymatic and progressive glycation of proteins and consequently an increase in the formation of glucose derived AGEs, enhanced glucose flux through the polyol pathway and PKC activation. Due to the unrelenting rise in the prevalence of diabetes worldwide, limiting the development of this disease and its deleterious complications is of great
importance to global health and economy. It is, therefore, not only important to treat the disease effectively, but more importantly to prevent its occurrence.

One of the most common complications associated with diabetes is an abnormal lipid profile, predominantly caused by oxidative stress, altered insulin release and insulin resistance. This dyslipidaemia is characterized by an increased TG, TC, LDL-C, VLDL-C and FFA levels and decreased HDL-C levels. These complications in turn result in the micro vascular and macro vascular complications seen in diabetic patients.

Even though type 2 diabetes is reversible if treated in its early stages, type 1 diabetes and advanced type 2 diabetes currently result in enormous psychological and economical strain to not only diseased individuals and their immediate dependents due to the lifestyle changes and medical expenses accompanying the management of this disease, but also to global health care systems. For this reason, extensive research is still being done on the discovery of inexpensive, more effective treatments for the management of this disease. In modern medicine, while effective treatment is available to manage diabetes mellitus, the drugs are often very expensive or have undesirable side effects. Personal communications with traditional healers in the North-West Province of South Africa, in addition to the information gained from ample scientific literature generated worldwide, suggest the potential use of indigenous botanicals to treat diabetes. For this reason, global science is aiming at providing scientific evidence to support the medicinal use of plants in underdeveloped regions, as well as to identify new plants with biochemically active components to treat diabetes and other ailments.

Currently, many different botanicals are being investigated for their possible anti-diabetic effects and mechanisms of action. Literature has shown various Aloe species to be valuable in the management of diabetes. There are 130 species of Aloe naturally occurring in South Africa, of which A. greatheadii
var davyana and A. ferox are the most common. Despite their extensive use for treating diabetes traditionally, very little, if any research has been done on the phytochemical composition and health applications of these two indigenous Aloe species. Hence, it would be of great value to both the commercial sectors and rural communities to define the phytochemical compositions of Aloe ferox and Aloe greatheadii, and to confirm their possible biological action and potential antidiabetic health benefits, using an STZ induced diabetic rat model.

11. LITERATURE CITED


Chapter 2: Literature review


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Chapter 2: Literature review


Chapter 2: Literature review


Chapter 3

Aloe ferox Leaf Gel
Phytochemical Content,
Antioxidant Capacity and
Possible Health Benefits
Chapter 3: *A. ferox* phytochemical content

*Aloe ferox* Leaf Gel Phytochemical Content, Antioxidant Capacity and Possible Health Benefits

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ABSTRACT

In this study we identified, quantified and compared the phytochemical contents and antioxidant capacities of *Aloe ferox* lyophilized leaf gel (LGE) and 95% ethanol leaf gel extracts (ELGE) using GC-MS and spectrophotometric methods. Analytically, 95% ethanol is less effective than ethylacetate-diethylether or hexane (in the case of fatty acids) extractions in separating phytochemicals for characterization purposes. However, although fewer compounds are extracted in the ELGE, they are approximately 345 times more concentrated as compared to the LGE, hence justifying ELGE use in biological efficacy studies *in vivo*. Individual phytochemicals identified included various phenolic acids/polyphenols, phytosterols, fatty acids, indoles, alkanes, pyrimidines, alkaloids, organic acids, aldehydes, dicarboxylic acids, ketones and alcohols. Due to the presence of the antioxidant polyphenols, indoles and alkaloids, the *Aloe ferox* leaf gel shows antioxidant capacity as confirmed by oxygen radical absorbance capacity (ORAC) and FRAP analyses. Both analytical methods used show the non-flavonoid polyphenols to contribute to the majority of the total polyphenol content. Due to its phytochemical composition, *Aloe ferox* leaf gel may show promise in alleviating symptoms associated with/or prevention of cardiovascular diseases, cancer, neurodegeneration, and diabetes.
Keywords: Aloe ferox leaf gel; phytochemical; polyphenols, antioxidant capacity; gas chromatography mass spectrometry, spectrophotometry, leaf gel extract, ethanol extract, ORAC, FRAP.

INTRODUCTION

The utilization of plants in various parts of the world is receiving more and more prominence, not only due to their health benefits, but also the opportunities they present to rural based economics. Mainly due to economic constraints, the populations of developing countries worldwide continue to rely heavily on the use of traditional medicine as their primary source of healthcare. Apart from Aloe being used extensively in the cosmetic industry, it has been described for centuries for its laxative, anti-inflammatory, immuno-stimulant, antiseptic (1), wound and burn healing (2), anti-ulcer (3), anti-tumor (4) as well as for its anti-diabetic (5) activities. The majority of the scientifically based research on this topic was done on Aloe vera (or Aloe barbadensis) and Aloe arborescens. However, in the rural communities, the type of Aloe which is chosen as a traditional medicine would depend on its immediate availability to the specific community. Hence, various communities in different parts of the world would use the species of Aloe indigenous to their immediate surroundings. In South Africa for instance, various traditional communities and local industries are using a variety of location specific Aloe species, e.g. Aloe ferox in the Eastern and Western
Cape Provinces and *Aloe greatheadii var Davyana* in the northern regions of South Africa. These *Aloe* species are used in the treatment of arthritis, skin cancer, burns, eczema, psoriasis, digestive problems, blood pressure problems and diabetes. These treatments are based on anecdotal evidence or research findings done almost exclusively on *Aloe vera*. Different *Aloe* species would have varying phytochemical contents, health benefits and possible toxicities. Hence, it is of relevance for scientists, industry and rural communities to not only research the relevant medicinal uses of their indigenous *Aloe* species, but also to determine the active components and their individual or combined mechanisms of biological function. The use of 95% ethanol extracts of various *Aloe* species is extensively described in the literature for determining biological activity in the treatment and prevention of a variety of health conditions (6, 7), particularly diabetes (8-11). In this study we determined and compared the phytochemical contents and antioxidant capacities of *Aloe ferox* lyophilized leaf gel and 95% ethanol leaf gel extracts using gas chromatography mass spectrometry (GCMS) and spectrophotometric methods of analysis. This was done not only to describe, *Aloe ferox* leaf gel extracts with regards to phytochemical contents and possible health benefits, but to compare various extraction methods for both analytical efficacy and possible biological relevance.
MATERIALS AND METHODS

Samples. Whole, freshly cut, Aloe ferox leaves (100 kg) were kindly supplied by the Aloe Ferox Trust. These leaves were harvested in the month of September from farms in the Albertinia region in the Western Cape of South Africa. The inner leaf gel was removed, homogenized, freeze dried and stored at -20°C until analysis. This was termed the leaf gel extract (LGE) for the purpose of this study. Approximately half of the LGE was used for preparation of a 95% ethanol extract as described previously (11). This was termed the 95% ethanol leaf gel extract (ELGE).

Materials. All analytical standards were purchased from Sigma-Aldrich (St Louis, MO, USA) and all the organic solvents used were of ultra high purity purchased from Burdick and Jackson (USA). Folin-Ciocalteu’s phenol reagent and other reagent chemicals were purchased from Merck (Darmstadt, Germany).

Ethylacetate-Diethylether Extraction. The internal standard, 3-phenylbutyric acid (25 mg/50 ml) was added to 25 mg of finely ground LGE and ELGE, followed by the addition of 1 mL sodium acetate buffer (0.125 M). β-Glucuronidase (30 μL) was added, the sample vortexed and incubated overnight at 37°C. The sample was extracted with 6 mL ethyl acetate followed by 3 mL diethylether. The organic phase was collected after each extraction via centrifugation. The organic phase from each extraction was pooled and dried under nitrogen. The dried extract was derivatized with bis
(trimethylsilyl) trifluoroacetamide (BSTFA) (100µL), trimethylchlorosilane (TMCS) (20µL) and pyridine (20µL) at 70°C for 30 min. After cooling, 0.1µL of the extract was injected into the GCMS via split-less injection.

**Fatty Acid Extraction.** Heptadecanoic acid (72mM), as an internal standard, was added to 25mg of LGE and ELGE followed by 100µL of a 45mM solution of butylated hydroxytoluene and 2ml methanolic HCL (3N). The samples were then vortexed and incubated for 4 hours at 90°C. After cooling to room temperature, the sample was extracted twice with 2ml of hexane, dried under a nitrogen stream and finally re-suspended with 100µL of hexane, 1µL of which was injected onto the GC-MS via split-less injection.

**Gas Chromatography Mass Spectrometry.** An Agilent 6890 GC ported to a 5973 Mass Selective detector (California, USA) was used for identification and quantification of individual fatty acids. For the acquisition of an electron ionization mass spectrum, an ion source temperature of 200°C and electron energy of 70 eV was used. The gas chromatograph was equipped with a SE-30 capillary column (Agilent, USA), a split/split-less injection piece (250°C) and direct GC-MS coupling (260°C). Helium (1ml/min) was used as the carrier gas. The oven temperature program for analyzing the ethylacetate-diethylether extract was with an initial oven temperature of 40°C and was maintained for 2min, followed by a steady climb to 350°C at a rate of 5 °C/min. For the fatty acid analysis, an initial oven temperature of 50°C
was maintained for 1.5 min and then allowed to increase to 190°C at a rate of 30°C/min. The oven temperature was maintained at 190°C for 5 min and then allowed to increase to 220°C at a rate of 8°C/min. The oven temperature was again maintained for 2 min and finally ramped to 230°C at a rate of 3°C/min and maintained for 24 min at this temperature.

**Total Polyphenol Assay.** The total polyphenol content of the extracts were determined according to the Folin-Ciocalteu procedure (12). Briefly, 10mg of finely ground LGE or ELGE was dissolved in 200μL H₂O in a test tube followed by 1mL Folin-Ciocalteu's reagent. This was allowed to stand for 8 min at room temperature. Next, 0.8mL of sodium carbonate (7.5%, w/v) was added, mixed and allowed to stand for 30 min. Absorption was measured at 765nm (Shimadzu UV-1601 spectrophotometer). The mean total phenolic content (n = 3) was expressed as milligram gallic acid (Sigma-Aldrich, St Louis, MO, USA) equivalents per 100g wet and dry mass (mg gallic acid equivalents (GAE)/100g) ± standard deviation.

**Total Flavonoid Assay.** The total flavonoid content was measured by using the AlCl₃ colorimetric assay (13) with some modifications. Briefly, 10mg of LGE or ELGE was dissolved in 1mL H₂O, to which 60μL of 5% (w/v) NaNO₂ was added. After 5 min, 60μL of a 10% (w/v) AlCl₃ was added. On the 6th min, 400 μL 1M NaOH was added and the total volume was made up to 2mL with H₂O. The solution was mixed well and the absorbance measured at 510nm against a reagent blank. Concentrations were determined
using a catechin (Sigma-Alrich, St Louis, MO, USA) solution standard curve. The mean total flavonoid content \( n = 3 \) was expressed as milligrams catechin equivalents (CE) per 100g wet and dry mass \( \pm \) standard deviation.

**Oxygen Radical Absorbance Capacity (ORAC).** ORAC analyses of hydrophilic and lipophylic compounds in LGE and ELGE were performed as described previously \( (14) \). The analysis of lipophylic compounds was aided by the addition of randomly methylated \( \beta \)-cyclodextrin as a solubility enhancer as described before \( (15) \). Briefly, in a volume of 200\( \mu \)L, the reaction contained 56nM fluorescein (Sigma-Alrich, St Louis, MO, USA) as a target for free radical attack by 240nM 2,2'-azobis(2-amidino-propane) dihydrochloride (Sigma-Alrich, St Louis, MO, USA). A BioTEK fluorescence plate reader (FL-600, UK) was used and the decay of fluorescence of fluorescein (excitation 485nm, emission 520nm) was measured every 5 min for 2 hours at 37°C. Costar black opaque (96-well) plates were used in the assays. Trolox (Sigma-Alrich, St Louis, MO, USA) was used as standard at a range between 0-20\( \mu \)M with a polynomial (2nd order) curve fit analysis. Mean values \( n = 3 \) of antioxidant capacities were expressed as \( \mu \)moles trolox equivalents (TE)/g wet and dry mass \( \pm \) standard deviation.

**Ferric Reducing Antioxidant Power (FRAP).** FRAP values were determined essentially as described previously \( (16) \). Briefly, the reduction of
a $\text{Fe}^{3+}$-2,3,5-triphenyltetrazolium (Sigma-Aldrich, St Louis, MO, USA) complex in the assay by the antioxidants in the samples was monitored at 593 nm. As a standard, $\text{FeSO}_4$ (Sigma-Aldrich, St Louis, MO, USA) was used and the FRAP activities of the samples expressed as the mean ($n = 3$) μmol $\text{Fe}^{2+}$/g wet and dry mass ± standard deviation.

RESULTS AND DISCUSSION

The compounds identified and their quantities in the *Aloe ferox* LGE and ELGE are summarized in Table 1. Of all the compounds identified, the groups of compounds best described for their health benefits are the phenolic acids/polyphenols, sterols, fatty acids and indoles. Apart from these, various alkanes, pyrimidines, alkaloids, organic acids, aldehydes, dicarboxylic acids, ketones and alcohols were also identified. Although the extraction methods used in this study were not selected to target alcohols, a few of these were also identified. One would, however, expect a far larger variety of alcohols to occur in *Aloe* and in far higher concentrations. For better extraction of these, headspace isolation by simultaneous purging should be used as described previously (17). However, by employing this method one would extract far less of the other biologically important health associated compounds. Therefore, to accomplish the aims of our study, alternative extraction procedures were used as described in the methods section, using ethylacetate-diethylether and hexane.
A general comparison of the phytochemical contents of the LGB and ELGE, calculated as per LGE dry mass, shows that, with the exception of a few compounds, far less compounds and at lower concentrations are extracted from 95% ethanol extracts than directly from the LGE using ethylacetate-diethylether or hexane. The occurrence of higher concentrations of a few compounds from the ELGE is most probably due to matrix protein conformation changes and precipitation by the ethanol, hence making extraction of these protein associated compounds easier (18). However, when quantifying the concentrations for the individual compounds occurring in the ELGE as per dry mass ELGE, the concentrations for the compounds extracted are approximately 345 times higher than that for the same compound occurring in the lyophilized LGE. Similarly, higher concentrations of total polyphenols, total flavonoids and total non-flavonoids, as well as higher antioxidant capacities using ORAC and FRAP analyses (Table 2) are seen in the ELGE extracts. Additionally, these values are again far less when quantified as per LGE dry mass. This indicates that from an analytical perspective, 95% ethanol is in general less effective than direct ethylacetate-diethylether or hexane extractions (in the case of fatty acids) for the phytochemical characterization of *Aloes*. However, the results also indicate the ELGE allows for effective concentration of a number of biologically active ingredients from LGE, confirming its popularity for use for testing biological activity for certain components *in vivo* and *in vitro*. Additionally, polyphenols are generally classified into flavonoids and non-
flavonoids (19). In Table 1, GCMS analyses indicate the majority of the polyphenol compounds identified in the *Aloe ferox* leaf gel belongs to the non-flavonoid group of polyphenols. This was confirmed by the spectrophotometric analysis of polyphenols summarized in Table 2, indicating the non-flavonoid components to contribute to 93% of the total polyphenols in the LGE and 92% in the ELGE.

