Evaluation of the anti-diabetic potentials of some African medicinal plants: A multimode study

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December 2018
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Submitted in fulfilment of the academic requirements for the degree of Doctor of Philosophy in Biochemistry, School of Life Sciences, University of KwaZulu-Natal (Westville campus), Durban 4000, South Africa

Supervisor: Professor MS Islam, PhD
ABSTRACT

The present study investigated the effects of five African medicinal plants (Alstonia boonei, Acalypha wilkesiana, Boerhaavia diffusa, Bridelia ferruginea, and Crassocephalum rubens) for their antioxidative and antidiabetic potentials by using several experimental protocols. The crude extracts (ethyl acetate, ethanol and aqueous) of the different parts (leaves, stem bark, root bark or aerial parts when applicable) were initially investigated for their detailed antioxidant and antidiabetic activity using in vitro, ex vivo and in silico experimental models. Then the most active crude extract from each plant was chosen for further fractionation with the solvents of increasing polarity. The solvent obtained fractions were then subjected to screening in terms of their antioxidant, α-glucosidase, α-amylase and lipase inhibitory activity in vitro and intestinal glucose absorption and muscle glucose uptake ex vivo. Results from these assays revealed that the butanol and aqueous fractions of A. boonei, butanol fraction of B. ferruginea, ethanol extract of A. wilkesiana and B. diffusa and the aqueous extracts of C. rubens showed the best activities in terms of all the tested models. The most active crude extracts and fractions were consequently subjected to GC-MS and LC-MS analyses in order to identify their bioactive components. Then the structures of the most bioactive components were docked with the tested enzymes using in silico modelling. The anti-diabetic effect of the butanol fractions of A. boonei and B. ferruginea together with the aqueous extract of C. rubens were investigated in an in vivo intervention trial using a type 2 diabetes rat model. The in vivo experiment revealed that the fractions and extract exhibited potent in vivo hypoglycaemic activity. Interestingly, these fractions were also able to alleviate T2D-associated complications involving oxidative stress. Analysis of in vivo oxidative stress markers such as superoxide dismutase, catalase, glutathione and thiobarbituric acid reactive substances, in the serum, liver, kidney, heart and pancreas of the animals also suggested their strong antioxidative effects. The results of this study suggest that the different extracts/fractions of the above-mentioned plants have promising anti-diabetic potentials; however further clinical trials are required in order to justify the usefulness of these plants for the development of potent and cost effective anti-diabetic drugs.
PREFACE

The information presented in this thesis is an original work by the candidate. It was carried out in the Department of Biochemistry, School of Life Sciences, College of Agriculture, Engineering and Science, University of KwaZulu-Natal, Westville Campus, Durban, South Africa from January 2016 to December 2018 under the supervision of Prof. MS Islam and has not otherwise been submitted in any form for any degree or diploma to any other University. Where use has been made of the work of others, it has been duly acknowledged in the text in the form of reference.

Candidate: Olajumoke Arinola Oyebode

Supervisor: Prof. MS Islam
DECLARATION 1 – PLAGIARISM

I, Olajumoke Arinola Oyebode, declare that

1. The research reported in this thesis, except where otherwise indicated, is my original research.

2. This thesis has not been submitted for any degree or examination at any other university.

3. This thesis does not contain other persons’ data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.

4. This thesis does not contain other persons' writing, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:
   a. their words have been re-written, but the general information attributed to them has been referenced.
   b. where their exact words have been used, then their writing has been placed in italics and inside quotation marks and referenced.

5. This thesis does not contain text, graphics or tables copied and pasted from the Internet, unless specifically acknowledged, and the source being detailed in the thesis and in the References sections.

Signed

.................................................
DECLARATION 2 - PUBLICATIONS AND PRESENTATIONS

Details of contribution to publications that make up part of the research presented in this thesis (include publications in preparation, submitted, in press and published and give details of the contributions of each author to the experimental work and writing of each publication).