Over the past 10 years there has been a growing interest in the value of polyphenols among researchers and food manufacturers. This is mainly because of their antioxidant properties, their abundance in the diet and their role in the prevention of various diseases associated with oxidative stress such as cancer, cardiovascular disease, neurodegeneration (20) and diabetes (21). Polyphenols constitute a large class of molecules containing a number of phenolic hydroxyl groups attached to ring structures allowing for their antioxidant activities. These compounds are multifunctional and can act as reducing agents, hydrogen donating antioxidants, and singlet oxygen quenchers (19). All of the individual *Aloe ferox* leaf gel antioxidant polyphenols identified in Table 1 may contribute to the prevention of the above-mentioned diseases to a greater or lesser extent. The individual contributions of these to disease prevention would, however, depend on their concentrations, antioxidant capacities, bioavailabilities and specific mechanisms of action. Although the individual phenolic acids/polyphenols occurring in the highest concentrations where benzoic acid, *p*-toluic acid, *p*-coumaric acid, *p*-salicylic acid, protocatechuic acid, hydroxyphenylacetic...
Acid, ferulic acid, aloe emodin and vanillic acid, it is well known that the protective health benefits of polyphenols are mainly through a combination of additive and/or synergistic effects between the individual compounds (22). Consequently, those polyphenol/phenolic compounds identified in lower concentrations may also be of value.

Due to the fact that the majority of the phenolic acids/polyphenols identified in Aloe ferox leaf gel in Table 1 are antioxidants (19) and these compounds as a group occur in the highest concentrations, one would expect these to contribute to the majority of the antioxidant capacity measured in these extracts (Table 2). However, apart from these polyphenols, the indoles (23) and alkaloids identified (24) are also known to possess antioxidant activities and may consequently also contribute to the ORAC and FRAP values of these extracts. When interpreting data of this nature, one should keep in mind that using the concentrations of these antioxidant compounds alone is insufficient criteria for making predictions of individual contributions to oxidative stress. As previously described, this is due to the fact that the concentrations of individual polyphenol antioxidants are not the only factor influencing antioxidant capacity, but the structural arrangements (number and position of hydroxyl groups, double bonds and aromatic rings) of these compounds also play a role (19). Additionally, their individual contributions to ORAC and FRAP may also differ. Due to the FRAP analysis being an indication of the ferric ion reducing power of a compound or mixture, and the ORAC analysis indicating the ability of a compound or
mixture to scavenge free radicals, the various individual polyphenol
components of the mixture may have stronger free radical scavenging
abilities than reducing power, or *vis a versa*, dependent on their chemical
structures (25).

Phytosterols are another group of compounds well known for their health
benefits. Of the four phytosterols identified in Table 1, \( \beta \)-sitosterol occurred
in by far the highest concentrations in the LGE, contributing to 93% of the
total phytosterols identified. The ELGE was once again less effective in
extracting these compounds and only cholestanol was identified. However,
the levels normalized to dry mass ELGE were not insignificant. Phytosterols
are best described for their total cholesterol and low-density lipoprotein
cholesterol (LDL-C) lowering effects, consequently associated with reducing
the risk for cardiovascular disease (26). As summarized by Devaraj and
Jialal (2006), evidence for this has been observed in hypercholesterolemic,
diabetic and healthy volunteers. The mechanism proposed by which
phytosterols accomplish this is by lowering cholesterol absorption due to the
structural similarities these compounds share with cholesterol (27-29). Apart
from lowering cardiovascular risk factors associated with diabetes,
phytosterols (\( \beta \)-sitosterol in particular) have been shown to affect diabetes
positively by directly lowering fasting blood glucose levels by cortisol
inhibition (30). Additionally, phytosterols have been shown to reduce
biomarkers for oxidative stress and inflammation (31), as well as to reduce
cancer development by enabling anti-tumor responses by increasing immune
recognition of cancer, influencing hormonal dependent growth of endocrine
tumors and altering sterol biosynthesis due to the structural similarities of the
phytosterols with these compounds and their substrates (32). Phytosterols
have also been shown to directly inhibit tumor growth by slowing cell cycle
progression, induction of apoptosis and by the inhibition of tumor metastasis
(32).

Long chain poly-unsaturated fatty acids (PUFA’s) also have important
biological functions noted to modulate risks of chronic degenerative and
inflammatory diseases, of which the essential PUFA’s, linolenic (C18:3 n-3)
and linoleic (C18:2 n-6) acids are best described (33, 34). Both these were
present in the *Aloe ferox* leaf gel extracts with linoleic acid being the major
fatty acid present. However, despite this, the concentrations of these are still
very low in comparison to the other compounds identified with possible
health benefits and were not even detectable in the lipophylic ORAC
analysis. These fatty acids may probably be too low for the *Aloe ferox* leaf
gel to contribute to health through its fatty acid composition.

**CONCLUSIONS**

In conclusion, the results of this study show that from an analytical
perspective, 95% ethanol is a less efficient solvent for the extraction of the
phytochemical components of *Aloe ferox* leaf gel for descriptive purposes as
compared to ethylacetate-diethylether or hexane (in the case of fatty acids).

Although the 95% ethanol extracts contain a smaller variety of extracted compounds, their concentrations are, however, approximately 345 times higher than that of the lyophilized *Aloe ferox* leaf gel when quantified as dry mass ELGE extract. This justifies the popularity of the ELGE for applications testing biological efficacy *in vivo* and *in vitro*. For the purpose of determining possible biological application, *Aloe ferox* leaf gel was characterized. Various phenolic acids/polyphenols, phytosterols, fatty acids, indoles, alkanes, pyrimidines, alkaloids, organic acids, aldehydes, dicarboxylic acids, ketones and alcohols were identified and quantified. Due to the presence of the antioxidant polyphenols, indoles and alkaloids, the *Aloe ferox* leaf gel shows antioxidant capacity as confirmed by ORAC and FRAP analyses. Both GC-MS and spectrophotometric analyses show the non-flavonoid polyphenols to contribute to the majority of the total polyphenol content. Due to the occurrence of the polyphenols, phytosterols and perhaps the indoles present, *Aloe ferox* leaf gel may show promise in alleviating or preventing the symptoms associated with cardiovascular diseases, cancer, neurodegeneration, and diabetes. This may be due to the well-documented lowering effects of these compounds on total cholesterol, LDL-C and fasting blood glucose. These results support the current use of *Aloe ferox* by both industry and traditional healers for the treatment of the above-mentioned diseases. However, further clinical trials regarding these claims are
necessary before accurate conclusions regarding these health benefits can be
made.

ABREVIATIONS

LGE, leaf gel extract; ELGE, ethanol leaf gel extract; GC-MS, gas chromatography coupled mass spectrometry; ORAC, oxygen radical absorbance capacity; FRAP, ferric reducing antioxidant power; LDL-C, low-density lipoprotein cholesterol; BSTFA, bis (trimethylsilyl) trifluoroacetamide; TMCS, trimethylchlorosilane; GAE, gallic acid equivalent; CE catechin equivalent; TE, trolox equivalent

ACKNOWLEDGEMENT

Special thanks to the Aloe Ferox Foundation in Albertinia, South Africa, for supplying the sample material for this study.
LITERATURE CITED


Table 1. Concentrations (particles per million) of GCMS identified compounds from lyophilized *Aloe ferox* leaf gel (LGE) and 95% ethanol leaf gel extract (ELGE).

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<tr>
<th>Compound</th>
<th>Concentration (ppm)</th>
<th>Concentration (ppm)</th>
<th>Concentration (ppm)</th>
<th>Concentration (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LGE (per dry mass LGE)</td>
<td>ELGE (per dry mass ELGE)</td>
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<tr>
<td>Phenol</td>
<td>15.37</td>
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<td>Gentisic</td>
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<td>o-Hydroxyeinnamic</td>
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<tr>
<td>3,4-</td>
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Phenolic acids / Polyphenols

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<td>Suberic</td>
<td>-</td>
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Sugars

<p>|                      | D-Ribonol | 7.09 | - | - |
|                      | 2-Hydroxyglutaryl | - | 20.21 | 7.0 \times 10^4 |
|                      | 3-Hydroxy-3- methylglutaryl | - | 20.66 | 7.2 \times 10^4 |
|                      | 2-Ketoglutaryl | - | 17.25 | 6.0 \times 10^3 |
|                      | Tartaric | - | 18.82 | 6.3 \times 10^3 |
|                      | Suberic | 12.19 | - | - |</p>
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<td>-</td>
<td>-</td>
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<tr>
<td>6,7</td>
<td>38.40</td>
<td>-</td>
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</tr>
<tr>
<td>1,3-dihydroxybutane</td>
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<td>m-Tolualdehyde</td>
<td>18.46</td>
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Table 2. Concentrations of total polyphenols (mg GAE/100g ± stdev), flavonoids (mg CE/100g ± stdev) and non-flavonoids (by calculation) as well as antioxidant capacity via oxygen radical absorbance capacity (ORAC, μmol TE/g) and ferric reducing antioxidant power (FRAP, μmol/g) analyses in lyophilized *Aloe ferox* leaf gel (LGE) and 95% ethanol leaf gel extracts (ELGE).

<table>
<thead>
<tr>
<th>Compounds</th>
<th>LGE (dry mass)</th>
<th>LGE (wet mass)</th>
<th>ELGE (expressed as dry mass ELGE)</th>
<th>ELGE (expressed as dry mass LGE)</th>
<th>ELGE (expressed as wet mass LGE)</th>
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</thead>
<tbody>
<tr>
<td>Total polyphenols</td>
<td>79.2 ± 4.03</td>
<td>2.74 ± 0.14</td>
<td>413 ± 9.89</td>
<td>26.8 ± 0.64</td>
<td>0.94 ± 0.02</td>
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<td>Total flavonoids</td>
<td>5.5 ± 0.38</td>
<td>0.19 ± 0.01</td>
<td>33.6 ± 1.99</td>
<td>2.18 ± 0.13</td>
<td>0.08 ± 0.004</td>
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<td>Total non-flavonoids</td>
<td>73.7 ± 0.45</td>
<td>2.55 ± 0.23</td>
<td>379 ± 6.78</td>
<td>24.6 ± 1.5</td>
<td>0.87 ± 0.02</td>
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<tr>
<td>ORAC - hydrophilic</td>
<td>53 ± 1.2</td>
<td>1.83 ± 0.04</td>
<td>136 ± 2.5</td>
<td>8.83 ± 0.16</td>
<td>0.31 ± 0.006</td>
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<tr>
<td>ORAC - lipophylic</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>ORAC - Total</td>
<td>53 ± 1.2</td>
<td>1.83 ± 0.04</td>
<td>136 ± 2.5</td>
<td>8.83 ± 0.16</td>
<td>0.31 ± 0.006</td>
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<tr>
<td>FRAP</td>
<td>4.9 ± 0.25</td>
<td>0.17 ± 0.08</td>
<td>19.0 ± 0.3</td>
<td>1.23 ± 0.02</td>
<td>0.05 ± 0.001</td>
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</table>

ND, not detected
Chapter 4
Phytochemical Contents and Antioxidant Capacities of Two Aloe greatheadii var. davyana Extracts
Article

Phytochemical Contents and Antioxidant Capacities of Two Aloe greatheadii var. davyana Extracts

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² School for Physical and Chemical Sciences, North-West University, Private Bag X6001, Box 269, Potchefstroom, 2531, South Africa; E-mails: francois.vanderwesthuizen@nwu.ac.za (Francois H. van der Westhuizen)

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Abstract: Aloe greatheadii var. davyana (Asphodelaceae) is used among rural South African communities to treat arthritis, skin cancer, burns, eczema, psoriasis, digestive problems, high blood pressure and diabetes, despite very little supporting scientific evidence. Due to increased interest by both the scientific community and industry regarding the medicinal uses of this plant species, we identified, quantified and compared the phytochemical contents and antioxidant capacities of two extracts of A. greatheadii; a leaf
gel extract (LGE) and a 95% aqueous ethanol leaf gel extract (ELGE), using various modified extraction procedures, GC-MS and spectrophotometry. Apart from extensively characterizing this medicinal plant with regards to its organic acid, polyphenols/phenolic acid, alcohol, aldehyde, ketone, alkane, pyrimidine, indole, alkaloid, phytosterol, fatty acid and dicarboxylic acid contents and antioxidant capacities, we describe a modified extraction procedure for the purpose of general phytochemical characterization, and compare this to a 95% aqueous ethanol extraction technique. From the results it is clear that \textit{A. greatheadii} contains a variety of compounds with confirmed antioxidant capacity and other putative health benefits (such as blood glucose, cholesterol and cortisol lowering properties) relating to the prevention or treatment of diabetes, cardiovascular disease, cancer and hypertension. The results also indicate that separate ethyl acetate/diethyl ether and hexane extractions of the LGE, better serve for general phytochemical characterization purposes, and 95% aqueous ethanol extraction for concentrating selective groups of health related compounds, hence justifying its use for biological \textit{in vivo} efficacy studies.

\textbf{Keywords:} Phytochemical content; antioxidant capacity; polyphenols; phytosterols.

\section*{Introduction}

Populations in developing countries worldwide rely heavily on the use of traditional medicine as their primary source of healthcare \cite{1}. In South Africa alone over 27 million people use indigenous plant based medicines and up to 60% of the population consult with one of 200 000 traditional healers \cite{2}. Additionally, there is an increased global commercial interest in the use of these plant species for their proposed health benefits despite little or no scientific evidence justifying the anecdotal claims accompanying many of these products.

\textit{Aloes} have been used therapeutically since ancient times \cite{3, 4} and interest in the inner, colourless leaf gel has increased over the last two decades \cite{5}. Partial characterization and biological action of various extracts of the leaf gel have been described, especially pertaining to diabetes \cite{6}. The majority of the scientifically based research on this topic has to date, however, been done
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Chapter 4: A. greatheadii phytochemical content

exclusively on two *Aloe* species namely; *Aloe vera* (or *Aloe barbadensis*) and *Aloe arborescens*. *Aloe* gel (from a variety of *Aloe* species) is sold commercially worldwide as an ingredient of a wide range of health care, cosmetic and therapeutic products [6]. This commercial activity, and widely distributed use of *Aloe* as used in traditional medicine, has led to an upsurge of both clinical and chemical research focusing on the active ingredients in these plants, as well as their biological activities.

The species of *Aloe* selected for commercial exploitation or selected by the traditional healer, would be based on its local availability and distribution. In South Africa the most widely distributed *Aloe* species are *Aloe greatheadii* var. *daviana* (Asphodelaceae) and *Aloe ferox* Mill. (Asphodelaceae). *A. greatheadii* grows wild in the northern parts of South Africa, whereas *A. ferox* grows wild primarily in the Eastern and Western Cape Provinces. Various extracts of these *Aloe* species are traditionally used and commercially sold as creams, ointments and tonics for the purpose of treating a variety of ailments, of which their applications to arthritis, skin cancer, burns, eczema, psoriasis, digestive problems, high blood pressure and diabetes are most common. There is, however, very little or no scientific evidence to support these claims, with many of the claims being based on research done on *A. vera*. As different *Aloe* species would have varying phytochemical contents due to inter-species variation, and varying climate and soil conditions, direct correlation of biological activity would be inaccurate. Consequently, it is of relevance for scientists, industry and rural communities to not only research the relevant medicinal uses of these indigenous *Aloe* species, but also to determine the active components and their individual or combined mechanisms of biological function.

This paper will primarily focus on identifying, quantifying and comparing the phytochemical composition of two *A. greatheadii* var. *daviana* extracts: a leaf gel extract (LGE) and a 95% aqueous ethanol leaf gel extract (ELGE), obtained using a modified extraction procedure, and analysis on GC-MS and spectrophotometry. This is done in order to determine whether this plant species contains any individual compound or group of compounds which may substantiate its current commercial and traditionally use as a herbal medicine, in addition to determining the most appropriate methods of extracting these compounds. The results will consequently be discussed in the light of their putative biological or therapeutic relevance.
Results and Discussion

The individual compounds identified via GC-MS in the LGE and ELGE of *A. greatheadii* var. *davyana* are arranged according to their structural classifications and summarized in Table 1. Of the individual compounds identified, those best described for their health benefits include the polyphenols/phenolic acids, sterols, fatty acids and indoles. Other compounds identified include various alkanes, pyrimidines, alkaloids, organic acids, aldehydes, dicarboxylic acids, ketones and alcohols.

**Table 1.** Concentrations of GC-MS Identified Compounds from Lyophilized *Aloe greatheadii* var *davyana* Leaf Gel (LGE) And 95% Aqueous Ethanol Leaf Gel Extracts (ELGE).

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Concentration (ppm)</th>
<th>Concentration (ppm)</th>
<th>Compounds</th>
<th>Concentration (ppm)</th>
<th>Concentration (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LGE (per dry mass)</td>
<td>ELGE (per dry mass)</td>
<td></td>
<td>LGE (per dry mass)</td>
<td>ELGE (per dry mass)</td>
</tr>
<tr>
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<td>LGE</td>
<td>ELGE</td>
<td></td>
<td>LGE</td>
<td>ELGE</td>
</tr>
<tr>
<td>Organic acids</td>
<td></td>
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<td>Alcohols</td>
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<td></td>
<td></td>
<td>propanediol</td>
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121
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<th>Chapter 4: A. greathedii phytochemical content</th>
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<tbody>
<tr>
<td><strong>Vanillic</strong></td>
<td>60.7</td>
</tr>
<tr>
<td><strong>Homovanillic</strong></td>
<td>23.4</td>
</tr>
<tr>
<td><strong>Gentisic</strong></td>
<td>55.6</td>
</tr>
<tr>
<td><strong>6,7-</strong></td>
<td>31.3</td>
</tr>
<tr>
<td><strong>Dihydroxyeoum</strong></td>
<td>ariec</td>
</tr>
<tr>
<td><strong>Hydroxychinami</strong></td>
<td>e</td>
</tr>
<tr>
<td><strong>Protocatechuic</strong></td>
<td>162</td>
</tr>
<tr>
<td><strong>3,4-</strong></td>
<td>2.76</td>
</tr>
<tr>
<td><strong>Dihydroxyphery</strong></td>
<td>lasetic</td>
</tr>
<tr>
<td><strong>Syringic</strong></td>
<td>14.4</td>
</tr>
<tr>
<td><strong>Sinapic</strong></td>
<td>37.8</td>
</tr>
<tr>
<td><strong>Caffeic</strong></td>
<td>107</td>
</tr>
<tr>
<td><strong>Isoferulic</strong></td>
<td>38.4</td>
</tr>
<tr>
<td><strong>Ferulic</strong></td>
<td>60.1</td>
</tr>
<tr>
<td><strong>Benzoic</strong></td>
<td>420</td>
</tr>
<tr>
<td><strong>Phenylacetic</strong></td>
<td>71.3</td>
</tr>
<tr>
<td><strong>2-</strong></td>
<td>233</td>
</tr>
<tr>
<td><strong>Methoxybenzoc</strong></td>
<td>(C21:0)</td>
</tr>
<tr>
<td><strong>s-Toluic</strong></td>
<td>162</td>
</tr>
<tr>
<td><strong>Phenylproionic</strong></td>
<td>37.5</td>
</tr>
<tr>
<td><strong>4-Phenyllactic</strong></td>
<td>613</td>
</tr>
<tr>
<td><strong>4-</strong></td>
<td>223</td>
</tr>
<tr>
<td><strong>Hydroxybenzoc</strong></td>
<td>(C20:4)</td>
</tr>
<tr>
<td><strong>2,3-</strong></td>
<td>12.1</td>
</tr>
<tr>
<td><strong>Hydroxybenzoic</strong></td>
<td>(C14:1)</td>
</tr>
<tr>
<td><strong>4-</strong></td>
<td>318</td>
</tr>
<tr>
<td><strong>Hydroxyphenylacetoc</strong></td>
<td>-</td>
</tr>
<tr>
<td><strong>Hydro-p-</strong></td>
<td>13.9</td>
</tr>
</tbody>
</table>

**Fatty acids**

- **Lauric (C12:0)**: 0.35
- **Tridecanoic**: 0.02
- **Undecanoic (C11:0)**: 0.03
- **Pentadecanoic (C15:0)**: 1.16
- **Palmitic (C16:0)**: 43.0
- **Stearic (C18:0)**: 3.24
- **Nonadecanoic (C19:0)**: 3.14
- **Heneicosanoic (C21:0)**: 0.28
- **Beheno (C22:0)**: 5.39
- **Tricosanoic (C23:0)**: 1.74
- **Lignoceric (C24:0)**: 5.11
- **Arachidonic (C20:4)**: 0.57
- **Myristoleic (C14:1)**: 0.20
- **10-Pentadecenoic (C15:1)**: 1.44
As shown in Table 1, the individual phenolic compounds identified in the highest concentrations in *A. greatheadii* LGE include 4-phenyllactic acid, benzoic acid, 4-hydroxyphenylacetic acid, 4-hydroxy-benzoic acid and 4-methoxymandelic acid. Of the phenolic compounds best known for their health benefits and associated antioxidant properties, 4-hydroxyphenylacetic acid, 2,3-dihydroxybenzoic acid, protocatechuic acid, p-coumaric acid and caffeic acid were the most abundant. Of the four phytosterols identified, camp esterol and β-sitosterol were by far the most abundant. Only one indole, indole-5-acetic acid was, however, identified in the LGE. Comparatively, higher concentrations of the above-mentioned polyphenols were also detected in the ELGE (when quantified as per dry mass ELGE). Different to the ethyl acetate/diethyl ether extracts of the however, hexahydrobenzoindole was the only indole identified in the ELGE, and surprisingly, no phytosterols were detected in this extract.

As shown in Table 2, ORAC and FRAP analyses of LGE and ELGE revealed the ELGE to have greater antioxidant capacity compared to the
LGB, which is supported by the higher total polyphenol contents detected in ELGE. The majority of the polyphenols in both the LGE and ELGE, as identified by GC-MS and confirmed spectrophotometrically, are non-flavonoids, making-up 83.8% of the total polyphenol content of the LGE and 92.4% of the ELGE. An interesting phenomenon was the absence of Aloe-emodin in the A. greatheadii extracts. This is of importance as many of the health benefits associated with other Aloe species (including similar leaf gel extracts of A. ferox, also indigenous to South Africa), are attributed to the presence of, amongst other compounds, Aloe-emodin [7]. Finally, the total sugar contents of these extracts were 5.43 g/100 g for the LGE and 83.76 g/100 g for the ELGE, 36% of which was quantified as glucose, 18% as fructose and the remainder as maltose and sucrose.

**Table 2.** Concentrations of Total Polyphenols, Flavonoids, Non-Flavonoids, Oxygen Radical Absorbance Capacity (ORAC) and Ferric Reducing Antioxidant Power (FRAP) in Lyophilized Aloe greatheadii var davyana Leaf Gel (LGE) and 95% Aqueous Ethanol Leaf Gel Extracts (ELGE).

<table>
<thead>
<tr>
<th>Compound</th>
<th>LGE (dry mass)</th>
<th>LGE (wet mass)</th>
<th>ELGE (expressed as dry mass ELGE)</th>
<th>ELGE (expressed as dry mass LGE)</th>
<th>ELGE (expressed as wet mass LGE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total polyphenols (mg of GAE/100g ± SD)</td>
<td>45.1 ± 0.94</td>
<td>1.20 ± 0.03</td>
<td>263 ± 6.51</td>
<td>30.9 ± 0.77</td>
<td>0.82 ± 0.02</td>
</tr>
<tr>
<td>Total flavonoids (mg of CE/100g ± SD)</td>
<td>7.66 ± 0.26</td>
<td>0.20 ± 0.01</td>
<td>20.2 ± 0.50</td>
<td>2.37 ± 0.06</td>
<td>0.06 ± 0.001</td>
</tr>
<tr>
<td>Total non-flavonoids (by calculation)</td>
<td>37.8 ± 0.99</td>
<td>0.99 ± 0.03</td>
<td>243 ± 6.96</td>
<td>28.6 ± 0.82</td>
<td>0.75 ± 0.02</td>
</tr>
<tr>
<td>ORAC - hydrophilic (µmol of TE/g)</td>
<td>59.0 ± 1.16</td>
<td>2.05 ± 0.4</td>
<td>83.0 ± 1.32</td>
<td>5.42 ± 1.21</td>
<td>0.19 ± 0.04</td>
</tr>
<tr>
<td>ORAC - lipophilic (µmol of TE/g)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ORAC - total (µmol of TE/g)</td>
<td>59.0 ± 1.16</td>
<td>2.05 ± 0.4</td>
<td>83.0 ± 1.32</td>
<td>5.42 ± 1.21</td>
<td>0.19 ± 0.04</td>
</tr>
<tr>
<td>FRAP (µmol/g)</td>
<td>2.63 ± 0.21</td>
<td>0.09 ± 0.01</td>
<td>8.98 ± 0.21</td>
<td>0.58 ± 0.01</td>
<td>0.02 ± 0.001</td>
</tr>
</tbody>
</table>

"-" denotes nothing detected.

We previously reported on the phytochemical composition and antioxidant capacities of A. ferox LGE and ELGE [7]. Compared to this species, A. greatheadii generally shows less variety and lower concentrations of the
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above mentioned health-associated antioxidant compounds. Similarly, Table 2 indicates the total polyphenol and non-flavonoid contents, as well as the antioxidant capacities of A. greatheadii LGE and ELGE, as measured by FRAP, to be lower than that as previously reported for A. ferox. The total flavonoid contents and the antioxidant capacities of A. greatheadii LGE, as measured by ORAC were, however, higher comparatively, which may be indicative of the types of polyphenols in A. greatheadii having stronger scavenging ability than ferric ion reducing potential. Apart from inter-species variation explaining these differences, soil conditions and climate may also play a role in the varying phytochemical contents of these plants.

From an analytical perspective, GC-MS analyses identified a larger number of compounds using direct LGE ethyl acetate/diethyl ether and hexane extractions, as compared to using the same extraction procedures on the ELGE, when quantified as per dry mass LGE (Table 1). This indicates that direct LGE ethyl acetate/diethyl ether and hexane extractions are best suited for general phytochemical characterization purposes. However, despite there being fewer compounds identified in the ELGE, the concentrations for many of the compounds with associated health benefits, and total sugars, were found to be between 1.2 to 1,250 times higher than the same compounds identified in the LGE, when expressed as per dry mass ELGE. This was further confirmed by the antioxidant capacity analyses showing higher activities in the latter extracts. This justifies the use of the latter approach for preparing extracts for use in in vivo or in vitro biological efficacy and mechanistic studies.

Epidemiological evidence supports the hypothesis that the consumption of foods rich in natural antioxidants plays an important role in the prevention of several chronic diseases associated with oxidative stress, including diabetes, cancer, hypertension and cardiovascular diseases [8]. Due to the fact that the majority of the phytochemicals identified in A. greatheadii were polyphenols/phenolic acids, one would expect these to be the major contributors to this plant’s antioxidant capacity and its proposed use for alleviating or preventing diseases associated with oxidative stress [9, 10, 11].

Considering that the mechanism proposed for developing diabetes is hyperglycaemia induced oxidative stress [5, 12-14], the current use of A. greatheadii in traditional and commercial tonics for treating this disease may be justified. To date, various authors have reported on the anti-diabetic properties of a variety of other Aloe species from both human and animal trials, of which Aloe vera and Aloe arborescens are by far the best.
described. Interventions involving various extracts, including a 95% aqueous ethanol extract of these, have been shown to alleviate the diabetic state. By preventing hyperglycaemia induced oxidative stress and the associated pancreatic β-cell destruction, these plant extracts have been shown to increase insulin secretion by the pancreas, and in so doing correct the diabetes associated hyperglycaemia and dyslipidaemia [5, 12-14]. This action is ascribed to the various polyphenols present in these extracts, which alleviate the diabetic condition by lowering glucose uptake, and in doing so prevent hyperglycaemia [15, 16]. Additionally, the plant sterols identified in A. greatheadii also possess similar glucose lowering effects. Tanaka et al. (2006) reported reductions in both fasting and random blood glucose levels of db/db diabetic mice chronically treated with the same phytosterols from A. vera leaf gel [17]. Apart from these glucose lowering effects, phytosterols are better known for their total cholesterol and low-density lipid cholesterol (LDL-C) lowering effects [18]. As summarized by Devaraj and Jialal [19], evidence for this has been observed in hypercholesterolemic, diabetic and healthy volunteers [19]. The mechanism proposed by which phytosterols accomplish this, is by lowering cholesterol absorption due to the structural similarities that these compounds share with cholesterol [20-22]. Apart from lowering cardiovascular risk factors associated with diabetes and other diseases, β-sitosterol has been shown to influence a diabetic state positively by directly lowering fasting blood glucose levels by cortisol inhibition [23]. Furthermore, phytosterols have been shown to reduce biomarkers for oxidative stress and inflammation [19], as well as to reduce cancer development by a variety of mechanisms [24]. Based on the presence of the high amounts of many of the same polyphenols and phytosterols in A. greatheadii LGE and ELGE, this Aloe species may show promise in preventing or alleviating the progression of diseases associated with oxidative stress, including diabetes, cancer, hypertension and cardiovascular diseases.