In all the publications included in this thesis, I (with guidance from my supervisor) designed the work, performed all the experiments and wrote all the publications. The co-authors contributed by conducting an editorial work, checking the scientific content of the work and my correct interpretation of the findings.

Already published papers from this thesis:

Publication 1
https://doi.org/10.2478/acph-2018-0037

Publication 2

Pending and prospective publications:

Publication 3

Publication 4
**Publication 5**

**Publication 6**

**Publication 7**
Olajumoke A. Oyebode, Ochuko L. Erukainure, and Md. Shahidul Islam. Butanol fraction of *Alstonia boonei* leaves ameliorates oxidative stress in a type 2 diabetes model of rats. *(In preparation).*

**Publication 8**

**Publication 9**

**Publication 10**

**Publication 11**
Publication 12

Publication 13

Other significant research output
Publication 14

Publication 15

Publication 16
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Presentation 1

Presentation 2

Presentation 3

Signed:

Date:…………………………
DEDICATION

To God Almighty

The Everlasting Father

The one who knows the end even before the beginning

The Ultimate Teacher

And above all, the Fountain of Knowledge
ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my supervisor Prof. Md. Shahidul Islam for the continuous support of my Ph.D. study and research, for his patience, motivation, enthusiasm, and immense knowledge. His guidance helped me all through my research and writing of this thesis. I could not have imagined having a better advisor and mentor for my Ph.D. study. My sincere thanks also go to Prof. Neil Koobanally, Prof. Mogie Singh, Mrs Soohana and Dr. Linda Bester, for guiding and directing me all through the diverse and exciting complexities of my Ph.D. research.

I thank my fellow lab mates in the biomedical research unit: Ochuko, Sanni, Xin Xiao, Rebecca, Shazia, Kovania, Lindiwe, Veronica, Brian, and Nonto, for the stimulating discussions and constructive criticisms in our seminars, as well as laborious efforts put in during our lab work in particular animal study. All the fun times we had together in the course of my study will always be cherished. Hope to do more of the fun part! Also, I will like to appreciate my friends Jude, Olakunle, Jeremiah, Olayide, Nneka, Olayemi, Olamide, Nokhu, and Mr and Mrs Babatunde, as well as my senior colleagues Dr. Chika, Dr. Mopuri, Dr. Collins and a host of others for their encouragement and unfailing support.

How can I fail to mention the prayers and support of my spiritual family: Pastor and Mrs Adejimi who with the members of the RCCG Durban stood in the gap for me in prayers for successful completion of my degree? May God reward you immensely.

Utmost thanks go to my husband and my lead cheerleader Engr. Oluwaseun Oyebode (Ph.D), who put up with my aloofness and unavailability which I exhibited sometimes because of my research, and to my Son Master David Oyebode, your entrance into this world encouraged me to finish this program in giant strides, I will be forever grateful to you both.

I cannot fail to mention the endearing support of my in-laws, colleagues and friends in the University of Ibadan. Thanks for your encouragement.

Last but not the least, I would like to thank my parents – Lt. Col. (Rtd.) and Chief Mrs Daramola, for encouraging me to pursue my dreams and supporting me spiritually and financially all throughout my academic pursuit. I am forever grateful. To my siblings, Arch. Ayotilerewa Daramola, the Olokobas and Tinuola Adegbemile, many thanks for your continuous advice and prayers.

A very special gratitude goes out to the National Research Foundation-The world academy of science (NRF-TWAS) and the UKZN postgraduate office for providing the funding necessary for this work.
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## LIST OF ACRONYMS