On the other hand, the total sugar/glucose contents of these extracts may be of concern, especially when considering using these extracts in the context of a diabetes intervention, as carbohydrate intake is considered a major factor in glycaemic control. Nielsen and co-workers report that a low carbohydrate diet, containing 20 % carbohydrates, is superior to a diet containing 55 - 60 % carbohydrates, with regards to controlling bodyweight, blood glucose levels and reducing in HbA1c [25]. The American Diabetes Association (ADA) defines a low carbohydrate diet as less than 130g/d or 26% of a nominal 2,000 kcal (8,400 kJ) diet [26]. Considering the above-mentioned
recommended carbohydrate intakes for diabetics and the mechanisms by which the polyphenols and phytosterols elicit their anti-diabetic actions (by lowering glucose absorption and protecting pancreatic β-cells from oxidative destruction), the sugar contents of these extracts may not necessarily be problematic due to the small amounts that would be additionally ingested during an intervention using these extracts. This, however, should be investigated, in addition to other methods of extraction which could potentially eliminate these sugars.

Conclusions

Analytically, direct LGE ethyl acetate/diethyl ether and hexane extractions produce better phytochemical characterization, whereas 95% aqueous ethanol extraction concentrates a number of health related compounds, justifying its applications to in vivo efficacy studies. From a medicinal application perspective, *A. greatheadii* contains a variety of compounds (esp. polyphenols and phytosterols) with confirmed antioxidant capacity, and putative therapeutic actions (including blood glucose, cholesterol and cortisol lowering properties) relating to the prevention or treatment of diabetes, cardiovascular disease, cancer and hypertension. No toxic compounds were detected in these *Aloe* extracts, however, due the presence of other confounders which may have been missed in this study, further confirmation of the proposed health benefits of these extracts through in vivo animal experimentation is strongly suggested.

Experimental

*General*

All analytical standards and reagents used for quantification, and those used for generating mass spectra for GC-MS identification, were purchased from Sigma-Aldrich (St Louis, MO, USA). All organic extraction solvents used were of ultra high purity purchased from Burdick and Jackson (USA). Folin-Ciocalteu’s phenol reagent and other reagent chemicals were purchased from Merck (Darmstadt, Germany).
Plant material

Whole, freshly cut, *A. greatheadii* var. *davyana* leaves (100kg) were harvested from approximately 200 plants in the month of May (2007) from a rural area in the Potchefstroom district of the North West Province in South Africa (herbarium deposit site: AP Goossens Herbarium (code: PUC), Potchefstroom South Africa; voucher number : PUC 7951). All leaves were collected from mature plants with a circular diameter greater than 50 cm.

Sample preparation

The leaf skin was removed by hand and the leaf gel homogenized, lyophilized and stored at -20°C until analysis. This was termed the leaf gel extract (LGE). A large portion of the LGE was used for the preparation of a 95% aqueous ethanol leaf gel extract (ELGE). Batches (420g) of finely ground lyophilized *Aloe* gel were extracted using 95% aqueous ethanol (500mL), followed by sonication for 10 min and shaking for 1 hour. The solvent was collected following centrifugation at 3000 x g for 10 min. This was repeated 10 times to ensure total extraction of all compounds from the lyophilized extracts. The supernatants were pooled and evaporated to dryness under reduced pressure in a rotary evaporator. The residue was stored in dry sterilized containers at -20°C until further use.

Ethyl acetate/diethylether extraction

3-Phenylbutyric acid (5mg/mL, 100μL) was added as an internal standard to LGE and ELGE (25mg) followed by sodium acetate buffer (0.125 M, 1mL) and β-glucuronidase (60μL). Samples were then incubated at 37°C for 3 hours. Incubated samples were subsequently extracted with ethyl acetate (6mL) followed by diethyl ether (3mL). After centrifugation, collected supernatants were pooled and dried under a nitrogen stream. Derivatization with bis(trimethylsilyl) trifluoroacetamide (BSTFA, 100μL), trimethylchlorosilane (TMCS, 20μL) and pyridine (20μL) at 70°C for 30 min followed. After cooling to room temperature, 0.1μL of the derivatized extract was injected into the GC-MS via splitless injection.
**Hexane Extraction for Fatty Acids**

The internal standard, heptadecanoic acid (72 mM), was added to LGE and ELGE (25 mg) followed by a 45 mM solution of butylated hydroxytoluene (100 μL) and methanolic HCL (3N, 1 mL). The samples were vortexed and incubated for 4 hours at 90°C. After cooling to room temperature, the samples were extracted twice with 2 mL of hexane, dried under a nitrogen stream and finally re-suspended with hexane (100 μL), 0.1 μL of which was injected onto the GC-MS via splitless injection.

**Phytochemical Characterization via GC-MS**

An Agilent 6890 GC ported to a 5973 MS detector (California, USA) was used for identification and quantification of individual phytochemicals, using MS libraries previously compiled from purchased standards. For the acquisition of an electron ionization mass spectrum, an ion source temperature of 200°C and electron energy of 70 eV was used. The GC was equipped with a SE-30 capillary column (Chemetrix, USA), a split/splitless injection piece (250°C) and direct GC-MS coupling (260°C). Helium (1 mL/min) was used as the carrier gas. The oven temperature program for analyzing the ethyl acetate/diethyl ether extracts utilized an initial oven temperature of 40°C, maintained for 2 min, followed by a steady climb to 350°C at a rate of 5°C/min. For the fatty acid analysis, hexane extracts were analyzed using an initial oven temperature of 50°C, maintained for 1.5 min, and then allowed to increase to 190°C at a rate of 30°C/min. This oven temperature was again maintained at 190°C for 5 min and then allowed to increase to 220°C at a rate of 8°C/min. This oven temperature was maintained for 2 min and finally ramped to 230°C at a rate of 3°C/min and maintained for a further 24 min.

**Total Polyphenols**

The total polyphenol contents of the LGE and ELGE were determined according to the Folin-Ciocalteu procedure [27]. Briefly, finely ground LGE or ELGE (10 mg) was dissolved in H₂O (200 μL) in a test tube and Folin-Ciocalteu’s reagent (1 mL) was added. This was allowed to stand for 8 min at room temperature. Next, sodium carbonate (7.5%, w/v, 0.8 mL) was added, mixed and allowed to stand for 30 min. Absorption was measured at 765 nm.
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(Shimadzu UV-1601 Spectrophotometer). The mean total phenolic contents (n = 3) were expressed as milligram gallic acid equivalents per 100g (mg GAE/100g dry mass or wet mass ± standard derivatives).

Total flavonoids

The total flavonoid contents of the LGE and ELGE were measured using the aluminium chloride assay as described by Zhishen and co-workers with slight modifications [28]. Briefly, LGE or ELGE (10mg) was dissolved in H₂O (1mL) in a test tube, to which 5% (w/v) NaNO₂ (60µL) was added. After 5 min, a 10% (w/v) AlCl₃ solution (60µL) was added. After 6 min, 1 M NaOH (400µL) was added and the total volume made up to 2mL with H₂O. The solution was mixed well and the absorbance measured at 510 nm against a reagent blank. Concentrations were determined using a catechin standard curve. Mean total flavonoid contents (n = 3) were expressed as milligrams catechin equivalents (CE) per 100g (mg CE/100g dry or wet mass ± standard deviation).

Oxygen Radical Absorbance Capacity (ORAC)

ORAC analyses of hydrophilic and lipophylic fractions of the LGE and ELGE were performed essentially as described by Prior et al. [29]. The analyses of lipophylic compounds were aided by the addition of randomly methylated β-cyclodextrin (kind gift from Dr R Prior) as a solubility enhancer as described by Huang et al. [30]. Briefly, in a volume of 200µL, the reaction contained 56 nM fluorescein as a target for free radical attack by 240 nM 2,2'-azobis(2-amidinopropane) dihydrochloride. A fluorescence plate reader (BioTEK FL-600, UK) was used and the decay of fluorescence of fluorescein (excitation 485 nm, emission 520 nm) was measured every 5 min for 2 hours at 37°C. Costar black opaque (96-well) plates were used in the assays. Trolox was used as a standard at a range of between 0-20 µM, giving a polynomial (2nd order) curve fit analysis. Mean values (n = 3) of antioxidant capacities were expressed as µmole trolox equivalents (TE) / g wet and dry mass ± standard deviation.
**Ferric Reducing Antioxidant Power (FRAP)**

FRAP values of the LGE and ELGE were determined essentially as described previously [31]. Briefly, the reduction of a Fe(III)-2,3,5-triphenyltetrazolium complex in the assay, by the antioxidants in the samples, was monitored at 593 nm. As a standard, FeSO₄ was used and the FRAP activities of the samples were expressed as the mean (n = 3) μmol Fe²⁺/g wet and dry mass ± standard deviation.

**Sugar content**

The total sugar content as well as the type of sugar present in the LGE and ELGE were determined at the Department of Agriculture, Directorate Food Safety and Quality Assurance, Division Analytical Services North, Pretoria, South Africa. The total sugar content was determined by Refractive index (RI) as previously described [32]. The concentrations of fructose and glucose were determined via HPLC using a Supelcosil LC-NH₂ column (Supelco, 250 x 4.6 mm, 5 μm, Sigma-Aldrich Catalogue No: 58338) and 75% Acetonitrile as mobile phase with a flow rate of 1.5 mL/min at 30°C and detected by a Refractive Index (RI) detector. The results are expressed as percentage sugars (m/m) in LGE and ELGE.

**References**


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Chapter 5

Antidiabetic Effects of Aloe ferox and Aloe greatheadii var. davyana Leaf Gel Extracts in a Streptozotocin Diabetes Rat Model
Chapter 5: Antidiabetic effects of Aloe ethanol extracts

Antidiabetic Effects of Aloe ferox and Aloe greatheadii var. davyana Leaf Gel Extracts in a Streptozotocin Diabetes Rat Model

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Chapter 5: Antidiabetic effects of Aloe ethanol extracts

ABSTRACT

The purpose of this study was to investigate the antidiabetic effects of ethanol extracts of *Aloe ferox* and *Aloe greatheadii* var. *davyana* leaf gel in a streptozotocin (STZ) diabetic rat model. Compared to the normal control group, STZ resulted in increased relative liver and kidney weight, end-point plasma glucose values, fructosamine, oxidative stress, liver enzymes, total cholesterol, triglycerides, VLDL-C and TC:HDL-C values and reduced serum insulin levels. Treatment with *A. greatheadii* moderately increased serum insulin accompanied by a modest decreased end-point plasma glucose and decreased liver enzyme ALP, in addition to moderately increased HDL-C and decreased TC:HDL-C values. *A. ferox* supplementation resulted in moderately increased serum insulin, accompanied by slight corrections in ALP and HDL-C, however, without a decrease in end-point plasma glucose. Little effect was seen on other diabetes markers. Glibenclamide resulted in correction of almost all diabetic markers, with the increased insulin secretions resulting in a normalization of end-point blood glucose values and a correction of the diabetes induced hyperlipidemia. Therefore, oral administration of the Aloe extracts, *A. greatheadii* in particular, resulted in moderate improvements in the STZ induced diabetic state, as evaluated by the measurement of various biochemical diabetes markers related to diabetes induced abnormalities in glucose, lipid, insulin and liver enzyme levels,
justifying further investigations into the use of these traditional remedies for the treatment of diabetes.

Keywords: Antidiabetic effects, *Aloe ferox*, *Aloe greatheadii* var. *davyana*, Streptozotocin-induced diabetes.

INTRODUCTION

The increase in the number of individuals with type 2 diabetes in developing countries especially has resulted in an upsurge in interest in the use of natural and traditional remedies for treating this disease (1). The total prevalence of type 2 diabetes is thought to be as high as 6% of the global population and is likely to rise as the population ages and becomes more obese (2). The significant worldwide increase in the prevalence of childhood obesity further amplifies the diabetes epidemic (3).

Diabetes mellitus is a non-communicable disease considered to be one of the five leading causes of death worldwide (4), characterized by hyperglycemia and hyperlipidemia as a result of altered glucose and lipid metabolism (5). Recently, the search for suitable antidiabetic agents has focused on plants used in traditional medicine. Although diabetes is being managed and treated in many first world countries by exclusively using conventional, synthetic drugs, in many developing countries, diabetic patients have resorted to traditional medicinal herbs for the treatment of this disease,
largely due to these being more easily accessible and less expensive for those
living in poor socioeconomic conditions (6).

Of the many traditional treatments for diabetes, Aloe is probably one most
used by these traditional healers. Beppu et al., Rajasekaran et al., and others
confirmed the antidiabetic effects of certain Aloe species (A. arborescens and
A. vera) in streptozotocin (STZ)-induced diabetic rats (4, 5, 7, 8), however,
these beneficial effects were not seen by all groups (9). Aloe ferox and Aloe
greatheadii var. davyana are indigenous to South Africa and are widely used
by both traditional healers and sold as ingredients to a variety of
commercially available tonics for the treatment for a number of ailments,
including diabetes. The medicinal use and commercialization of A. ferox and
A. greatheadii is, however, primarily based on anecdotal evidence and / or
research done on A. vera and A. arborescens. The phytochemical
composition and antioxidant capacity of various extracts of A. ferox and A.
greatheadii have only recently been described and there is reason to believe
that these extracts may have possible health benefits, especially for treating
diabetes (10, 11).

This evidence subsequently motivated the current exploration of the
antidiabetic effects of the same extracts of A. ferox and A. greatheadii
prepared by ethanol extraction (10, 11), administered at a similar upper
dosage level as has been previously described by other groups using other
Aloe species (8, 12), in a STZ-induced diabetic rat model.
MATERIAL AND METHODS

Collection of plant materials. Whole, freshly cut, *A. greatheadii* var. *davyana* leaves (100 kg) were harvested from approximately 200 plants in the month of May (2007) from a rural area in the Potchefstroom district of the North West Province in South Africa (herbarium deposit site: AP Goossens Herbarium (code: PUC), Potchefstroom South Africa; voucher number: PUC 7951). All leaves were collected from mature plants with a circular diameter greater than 50 cm. Whole, freshly cut, *A. ferox* leaves (100 kg) were kindly supplied by the Aloe Ferox Trust (herbarium deposit site: AP Goossens Herbarium (code: PUC), Potchefstroom South Africa; voucher number: PUC 9940). These leaves were harvested in September 2007 from farms in the Albertinia region in the Western Cape of South Africa.

Preparation of Aloe extracts. The *A. ferox* and *A. greatheadii* var. *davyana* leaf gel extracts were prepared using the method previously described (12) with slight modifications as described by Loots et al., 2007 and Botes et al., 2008 (10, 11). An aqueous suspension was prepared by dissolving suitable amounts of ethanol free extract to the desired concentration. The solutions were prepared fresh daily and administered intragastrically once daily.

Animals, induction of diabetes and interventions. The study was approved by the Ethics Committee (Evaluation Sub-Committee for Experimental Animals) of the North-West University (Potchefstroom, South
Africa) (Ref: 06D06) and the study was conducted in accordance with the
principles of laboratory animal care (NIH Publication "Guide for the Care
and Use of Laboratory Animals", No. 85–23, revised 1985). Fifty male
Wistar rats weighing 200g–250g were randomly divided into five groups of
ten rats each as follows: normal control rats (NC), STZ-induced diabetic
control rats (DC), STZ-induced diabetic rats receiving 300mg/kg A.
greatheadii var. davyana leaf gel extract (DAG), STZ-induced diabetic rats
receiving 300mg/kg A. ferox leaf gel extract (DAF) and STZ-induced diabetic
rats receiving 600µg/kg glibenclamide (DG) as a positive control for 5 weeks
(8, 12).

Following a 12 hour fast, the rats in the DC, DG; DAF and DAG groups
were intraperitoneally injected with a single dose of STZ (40mg/kg) (Sigma,
St. Louis, MO., USA) prepared in 0.1 M of sodium citrate buffer (pH 4.5)
and left for one week for diabetes to develop (8, 12). STZ is used to induce
diabetes (13) via destruction of the β-cells as a result of oxidative stress, and
can be used to develop a diabetes model resembling that of type 1 or type 2
diabetes, depending on the dosage used. At this dosage of STZ used in this
study, a partial destruction of β-cells resulted in a mild insulin deficient state
more closely resembling that of type 2 diabetes due to the fact that the
resultant model is insulin independent (14). The rats in the NC group
received the same volume of citrate buffer intraperitoneally (8). Diabetes
was confirmed by tail prick using an Accu-Check® glucometer (Roche
diagnostics) and Onetouch Surestep glucose strips (Lot No. 285366A) by a
blood glucose value of > 14 mmol/L (12).

Aloe leaf gel extracts and glibenclamide were prepared using double
distilled sterile deionised water (8, 12). The rats then received either 300
mg/kg of one of the two leaf gel extracts or 600 μg/kg glibenclamide in a
volume of approximately 1 ml (8, 12) via intragastric tube once daily,
depending on their grouping. The solutions were prepared fresh prior to daily
dosing for the entire five week intervention period.

Blood sampling. Fasted rats were sacrificed at the end of the intervention
by cervical decapitation and blood collected as described previously (8, 12).
Blood samples were prepared according to the requirements of the various
analytical methods. Plasma was collected in heparin tubes and serum was
collected in tubes containing no anticoagulant. The blood was centrifuged at
4°C for 15 minutes at 2000g and plasma and serum were collected from the
respective tubes and frozen at -84°C until further analysis.

Biochemical analyses. Fructosamine was determined by a colorimetric
endpoint reaction (Cobas® (Roche diagnostics)).
Serum insulin was measured by using an Ultrasensitive Rat Insulin ELISA
kit (Mercodia). The assay was performed as instructed by the supplier.
Briefly, during incubation, insulin in the sample reacts with peroxidase-
conjugated anti-insulin antibodies and anti-insulin antibodies bound to the
microtitration well. The conjugate then reacts with 3,3',5,5'-tetramethylbenzidine in a colorimetric end-point reaction. Results were
obtained by measuring an increased absorbance at 450nm. This analysis was
performed on a Multiscan Ascent spectrophotometer (Wiesbaden, Germany).
Insulin resistance was calculated using the homeostasis model assessment
(HOMA) formula: HOMA = (fasting insulin (μIU/mL) \times fasting venous
glucose (mmol/L)) / 22.5 (8). Serum triacylglycerol (TG), total cholesterol
(TC), HDL-cholesterol, LDL-cholesterol and VLDL-cholesterol were
measured by Pathcare Laboratories (Potschefstroom, South Africa). Total
cholesterol (TC) was determined using a polychromatic end point technique
employing the horseradish peroxide (HPO), HDL-cholesterol using the
accelerator selective detergent method and LDL-cholesterol and triglycerides,
using a biochromatic end point technique. Alanine transaminase (ALT),
alkaline phosphatase (ALP) and venous blood glucose levels were measured
using a Vitros DT60 II Chemistry Analyser (Ortho-Clinical Diagnostics,
Rochester, New York, USA), with Vitros reagents and controls.
Ferric reducing antioxidant power in serum and tissue was determined
colorimetrically by the FRAP assay as previously described (15) using a
BioTek FL600 plate reader (Winooski, VT, USA) at a wavelength of 595nm.
The Diacron reactive metabolites (dROMs) test (DIACRON International,
Grosseto, Italy) was used to measure the serum reactive oxygen metabolite
pool. The assay was performed as instructed by the supplier. The
colorimetric assay was performed kinetically on a BioTek plate reader
measuring change in absorbance at 560nm over a period of 15 min at 25°C.
Samples were quantified using a standard and expressed as Carratelli units (CARR U) where 1 CARR U corresponds to 0.08 mg/100 ml H₂O₂.

**Statistical analysis.** The computer software package Statistica® (Statsoft Inc, Tulsa, OK, USA) was used for data analysis. Data are expressed as a mean and 95% CI. Differences between the groups were determined by using the analysis of variance (ANOVA) and analysis of co-variances (ANCOVA) when adjusting for possible confounders. Where significance between the groups was indicated with the ANOVA, the Tukey honest significant difference test for unequal N was used to determine between which groups the differences occurred. Statistical significance tests are dependent on sample size and have a tendency to yield smaller p-values as the size of the data sets increase (16). Small sample sizes (as is often the case in animal studies) may, however, lead to failure to detect a true effect (16). In these cases, effect size calculations, which are independent of sample size, are an objective measure of the likelihood of a difference having a practical/clinical significance, can be used (17). In order to determine whether the results may have practical relevance, effect sizes were calculated according to the following formula: \( d = \frac{|\bar{x}_1 - \bar{x}_2|}{s_{\text{max}}} \), where \( \bar{x}_1 \) is the mean of one group and \( \bar{x}_2 \) the mean of the other group and \( s_{\text{max}} \) the maximum standard deviation of the two groups. The likelihood of practical relevance is reported as an effect size (d) and can be interpreted as follows: \( d \leq 0.2 \) is a small likelihood, \( d = 0.5 \) is a medium likelihood, \( d = 0.8 \) is a large likelihood for effect for parametric data (16). Rosenthal et al. (17) state the following on
the use of effect sizes: "...suppose we were confronted with a "non-
significant" p and a "large" effect size - what should this tell us? Were we
simply to conclude on the basis of the significance level that "nothing
happened", we might be making a serious mistake. A small sample size may
have led to failure to detect the true effect, in which case, we should continue
this line of investigation with a larger sample size...".

RESULTS

Five rats did not develop diabetes and/or recovered spontaneously and
nine rats died during the study, resulting in unequal group sizes.

Body weight. The normal control rats gained 72.8g on average during the
five weeks while the diabetic control and glibenclamide group gained a mean
of 5.63g and 21.61g, respectively. The A. greatheadii and A. ferox groups,
however, showed a mean weight reduction of 9.7g and 10.9g during the five
week intervention (Table 1).

Relative organ weight. Only small differences were observed in the mean
pancreatic weights, expressed as percentage of body weight, between the five
groups (Table 1). While the mean pancreatic weight of the normal control
group was not significantly higher than that of the diabetic control group, the
effect size calculation indicated that the difference in organ weight may have
a moderate practical significance. The glibenclamide intervention group had
a significantly lower pancreatic weight than the normal control group (0.25%
The normal control rats had lower mean liver and kidney weights, expressed as percentage of body weight, than the diabetic control rats. All three interventions tested did little to change this, with all three groups having mean liver and kidney weights still significantly higher than that of the normal control rats.

**Diabetes markers.** As described in Table 2, baseline glucose determinations done via tail prick revealed no significant differences between groups (p=0.785), indicating all rats to be in the same state of normal glycemia prior to diabetes induction. End-point plasma glucose values, after the diabetes induction and the interventions that followed, show significant differences between the groups (p=0.0001) with a significant increase in the diabetic control group comparative to the normal control group as would be expected. *A. greatheadii* decreased the diabetes induced plasma hyperglycemia non-significantly but with clinical relevance as indicated by the effect size calculations. The *A. ferox* intervention had less of an influence comparatively. The glibenclamide intervention on the other hand had a large effect, lowering plasma glucose to values no longer significantly different from normal (p=0.954). Fructosamine concentrations were significantly elevated in the diabetic control group compared to the normal control group (p=0.0001) (Table 2). The interventions, however, had no effect on restoring these values. In fact, the fructosamine values in the glibenclamide group were not only increased compared to the normal control group but also compared to the diabetic control group. This increase is, however, not
considered to be of clinical relevance (d<0.2). As expected, serum insulin levels decreased significantly due to the STZ-induced diabetes, compared to the NC group (p=0.001). Effect size calculations also show high practical significance (d=1.49). Serum insulin concentrations in the *A. greatheadii*, *A. ferox* and glibenclamide groups corrected somewhat, with glibenclamide having the largest influence. The effect sizes indicate these corrections to be clinically relevant for all 3 interventions. These changes in insulin values correlate to the changes in end-point glucose values, as an inverse correlation can be expected between serum insulin and plasma glucose values. When comparing insulin values between the groups after adjusting for change in body weight during the intervention, the differences in serum insulin were no longer significant, suggesting that the effect of the treatments on insulin levels may be related to the weight changes observed in these groups. Furthermore, as expected, the diabetic group showed a non-significant increase in insulin resistance when compared to the normal control group. This increase, however, showed large practical significance. The glibenclamide intervention showed a normalization of insulin resistance (p=0.978, compared to normal control), however, neither of the Aloe interventions showed any correction to this (Table 2).

**Liver enzymes.** The STZ-induced diabetes resulted in a significant increase in the plasma ALP (p=0.0001). The Aloe interventions both resulted in a reduction in diabetes induced ALP, with a significant reduction in the *A. greatheadii* group (874.3U/L vs 11145U/L). Glibenclamide significantly
reduced the diabetes induced ALP to levels no longer significantly different from normal control levels (323.7U/L vs 105.3U/L) (p=0.077). Effects size calculations showed that these changes in all the intervention groups were large enough to have practical significance (Table 2). STZ-diabetes resulted in a clinically significant increase in ALT levels although not statistically significantly so. The Aloe interventions did not correct this and the glibenclamide significantly further increased plasma ALT levels (131.2U/L vs 74.6U/L).

Lipids. STZ-induced diabetes resulted in significantly increased levels of TC (p=0.001), Trig (p=0.015), VLDL (p=0.018) and TC:HDL-C (p=0.0001) levels in the diabetic control group when compared to the NC group. Additionally, effect size calculations indicated a high practical significance for all these changes (Table 2). The increase in HDL-C in the diabetic control group compared to the normal control group was unexpected. Even so, both Aloe interventions showed a further increase in the HDL-C values compared to that of the diabetic control group resulting in a modest improvement in the TC:HDL-C ratio in the A. greatheadii group compared to the diabetic control group. After adjustment for change in body weight during the intervention, there was no longer any significant difference in HDL-C between the five groups suggesting that the increases in HDL-C observed in both aloe groups may be related to the weight loss observed in these groups. The glibenclamide intervention, comparatively better restored the majority of these diabetic lipid markers to levels no longer significantly
different from the normal control group, except for TC (TC: p=0.060, Trig: p=0.219, HDL-C: p=0.992 and VLDL-C: p=0.235), although not significantly so compared to diabetic control, but with moderate to large effect sizes.

Oxidative stress and antioxidant markers. STZ-induced diabetes had no effect on dROM values, however, FRAP values decreased significantly in the diabetic control group compared to the normal control group. None of the three treatments significantly bettered the oxidative stress and antioxidant markers.

DISCUSSION

This is the first intervention study investigating the possible antidiabetic properties of A. greatheadii and A. ferox. Both aloe species have previously been shown to contain antidiabetic phytochemicals (10,11). An intervention dosage of 300mg/kg ethanol leaf gel extracts of A. greatheadii and A. ferox (upper dosage level comparative to other groups and identical to the dosage used for successfully testing ethanol leaf gel extracts of A. vera (8,12)), were administered to STZ-induced diabetic rats over a period of 5 weeks. Considering the STZ diabetes animal model used in this study, a single intraperitoneal dose of 40mg STZ per kg body weight, produced chronic hyperglycemia after seven days. As STZ selectively destroys the pancreatic β-cells, insulin deficiency occurs, which in turn results in the hyperglycemia
Furthermore, due to the uncontrolled diabetic state demonstrated by the chronic and end-point hyperglycemia in the DC group compared to the NC group, an expected increase in fructosamine levels was observed in the DC group as compared to the NC group (Table 2) (9). This chronic hyperglycemia may additionally result in hyperglycemia-induced oxidative stress (19), explaining the slightly increased dROM and significantly reduced FRAP values seen in this STZ diabetes model. As expected, insulin resistance occurred. Furthermore, the STZ diabetes model used in this study showed increased fasting levels of TC, TG, HDL-C, VLDL-C and TC:HDL-C, which is also characteristic of these models (18) in addition to increased levels of ALT and ALP in the bloodstream (20, 21). Apart from these biochemical changes, this model additionally showed considerably reduced weight gain, increased liver mass, increased kidney mass and a decreased pancreatic mass, which is consistent to previous findings (22, 23, 24, 25, 26, 27).

The fact that glibenclamide, a typical type 2 diabetes medication, almost entirely normalized the diabetic state, shows this STZ-diabetes model satisfactory for investigating non-insulin dependent diabetes, similar to what is seen in type 2 diabetes (except for investigations of insulin resistance, as will be discussed later) (18).

The previously reported phytochemical composition of *A. greatheadii* and *A. ferox* leaf gel extracts, shows the presence of a number of possible antidiabetic phytochemicals, including phenolic acids, polyphenols,
phytosterols and indoles \((10, 11)\), and hence these extracts are thought to potentially have benefit in the treatment of diabetes. The *A. greatheadii* leaf gel extract intervention resulted in a decreased hyperglycemic state when compared to the diabetic control group accompanied by increased insulin levels. Although not significant by ANOVA, the large effect size calculated, does, however, indicate that this change seen may have a high practical/clinical impact. The *A. ferox* intervention resulted in a similar insulin secretion effect, however, with no change to the hyperglycemia. The slightly stronger antidiabetic action of *A. greatheadii* comparative to the *A. ferox*, can be explained by the higher concentrations of the potentially protective phytochemicals previously identified in this extract comparative to that of *A. ferox* \((10)\). Despite the increased insulin secretion and the consequent reduced blood glucose levels seen in these intervention groups, insulin resistance was not improved. In fact, a slightly worsened (non-significant with large effect size) insulin resistance occurred. Similarly, the fructosamine concentrations (an indicator of blood glucose control over a 21 day period \((28)\) remained unchanged. These results suggest that *A. ferox* and *A. greatheadii*, may show some effect in ameliorating the STZ diabetes induced hyperglycemia by increasing insulin secretion by the pancreatic \(\beta\)-cells, however, the slightly worsened insulin resistance and unchanged fructosamine, may be indicative that longer interventions with these extracts may be necessary, or that higher dosages may be required. Although the Aloe interventions may have possible \(\beta\)-cell protective effects, which result in the
slightly increased insulin secretions and consequent lowering of the blood glucose levels (by \textit{A. greatheadii}), these interventions (administered as 300mg/kg for 5 weeks) may have been unable to activate the many other mechanisms responsible for lowering of a hyperglycemic state e.g. 5' adenosine monophosphate-activated protein kinase (AMPK) \cite{29}, ATP/AMP ratios \cite{30} and the effects of the increased FFA on peroxisome proliferator-activated receptors (PPAR) \cite{31} and retinol binding protein 4 \cite{32} and its influence on glucose transporter 4 (GLUT4) \cite{33}, hence, not being able to influence glucose uptake enough, compared to insulin secretion, to influence the diabetes induced insulin resistance positively. More importantly, however, it has been shown that excessive glucose production rather than insulin resistance accounts for hyperglycemia in recent-onset STZ-diabetic rats, hence, the calculation for determining insulin resistance as used in this instance, may not be appropriate for this particular model. \citet{2009} further support this suggesting that an STZ-diabetes model is not the best suited model for monitoring changes in insulin resistance and other models should be considered for more accurate investigations of this e.g. a high fat diet fed diabetes model \cite{18}. The glibenclamide intervention also resulted in increased insulin secretion with large effect size which totally ameliorated the hyperglycemic state after the five week intervention period ($p=0.0001$), with normalization of the STZ induced insulin resistance. Comparatively, glibenclamide, showed a far better effect in ameliorating STZ diabetes hyperglycemia than the Aloe interventions did. The fructosamine
levels on the other hand were unexpectedly, significantly increased, relative
to the diabetic control. As fructosamine is a marker for long term glucose
control, and the fact that the weekly glucose determinations indicated that the
glucose concentrations only returned to normal during the last week of the
intervention (data not shown), may explain the lack of improvement in the
fructosamine levels. Additionally, despite glibenclamide restoring the
diabetes induced hyperglycemia, it had little effect on normalizing the
oxidative stress markers (dROM and FRAP). Furthermore, as is the case
with the elevated fructosamine levels, a longer duration of stabilized blood
levels may be required before these markers return to normal.

With regards to the abnormal lipid profiles induced by the STZ-diabetic
state, the *A. ferox* and *A. greatheadii* interventions resulted in a non-
significant general increase in the lipid markers analyzed, with moderate
effect sizes calculated for the *A. greatheadii* intervention (except for TC,
showing increased values, however, without significance or effect sizes
comparative to DC). In the *A. greatheadii* group, the increased TC may
largely be a result of increased HDL-C as can be seen in the decreased
TC:HDL-C ratio. This generalized hyperlipidemia seen in these groups may
be attributed to an increase in fat absorption via the gut as a result of
abnormally increased levels of small intestinal acyl-CoA:cholesterol
acyltransferase (ACAT) activity (34), which is known to be elevated when
insulin is deficient (35). Unfortunately the exact effect of insulin on ACAT
is still unclear. Another possible explanation could be an increased activation
of hormone sensitive lipase (HSL) (35). Furthermore, adjustment for weight change during the intervention affected only HDL-C levels suggesting that the increased HDL-C levels observed in the Aloe treatment groups may be related to the observed weight loss in these groups. HDL-C has been shown to increase with weight reduction (37). From these results it is evident that although these Aloe interventions, at a dosage of 300 mg/kg for 5 weeks, have some merit in restoring hyperglycemia through increased insulin secretion, they show little to no effect in restoring the hyperlipidemia associated with STZ induced diabetes. However, due to the fact that these extracts have been previously described to contain lipid lowering phytochemicals (10, 11), it does beg the question as to whether a higher dosage, for a longer intervention period, may have shown significant changes to these parameters. The glibenclamide intervention on the other hand resulted in a general lowering of the elevated hyperlipidemia with moderate to large effects sizes. Considering the mechanism by which these are elevated during the diabetic state, the reduction in hyperglycemia due to the glibenclamide induced increased insulin secretion, could have resulted in an inhibition of lipase in the adipose tissue, hence, lowering the amount of FFA released into the blood stream and the consequent cascade leading to the production of the abnormal, diabetic, lipid profile (38).
CONCLUSIONS

Although not consistently so, many previous studies conducted in the same manner, using identically prepared extracts of other Aloe species, at similar dosages, have reported significant antidiabetic effects over shorter intervention periods. In our study, improvements were observed in end-point glucose, serum insulin, HDL-C and TC:HDL-C using A. greatheadii leaf gel extracts, in an STZ-induced diabetic rodent model. The A. ferox intervention also showed some positive effects, however, to a lesser extent compared to A. greatheadii. Although the intervention period used in this study was 1-2 weeks longer than most of those previously described and the dosages administered comparatively in the highest ranges, a longer intervention period and/or higher dosages of these particular Aloe extracts may have resulted in more significant results, especially when considering the changes observed in the end-point plasma glucose and serum insulin levels. Additionally, although the size of the groups chosen was similar to that of the other studies previously described, considering that a lack of statistical significance for many of the markers was accompanied by effect sizes indicating clinical relevance, larger sample sizes may have resulted in more pronounced effects, and studies of this nature in the future should take this into account. Furthermore, different extracts using other parts of these plants may also be considered for future antidiabetic intervention studies using A. ferox and A. greatheadii.
ABBREVIATIONS

A. ferox, Aloe ferox; A. greatheadii, Aloe greatheadii; STZ, streptozotocin; FRAP, ferric reducing antioxidant power; VLDL-C, very low-density lipoprotein cholesterol; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; ALP, alkaline phophatase; A. vera, Aloe vera; A. arborescens, Aloe arborescens; DC, STZ-induce diabetic control; DG, STZ-induced diabetic rats receiving 600μg/kg glibenclamide; DAF, STZ-induced diabetic rats receiving 300mg/kg A. ferox; DAG, STZ-induced diabetic rats receiving 300mg/kg A. greatheadii; NC, normal control group; HOMA, homeostasis model assessment; TG, triglycerides; HPO, horseradish peroxide; LDL-C, low-density lipoprotein cholesterol; ALT, alanine transaminase; dROM, diacron reactive metabolites; ANOVA, analysis of variance; ANCOVA, analysis of co-variance; HSL, hormone sensitive lipase; SREBPs, sterol regulatory element-binding proteins; FFA, free fatty acids; AMPK, 5'adenosine monophosphate-activated protein-kinase; PPAR, peroxisome proliferators-activated receptors; GLUT-4, glucose transporter-4; ACAT, acyl-CoA:cholesterol acyltransferase; ATP, adenosine triphosphate; AMP, adenosine monophosphate.
LITERATURE CITED


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Chapter 5: Antidiabetic effects of Nux vomica extracts


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Chapter 5: Antidiabetic effects of Aloe ethanol extracts


Table 1. Body weight and relative organ weight.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Normal control</th>
<th>Diabetic control</th>
<th>Diabetic + Aloe greatheadii</th>
<th>Diabetic + Aloe ferox</th>
<th>Diabetic + Glibenclamide</th>
<th>Comparison between groups (ANOVA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (baseline) (g)</td>
<td>Mean: 237.6</td>
<td>Mean: 226.0</td>
<td>Mean: 244.5</td>
<td>Mean: 253.2</td>
<td>Mean: 253.2</td>
<td>Mean: 184.2</td>
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<tr>
<td></td>
<td>95% CI: 226.2; 249.0</td>
<td>95% CI: 209.2; 242.7</td>
<td>95% CI: 235.2; 253.8</td>
<td>95% CI: 221.6; 284.8</td>
<td>95% CI: 221.6; 284.8</td>
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<td>Weight (end) (g)</td>
<td>Mean: 310.4</td>
<td>Mean: 231.6</td>
<td>Mean: 294.8</td>
<td>Mean: 217.1; 246.0</td>
<td>Mean: 212.6; 256.9</td>
<td>Mean: 205.8</td>
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<tr>
<td></td>
<td>95% CI: 290.7; 330.2</td>
<td>95% CI: 217.1; 246.0</td>
<td>95% CI: 212.6; 256.9</td>
<td>95% CI: 228.1; 295.5</td>
<td>95% CI: 228.1; 295.5</td>
<td>95% CI: 186.6; 224.9</td>
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<tr>
<td>Δ Weight (g)</td>
<td>Mean: 72.79</td>
<td>Mean: 5.63</td>
<td>Mean: -9.70</td>
<td>Mean: -10.91</td>
<td>Mean: -10.91</td>
<td>Mean: 21.6</td>
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<td>95% CI: 58.65; 86.93</td>
<td>95% CI: -6.11; 17.38</td>
<td>95% CI: -28.24; 8.84</td>
<td>95% CI: -40.67; 18.84</td>
<td>95% CI: -40.67; 18.84</td>
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<td>Weight (pancreas) (%) body weight</td>
<td>Mean: 0.29</td>
<td>Mean: 0.26</td>
<td>Mean: 0.26</td>
<td>Mean: 0.24</td>
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<td>95% CI: 0.23; 0.30</td>
<td>95% CI: 0.24; 0.29</td>
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<td>95% CI: 0.24; 0.26</td>
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<td>Weight (liver) (%) body weight</td>
<td>Mean: 2.47</td>
<td>Mean: 3.65</td>
<td>Mean: 3.88</td>
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<td>95% CI: 2.37; 2.58</td>
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<td>Weight (kidney) (%) body weight</td>
<td>Mean: 0.66</td>
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<td>Mean: 0.91</td>
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<td>95% CI: 0.84; 1.00</td>
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</table>

*: Differs significantly from DC; #: Treatment groups which differ significantly from NC; a: effect size calculations indicating high practical significance compared to DC; b: Effect size calculations indicating medium practical significance compared to DC.
### Table 2. Diabetic and antioxidant markers.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Normal control</th>
<th>Diabetic control</th>
<th>Diabetic + Aloe</th>
<th>Diabetic + Aloe</th>
<th>Diabetic + Glibenclamide</th>
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<td>(n=10)</td>
<td>(n=6)</td>
<td>(n=7)</td>
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<td>Mean 95% CI</td>
<td>Mean 95% CI</td>
<td>Mean 95% CI</td>
<td>Mean 95% CI</td>
<td>P-value</td>
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<tr>
<td>Plasma glucose (mmol/L)</td>
<td><strong>3.80 3.60; 4.00</strong></td>
<td>20.07 17.74; 22.41</td>
<td><strong>17.29 14.05; 20.53</strong></td>
<td><strong>19.51 14.03; 24.99</strong></td>
<td><strong>4.90 3.71; 6.09</strong></td>
<td>0.000</td>
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<td>Baseline Glucose (mg/dL)</td>
<td>4.27 3.43; 5.11</td>
<td>4.05 3.33; 4.77</td>
<td>3.77 3.41; 4.13</td>
<td>3.73 3.42; 4.04</td>
<td>4.02 3.16; 4.89</td>
<td>0.719</td>
</tr>
<tr>
<td>Serum insulin (uU/L)</td>
<td><strong>17.45 11.66; 23.25</strong></td>
<td>5.32 3.34; 7.31</td>
<td><strong>9.15 3.76; 14.54</strong></td>
<td><strong>7.49 5.49; 9.49</strong></td>
<td><strong>10.41 6.64; 14.18</strong></td>
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<td>Insulin resistance (IU/L)</td>
<td>2.94 1.97; 3.91</td>
<td>5.14 2.16; 8.12</td>
<td><strong>6.66 3.10; 10.23</strong></td>
<td><strong>6.31 3.98; 8.63</strong></td>
<td><strong>2.30 1.68; 2.93</strong></td>
<td>0.001</td>
</tr>
<tr>
<td>Fructosamine (μmol/L)</td>
<td><strong>232.6 231.1; 242.0</strong></td>
<td>296.9 282.9; 311.0</td>
<td><strong>296.1 287.2; 304.9</strong></td>
<td><strong>306.5 284.8; 328.2</strong></td>
<td><strong>333.00 317.3; 348.7</strong></td>
<td>0.000</td>
</tr>
<tr>
<td>ALP (U/L)</td>
<td><strong>105.3 95.3; 115.4</strong></td>
<td>1145.0 839.6; 1450.4</td>
<td><strong>874.3 757.2; 991.5</strong></td>
<td><strong>985.3 782.6; 1188.0</strong></td>
<td><strong>323.7 217.2; 430.2</strong></td>
<td>0.000</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td><strong>74.55 57.22; 91.88</strong></td>
<td>107.8 84.49; 131.0</td>
<td>117.3 84.81; 149.9</td>
<td>103.2 80.56; 125.8</td>
<td><strong>131.2 90.7; 171.7</strong></td>
<td>0.011</td>
</tr>
<tr>
<td>TC (mmol/L)</td>
<td><strong>0.94 0.85; 1.02</strong></td>
<td>1.67 1.22; 2.11</td>
<td><strong>1.82 1.28; 2.36</strong></td>
<td><strong>1.86 1.55; 2.17</strong></td>
<td><strong>1.28 0.91; 1.65</strong></td>
<td>0.000</td>
</tr>
<tr>
<td>Trig (mmol/L)</td>
<td><strong>0.76 0.65; 0.87</strong></td>
<td>2.02 1.25; 2.80</td>
<td><strong>2.77 1.59; 3.94</strong></td>
<td><strong>1.93 1.22; 2.63</strong></td>
<td><strong>1.58 1.29; 1.86</strong></td>
<td>0.000</td>
</tr>
<tr>
<td>HDL-C (mg/dL)</td>
<td><strong>0.92 0.85; 0.98</strong></td>
<td>1.11 0.87; 1.35</td>
<td><strong>1.32 1.03; 1.61</strong></td>
<td><strong>1.26 0.99; 1.52</strong></td>
<td><strong>1.00 0.87; 1.13</strong></td>
<td>0.003</td>
</tr>
<tr>
<td>VLDL-C (mg/dL)</td>
<td><strong>0.35 0.29; 0.40</strong></td>
<td>0.92 0.57; 1.27</td>
<td><strong>1.26 0.72; 1.79</strong></td>
<td><strong>1.00 0.61; 1.38</strong></td>
<td><strong>0.72 0.58; 0.85</strong></td>
<td>0.000</td>
</tr>
<tr>
<td>TCHDL-C (mg/dL)</td>
<td><strong>1.02 0.97; 1.07</strong></td>
<td>1.50 1.29; 1.72</td>
<td><strong>1.36 1.16; 1.56</strong></td>
<td><strong>1.51 1.28; 1.74</strong></td>
<td><strong>1.44 1.31; 1.56</strong></td>
<td>0.000</td>
</tr>
<tr>
<td>d-ROM (CARR U)</td>
<td>376.9 321.4; 432.4</td>
<td>399.7 247.5; 551.9</td>
<td>379.5 299.3; 459.8</td>
<td>397.3 343.5; 451.2</td>
<td><strong>374.5 442.9; 506.1</strong></td>
<td>0.149</td>
</tr>
<tr>
<td>FRAP</td>
<td><strong>548.3 312.6; 384.0</strong></td>
<td>285.7 265.3; 396.1</td>
<td><strong>293.1 246.5; 359.7</strong></td>
<td><strong>279.6 249.6; 309.6</strong></td>
<td><strong>500.86 248.9; 352.9</strong></td>
<td>0.021</td>
</tr>
</tbody>
</table>

*: Differs significantly from DC; #: Treatment groups which differ significantly from NC; a: effect size calculations indicating high practical significance compared to DC; b: Effect size calculations indicating medium practical significance compared to DC.
Chapter 6

Discussion and Conclusion
1. INTRODUCTION

In this chapter, the main findings of this study will be discussed and conclusions regarding these will be made. Additionally, the strengths, limitations and problems experienced will be discussed and recommendations for future research exploring the antidiabetic effects of *Aloe greatheadii* var. *davyana* and *Aloe ferox* will be made.

The aim of the study was to investigate the potential antidiabetic effects of two *Aloe* species (*A. ferox* and *A. greatheadii* var. *davyana*), indigenous to South Africa. The first objective was to determine and compare the phytochemical composition of the two different extracts of both *Aloe* species, in order to determine if these extracts contain any compounds which may justify further investigations of the antidiabetic effects in a diabetic animal model, and which extraction procedure extracts comparatively the most phytochemicals with previously described health benefits. Based on the comparative phytochemical composition and supported by the literature, the ethanol leaf gel extract was found most suitable for further investigations of the potential antidiabetic effects of these *Aloe* species by intragastric dosing in a STZ induced diabetic male Wistar rat model.

2. SUMMARY OF THE MAIN FINDINGS

Firstly, the phytochemical contents and antioxidant capacities of both *A. ferox* and *A. greatheadii* leaf gel (LGE) and 95% ethanol leaf gel extracts (ELGE) were identified, quantified and compared. This was accomplished using GCMS and spectrophotometric analyses. The identified phytochemicals with possible biological action in both *A. ferox* and *A. greatheadii* include polyphenols/phenolic acids, sterols, fatty acids, and indoles. Additionally, various other compounds including alkanes, pyrimidines, alkaloids, organic acids, aldehydes, dicarboxylic acids, ketones,
alcohols, and dicarboxylic acids, were also indentified. The ferric reducing antioxidant power (FRAP) and oxygen radical absorbance capacity (ORAC) analyses revealed strong antioxidant capacities of both *A. ferox* and *A. greatheadii* LGEs and 95% ELGEs. Furthermore, sugar determination revealed that the total sugar contents of the *Aloe* extracts were 5.43 g/100 g and 83.76 g/100 g for the *A. greatheadii* LGE and ELGE respectively, and 11.40 g/100 g and 96.91 g/100g for the *A. ferox* LGE and ELGE respectively. Of this, 36% vs. 40% (*A. greatheadii* vs. *A. ferox*) was quantified as glucose, 18% vs. 10.6% (*A. greatheadii* vs. *A. ferox*) as fructose and the remainder as maltose and sucrose.

A larger number of compounds were extracted with GC-MS analyses using direct leaf gel extract ethyl acetate/diethyl ether and hexane extractions compared to the same extraction method using ethanol leaf gel extract. Even so, when expressed as per dry mass, the concentrations of the compounds with known health benefits identified in the ethanol leaf gel extract were 1.20 to 1.25 times higher than the leaf gel extract. Additionally, antioxidant capacity analyses also showed higher activities in the ethanol leaf gel extract. This demonstrates that, even though the direct leaf gel extraction is best suited for general phytochemical characterization purposes, the use of ethanol leaf gel extract will be more effective in *in vivo* or *in vitro* biological efficacy and mechanistic studies (Loots et al., 2007; Botes et al., 2008).

Based on the above phytochemical compositions, the antidiabetic effects of the *A. ferox* and *A. greatheadii* ELGE were investigated in a STZ-induced diabetic rat model. Oral *A. greatheadii* supplementation resulted in moderately increased serum insulin together with modest decreased end-point plasma glucose and decreased liver enzyme alkaline phophatase (ALP) concentrations. Additionally, all lipids, including high-density lipoprotein cholesterol (HDL-C) increased, while total cholesterol (TC):HDL-C values decreased slightly in both treatment groups. *A. ferox* supplementation
resulted in moderately increased serum insulin, and slight corrections in ALP and low-density lipoprotein cholesterol (LDL-C). No change was, however, observed in end-point plasma glucose for this intervention. Oral *A. ferox* and *A. greatheadii* supplementation, however, had little effect on other diabetes markers tested. Glibenclamide (as a positive control) resulted in significant improvements of almost all diabetic markers (increased insulin secretion, subsequent normalization of end-point blood glucose values and a correction of the diabetes induced hyperlipidaemia).

### 3. DISCUSSION AND CONCLUSIONS

Chapters 2, 3 and 4 provide the necessary information to interpret the results of the animal study (Chapter 5). The phytochemical analyses of *A. ferox* and *A. greatheadii* ethanol leaf gel extracts (chosen above that of the LGE for the later intervention studies due to the higher concentrations of biologically active compounds when quantified as per dry mass ELGE), as discussed in Chapters 3 and 4 (Loots *et al.*, 2007; Botes *et al.*, 2008), revealed a wide range of compounds with previously proven antidiabetic as well as antioxidant properties. Compared to *A. ferox*, *A. greatheadii* showed fewer compounds in lower concentrations, with the exception of a few biologically active polyphenols. FRAP and ORAC analyses also revealed *A. greatheadii* ethanol leaf gel extract to have a lower antioxidant capacity than *A. ferox* ethanol leaf gel extract. However, the total flavanoid content of the *A. greatheadii* leaf gel extract showed greater antioxidant capacity as measured by ORAC analysis compared to *A. ferox* indicating that the flavanoids in *A. greatheadii* may have greater oxygen radical scavenging effects than ferric ion reducing potential.

A single intraperitoneal dose of STZ (40 mg/kg body weight) resulted in hyperglycaemia after seven days as a result of insulin deficiency due to the selective destruction of pancreatic β-cells (Islam & Loots, 2009).
Furthermore, due to the uncontrolled diabetic state demonstrated by the chronic end-point hyperglycaemia in the DC group compared to the NC group, an expected increase in fructosamine levels was observed (Chapter 5, Table 2) (Elliot et al., 1996). Brownlee showed that chronic hyperglycaemia may result in oxidative stress (Brownlee, 2005). This may explain the slight increase in dROM values and significantly reduced FRAP values seen in this STZ diabetes model. The STZ diabetes model also presented with increased insulin resistance as well as increased fasting levels of TC, TG, HDL-C, VLDL-C and TC:HDL-C, which are also characteristic of these models (Islam & Loots, 2009). However, contrary to the expected decrease in HDL-C levels in the STZ diabetes animal model (Mitra et al., 1995), the HDL-C levels increased in the DC compared to the NC group. The activation of hormone sensitive lipase (HSL) (Al-Shamaony et al., 1994) may explain these increased lipid levels. The activation of HSL is triggered by insulin deficiency resulting in the release of free fatty acids from adipose tissue (Al-Shamaony et al., 1994), which ultimately facilitates an increased synthesis of phospholipids and cholesterol in the liver. These phospholipids, together with the excess triglycerides, are released into the bloodstream as LDL-C and VLDL-C (Bopanna et al., 1997).

The STZ diabetes model also showed considerably reduced weight gain, increased liver mass, increased kidney mass, and a decreased pancreatic mass, which is consistent to previous findings (Whiting et al., 1977, Burcelin et al., 1995, Garcia-Compean et al., 2009, Satriano & Vallon, 2006, Satriano, 2007, Valentovic et al., 2006, Brownlee, 2003). The decreased weight gain can be explained by reduced rates of lipogenesis as a result of reduced insulin levels (Freed et al., 1988). Decreased hypothalamic-pituitary-adrenal activity, partly caused by decreased leptin levels, may explain the reduced weight gain in the diabetic rats (Akirav et al., 2004). The increased liver mass may partly be due to the formation of a fatty liver induced by hypoinsulinaemia as well as increased lipolysis of adipose tissue,
resulting in increased FFA and accumulation of these in the liver together with impaired exertion of these lipoproteins by the liver (Garcia-Compean et al., 2009; Zafar et al., 2009). The increased FFA, in combination with oxidative stress, may result in hepatocyte destruction, and hence, increase levels of ALT and ALP detected in the bloodstream (Neuschwander-Tetri & Caldwell, 2003, Grove et al., 1997). The increased kidney mass can be explained by an increased glomerular filtration rate at the onset of diabetes (Satriano & Vallon, 2006), as well as a compensatory response by the kidneys to the increased load imparted by hyperfiltration (Satriano, 2007), which may also be associated with STZ-induced diabetes (Valentovic et al., 2006). The loss in relative pancreatic mass can, of course, be explained by the STZ-induced \( \beta \)-cell destruction (Valentovic et al., 2006, Brownlee, 2003).

The fact that glibenclamide, a typical type 2 diabetes medication, almost entirely normalized the diabetic state, indicates this STZ-diabetes model to be satisfactory for investigating non-insulin dependent diabetes, similar to what is seen in type 2 diabetes (except for investigations of insulin resistance, as will be discussed later).

Considering the role of oxidative stress in the development of various diseases, including diabetes, as well as the proven positive outcomes of antioxidants in these diseases, the use of these \textit{Aloe} extracts, due to their high polyphenol contents and antioxidant capacities, could, therefore, be considered as a possible intervention for the hyperglycaemia-induced oxidative stress and related \( \beta \)-cell destruction which accompanies a diabetic state. Unfortunately the \textit{Aloe} interventions used in this study showed no improvement in the oxidative stress markers during the 5 week intervention period. This may be due to the fact that the antioxidant polyphenols present in these extracts are absorbed from the gut with maximum concentration in the blood approximately 2 hours after ingestion (Kivits et al., 1996; reviewed
The fact that fasting blood samples were analysed in this intervention (hence, blood collected long after ingestion of the Aloe extracts), means that the only antioxidant effects that one may measure are those due to the long term protective action of the absorbed polyphenols (i.e. protecting the pancreatic β-cells from further hyperglycaemia induced oxidative stress), and not due to the direct antioxidant capacity of the blood due to the presence of these compounds in the bloodstream at that moment in time.

Despite this, however, as described in Chapter 5, the A. greatheadii leaf gel extract intervention did result in a decreased hyperglycemic state when compared to the diabetic control group accompanied by increased insulin levels. The A. ferox intervention resulted in a similar insulin secretion effect, however, with no change to the hyperglycaemia. The slightly stronger antidiabetic action of A. greatheadii comparative to that of A. ferox can be explained by the higher concentrations of the potentially protective phytochemicals, including 4-hydroxybenzoic acid and 4-hydroxyphenyllactic, previously identified in this extract comparative to that of A. ferox (Chapters 3 and 4). A slight worsened insulin resistance was, however, observed following the Aloe interventions, despite the increased insulin secretion and subsequent decrease in glucose levels. In addition, the fructosamine concentrations (an indicator of blood glucose control over a 21 day period (Lugman et al., 1985) remained unchanged. Considering these results, A. ferox and A. greatheadii may possibly improve hyperglycaemia induced by STZ diabetes, by increasing insulin secretion by the pancreatic β-cells. On the other hand, the worsened insulin resistance and unchanged fructosamine levels may indicate that a longer intervention period or higher dosages of the Aloe extracts may be required to improve chronic glucose levels. However, it has been shown that excessive glucose production rather than insulin resistance accounts for hyperglycemia in recent-onset STZ-diabetic rats (Burcelin et al., 1995), hence, the calculation used for the determination of
insulin resistance as used in this instance, may not be appropriate for this particular model. This is supported by Islam and Loots (2009), who suggested that an STZ-diabetes model is not the best suited model for monitoring changes in insulin resistance. Other models should, therefore, be considered for more accurate investigations of this, e.g. a high fat diet fed diabetes model (Islam & Loots, 2009).

A. ferox and A. greatheadii interventions resulted in a non-significant general increase in the lipid markers. In the A. greatheadii group, the increased TC may largely be a result of increased HDL-C as can be seen in the decreased TC:HDL-C ratio. The generalized hyperlipidaemia seen in these groups may be due to increased fat absorption via the gut as a result of abnormally increased levels of small intestinal acyl-CoA:cholesterol acyltransferase (ACAT) activity (Jiao et al., 1988). This increase is associated with insulin deficiency (Kusunoki et al., 2000). Unfortunately the exact effect of insulin on ACAT is still unclear. Additionally, adjustment for weight change during the intervention affected only HDL-C levels. This may indicate that the increased HDL-C levels observed in the Aloe treatment groups may be related to the observed weight loss in these groups, as HDL-C has been shown to increase with weight reduction (Katcher et al., 2007). From these results it is evident that although these Aloe interventions, at a dosage of 300 mg/kg for 5 weeks, which relates to 116.08 g/kg and 96.46 g/kg wet gel for AF and AG respectively, have some merit in restoring hyperglycemia through increased insulin secretion, they show little to no effect in restoring the hyperlipidemia associated with STZ induced diabetes. However, due to the fact that these extracts have been previously described to contain lipid lowering phytochemicals (Loots et al., 2007; Botes et al., 2008), it does beg the question as to whether a higher dosage, for a longer intervention period, may have shown significant changes to these parameters.
Overall, oral administration of the Aloe extracts, A. greatheadii in particular, resulted in moderate improvements in the STZ induced diabetic state, as evaluated by the measurement of various biochemical diabetes markers related to diabetes induced abnormalities including glucose, lipids, insulin and liver enzyme levels, justifying further investigations into the use of these traditional remedies for the treatment of diabetes.

4. STRENGTHS OF THE STUDY

- The topic addressed in this study is unique with regard to the Aloe species used. The possible antidiabetic effects of these Aloe species are extremely relevant to the local community, as it is already being used to treat diabetes, among other diseases, despite the limited existing scientific evidence. The extremely high prevalence of diabetes in South Africa further strengthens the investigation of possible antidiabetic effects of indigenous plants already used by local communities.

- The phytochemical characterization of the Aloe leaf gel extracts and ethanol leaf gel extracts preceding the animal study served as an important foundation for the animal study, as various antidiabetic and antioxidant components were identified during this analysis.

- The study was conducted over a five week period. This extends the duration of former studies using the same study design by an average of two weeks.

- A preliminary dose response study was done to determine exactly the amount of STZ needed to induce the desired diabetes animal model.
5. LIMITATIONS AND PROBLEMS EXPERIENCED IN THE STUDY

- Regular intragastric dosing and handling of the animals may have caused increased stress. This may be a possible explanation for the apparent augmentations in oxidative stress markers. Different dosing methods may have eliminated this possible confounding factor.
- The study was designed to investigate the chronic effects of *Aloe*. However, a different study design focusing on the immediate effects of the *Aloe* extracts may exert different effects.

6. RECOMMENDATIONS

Previous studies done using a variety of extracts from various other *Aloe* species, showed mixed results regarding antidiabetic action (Agarwal, 1985; Beppu *et al.*, 1993; Koo *et al.*, 1994; Bunyanphatsara *et al.*, 1996; Yongchaiyudha *et al.*, 1996; Lee *et al.*, 2000; Okyar *et al.*, 2001; Yagi *et al.*, 2002; Beppu *et al.*, 2003; Can *et al.*, 2004; Rajasekaran *et al.*, 2005a; Rajasekaran *et al.*, 2005b; Beppu *et al.*, 2006; Chandan *et al.*, 2007). The current study was designed using a similar design as those studies where significantly pronounced antidiabetic action was achieved (using other *Aloe* species of course). Hence, this study was conducted in the same manner, using identically prepared extracts, at comparatively the highest dosages reported, over a slightly longer duration. Although, in this study we observed improvements to end-point glucose, serum insulin, HDL-C and TC:HDL-C, using *A. greatheadii* leaf gel extracts, and the same positive trend for the *A. ferox* intervention (however, to a lesser extent), we feel that a longer intervention period and/or higher dosages of these particular *Aloe* extracts may have resulted in more significant results, especially when considering the changes we observed in the end-point plasma glucose and serum insulin levels. Additionally, although the size of the groups chosen was similar to
that of the other studies previously described, considering that a lack of statistical significance for many of the markers was accompanied by effect sizes indicating clinical relevance, larger sample sizes may have resulted in more pronounced effects, and studies of this nature in the future should take this into account. Furthermore, different extracts using other parts of these plants may also be considered for future antidiabetic intervention studies using *A. ferox* and *A. greatheadii*. Additionally, as previously mentioned, the total sugar/glucose contents of these extracts may be of concern, especially when considering using these extracts in the context of a diabetes intervention, as carbohydrate intake is considered a major factor in glycaemic control. Nielsen and Jonsson (2006) reported that a low carbohydrate diet, containing 20% carbohydrates, is superior to a diet containing 55 - 60% carbohydrates, with regards to controlling body weight, blood glucose levels and reducing HbA1c. The American Diabetes Association (ADA) defines a low carbohydrate diet as less than 130 g/d or 26% of a nominal 2,000 kcal (8400 kJ) diet (American Diabetes Association, 2002). Considering the above-mentioned recommended carbohydrate intakes for diabetics and the mechanisms by which the pholyphenols and phytosterols elicit their anti-diabetic actions (by lowering glucose absorption and protecting pancreatic β-cells from oxidative destruction), the sugar contents of these extracts may not necessarily be problematic due to the small amounts that would be additionally ingested during an intervention using these extracts. This, however, should be investigated, in addition to other methods of extraction which could potentially eliminate these sugars.

Finally, oral dosing via gastric gavage may have caused excessive stress to the rats. Other methods such as training the rats to drink from a syringe (Rouke & Pemberton, GSK research and development) or dosing through an automated pre-programmed infusion pump (Woods *et al.*, 2009) may be more favourable dosing methods.
7. LITERATURE CITED


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KUSUNOKI J., ARAGANE K., KITAMINE T., Kozono H., KANO K., FUJINAMI K., KOJIMA K., CHIWATA T., SEKINE Y. 2000. Postprandial Hyperlipidemia In Streptozotocin-Induced Diabetic Rats Is Due To Abnormal Increase In Intestinal Acyl Coenzyme A:Cholesterol


Addendum
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Materials and Methods
Results
Discussion
Abbreviations Used
Safety
Acknowledgment
Supporting Information description
Literature Cited
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Gas Chromatographic Methods. For manuscripts in which gas chromatographic methods are used, see “Reporting of Gas Chromatographic Methods”, by Morton Beroza and Irwin Hornstein [J. Agric. Food Chem. 1973, 21, 7A (located at the back of the January 1973 issue or as a link from the Journal's Author Information page)].

Spectroscopic Data. This is a guide only; in certain cases different methods of data presentation may be more suitable. Authors are encouraged to consult examples of data presentation published in recent issues of the Journal for appropriate style and format. Complete infrared, NMR, mass, or other spectra will be published only if novel or necessary to substantiate points made under the Results or Discussion sections. Such presentations take up valuable space, and essentially the same information can frequently be put into a much more compact form by simply listing the position and intensity of the maxima. It is usually not necessary to list all of the maxima in the spectra to provide an adequate description. Report the type of instrument used (e.g., in mass spectrometry, whether magnetic, quadrupole, etc.) and also the type of cell, the solvent (if any), and the state of the sample (whether liquid, gas, solution, etc.).

Mass Spectra. List the molecular ion and about 10 of the major ions with their intensities in parentheses, or more preferably use the method outlined by H. S. Hertz, R. A. Hites, and K. Biemann (Anal. Chem. 1971, 43, 681-691). This method involves dividing the spectrum into consecutive regions of 14 mass units starting at m/z 6 (i.e., 6-19, 20-33, 34-47, 48-61, etc.). The two most intense ions in each region are then listed. Intensities, relative to the most intense ion, the intensity of which is taken as 100, are shown in parentheses immediately following the m/z value; for example: hexanal, mass spectrum found (70 eV, two most intense ions each 14 mass units above m/z 34): 43 (86), 44 (100), 56 (86), 57 (65), 71 (28), 72 (33), 82 (18), 85 (5), 97 (2), 100 (2). If the molecular ion does not appear in this presentation, the author should indicate it separately.

Proton Magnetic Resonance (PMR or 1H NMR) Spectra. The frequency used, the solvent, and also temperature (if other than ambient) are first specified. The type of unit used (δ or r) is then stated, followed by the position of the center of gravity of the sharp line, broad line, or spin–spin multiplet in these units. This is then followed by information in parentheses which (1) describes the type of splitting, that is, singlet as s, doublet as d, triplet as t, quadruplet as qd, multiplet as m; (2) gives the value of the number of protons the area represents; (3) gives the coupling constant J; and (4) gives the part of the molecule connected with the particular absorption with the protons involved underlined.

As an example that would be PMR for ethanol (60 MHz, CDCl3): δ 1.22 (t, 3, J = 7 Hz, CH2CH3), 2.58 (s, 1, OH), 3.70 (qd, 2, J = 7 Hz, OCH2CH3).

Other Spectra. In general, list position and intensity of the maxima. In some cases it may be desirable to list points of inflection.

A brief explanation should be given for any abbreviations not in common use.

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- Reporting X-ray data
  “Novel Compound Characterization. For a discussion of the Journal’s expectation for compound characterization, please read “Compound Identification: A Journal of Agricultural and Food Chemistry Perspective” by R. J. Moloney and P. Schlegerle. J. Agric. Food Chem. 2007, 55, 4625-4629 (DOI: 10.1021/jf070242j). It is essential that novel compounds, either synthetic or isolated from natural sources, be characterized rigorously and unequivocally. Supporting data normally include physical form, melting point (if solid), UV/IR spectra if appropriate, 1H and 13C NMR, mass spectrometric data, and optical rotation (when compounds have chiral centers).

Examples:
- Reporting X-ray data
- Reporting data in detail, including UV shifts
- Reporting data for previously known compounds
- Flavor Constituents. Manuscripts reporting on flavor constituents should conform to the recommendations made by the International Organization of the Flavor Industry [for details, see the Editorial in the October 1996 issue of J. Agric. Food Chem. (44, 2941-2941)]. In brief, any identification of a flavoring substance must pass scrutiny of the latest forms of available analytical techniques. In practice, this means that any particular substance must have its identity confirmed.
by at least two methods, for example, comparison of chromatographic and spectrometric data (which may include GC, MS, IR, and NMR) with those of an authentic sample. If only one method has been applied (MS data alone or retention index or Kovats index alone), the identification shall be labeled "tentative." In addition, authors are encouraged to include at least semiquantitative data on the concentration of an identified compound in the original source, for example, foodstuff or plant part. Ranges such as <1 μg/kg, 1–10 μg/kg, and 10–100 μg/kg are acceptable.

Flavor is evoked by smell (aroma) and taste. A good example showing the correct characterization of taste compounds is the study by Czepa and Hofmann (J. Agric. Food Chem. 2003, 51, 3865–3873). A good example for aroma compound identification is the study by Millo and Grosch (J. Agric. Food Chem. 1996, 48, 2266–2271).

The use of reference compounds is a must, if data on sensory properties of single compounds are reported. Odor, which is perceived during sniffing of a food extract at a certain retention index, may be indicative of the presence of a given compound, but not conclusive unless substantiated by chromatographic and/or spectrometric data and comparison with an authentic reference compound.

Soil Classification. Soils used in research should be described down to the family level according to the soil classification scheme given in Soil Taxonomy, A Basic System of Soil Classification for Making and Interpreting Soil Surveys, 2nd ed. (Agricultural Handbook 436; U.S. Government Printing Office: Washington, DC, 1999) (available on-line at http://soils.usda.gov/technical/classification/taxonomy/). Also give series name if known. This requirement is to allow comparison and extrapolation to other work giving similar soil classifications, as published in journals such as the Journal of Soil Science, Soil Science Society of America Journal, Journal of Environmental Quality, and Geoderma. If information is unavailable to classify the soils at the desired family level, classification should be described or estimated at least to the great group level in the same classification system.


Animal or Human Studies. Manuscripts describing studies in which the use of live animals or human subjects is involved must include under Materials and Methods a statement that such experiments were performed in compliance with the appropriate laws and institutional guidelines, and also name the institutional committee that approved the experiments. For experiments with human subjects, a statement that informed consent was obtained from each individual must be included and the consent forms made available to the Journal on request. Reviewers of manuscripts involving animal or human experiments will be asked to comment specifically on the appropriateness and conformity to regulations of such experiments.

Animal Subjects. The use of animals in a study should be employed only when there are no alternative methods for investigating the fundamental questions of the study. In such cases, it is the ethical responsibility of all authors to ensure that the care of animals is of the highest possible order, that pain and/or distress is minimized, and that the numbers involved are strictly limited to those essential to fulfill the experimental design. In the United States the care and use of laboratory animals is regulated by the U.S. Department of Agriculture (USDA) under the Animal Welfare Act. Links to the regulations, including a checklist of Institutional Animal Use and Care Committees (IAUC) guidelines, is available at http://www.aphis.usda.gov/animal_welfare/publications_and_reports.shtml. It is recognized that researchers in other countries may be governed by different laws and regulations. In such cases, experiments should be designed to conform either to the above USDA regulations or to the International Guiding Principles for Biomedical Research Involving Animals (1985), available at http://www.cions.ch/frame_1985_texts_of_guidelines.htm.

Human Subjects. The use of human subjects in experimental studies requires informed consent. Such consent requires that the subjects be informed completely not only about the procedures involved but also about the aims, design, and expected outcomes of the study. Consent must be obtained not only when subjects are involved directly in the study but also when samples (tissue, blood, plasma, etc.) are required for in vitro experiments. In the United States the protection of human research subjects is regulated by the U.S. Department of Health and Human Services (HHS). Regulations are available at http://www.hhs.gov/ohrp/humansubjects/guidance/45cfr46.htm#46.116. Laws and regulations governing researchers in other countries must be observed, but experiments should be designed to conform to the intent of the HHS regulations as far as possible.

In relation to the subject matter of the Journal, experiments involving taste and food quality evaluation and consumer acceptance are exempt from the above regulations [CFR 46.101 (b) (6)]. However, it should be noted that this would not exempt studies in which extracts, isolates, pure compounds, etc., obtained from conventional food sources are subjected to such evaluation.

The Journal will reject any manuscript for which there is a reason to believe that animals have been subjected to unnecessary pain or distress or when informed consent of human subjects is absent or incomplete.
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Correct identification of components of natural products

The correct identification of the various components of extracts from natural sources is of key importance, and as publishers we are keenly aware of our responsibility to the scientific community in this area. Consequently, for papers on this topic, we have adopted the recommendations of the Working Group on Methods of Analysis of the International Organization of the Flavour Industry (IOFI), as published in *Flavour Fragr. J.* 2006, 21, 185. These recommendations may be summarized as follows:
Any identification of a natural compound must pass scrutiny by the latest forms of available analytical techniques. This implies that its identity must be confirmed by at least two different methods, for example, comparison of chromatographic and spectroscopic data (including mass, IR and NMR spectra) with those of an authentic sample, either isolated or synthesized. For papers claiming the first discovery of a given compound from a natural source, the authors must provide full data obtained by their own measurements of both the unknown and an authentic sample, whose source must be fully documented. Authors should also consider very carefully potential sources of artifacts and contaminants resulting from any extraction procedure or sample handling.
Jer. 29 v 11

“For I know the plans I have for you, declares the Lord, plans to prosper you and not to harm you, plans to give you a hope and a future.”