<table>
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<th>Definition</th>
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<tbody>
<tr>
<td>6-HD</td>
<td>6-hydroxydopamine</td>
</tr>
<tr>
<td>ACE</td>
<td>associated chemical enterprise</td>
</tr>
<tr>
<td>ADA</td>
<td>American diabetes association</td>
</tr>
<tr>
<td>AGE</td>
<td>advanced glycation end products</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
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<tr>
<td>AUC</td>
<td>area under the curve</td>
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<tr>
<td>BC</td>
<td>Before Christ</td>
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<tr>
<td>CRP</td>
<td>C-reactive proteins</td>
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<tr>
<td>CVD</td>
<td>cardiovascular disease</td>
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<td>DABH</td>
<td>diabetic animals treated with high dose of <em>A. boonei</em></td>
</tr>
<tr>
<td>DABL</td>
<td>diabetic animals treated with low dose of <em>A. boonei</em></td>
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<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DBFL</td>
<td>diabetic animals treated with low dose of <em>B. ferruginea</em></td>
</tr>
<tr>
<td>DCRH</td>
<td>diabetic animals treated with high dose of <em>C. rubens</em></td>
</tr>
<tr>
<td>DCRL</td>
<td>diabetic animals treated with low dose of <em>C. rubens</em></td>
</tr>
<tr>
<td>DETAPAC</td>
<td>diethylenetriaminepentaacetic acid</td>
</tr>
<tr>
<td>DKA</td>
<td>diabetic ketoacidosis</td>
</tr>
<tr>
<td>DN</td>
<td>diabetic nephropathy</td>
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<td>DNS</td>
<td>dinitrosalicylic acid</td>
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<td>DPPH</td>
<td>1,1-diphenyl-2-picrylhydrazyl radical</td>
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<tr>
<td>DR</td>
<td>diabetic retinopathy</td>
</tr>
<tr>
<td>DTNB</td>
<td>5,5'-dithiobisnitrobenzoic</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>eNOS</td>
<td>endothelial nitric oxide synthase</td>
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</table>
ETC | electron transport chain  
---|---  
FADH$_2$ | flavin adenine dinucleotide  
FRAP | ferric (Fe$^{3+}$) reducing antioxidant power  
G6P | glucose-6-phosphatase  
GAD | glutamic acid decarboxylase  
GAE | gallic acid equivalent  
GC-MS | gas chromatography-mass spectrometry  
GDM | gestational diabetes  
GDP | gross domestic product  
GFAT | glutamine fructose 6-phosphate amidotransferase  
GIP | glucose-dependent insulino tropic peptide  
GLP-1 | glucagon like peptide-1  
GLUT4 | glucose transporter 4  
GSH | reduced glutathione  
GSSG | oxidized glutathione  
H$_2$O$_2$ | hydrogen peroxide  
HDL | high density lipoprotein  
HHNS | hyperosmolar hyperglycemic nonketotic syndrome  
HOMA-IR | homeostatic model assessment-insulin resistance  
HOMA-β | homeostatic model assessment-beta cell  
IDDM | Insulin dependent diabetes mellitus  
IDF | international diabetes federation  
IGF-IR | insulin growth factor-insulin receptor  
IL-1 | interleukin-1  
IL-2 | interleukin-2  
IRS | insulin receptor substrate  
K$^+$-ATP | potassium adenosine triphosphate  
LDL | low density lipoprotein
MAPK | mitogen-activated protein kinase
MCP-1 | monocytes chemoattractant protein-1
MDA | Malondialdehyde
MOPS | 3-(N-morpholino) propanesulfonic acid
mRNA | mitochondrial RNA
NAB | group of normal animals treated with high dose of *A. boonei*
NADH | nicotinamide adenine dinucleotide
NADPH | nicotinamide adenosine dinucleotide
NBF | group of normal animals treated with high dose of *B. ferruginea*
NCR | group of normal animals treated with high dose of *C. rubens*
NEFAs | non-esterified fatty acids
NIDDM | non-insulin dependent diabetes mellitus
NO | nitric oxide
OGTT | oral glucose tolerance test
OXPHOS | oxidative phosphorylation
PI3K | phosphoinositide 3-kinase
PIP2 | phosphatidylinositol 4,5-bisphosphate
PKA | protein kinase-A
PKB | protein kinase B
PKC | protein kinase-C
pNPG | p-nitrophenyl-α-D-glucopyranoside
PPARγ | Peroxisome proliferator-activated receptor gamma
ROS | reactive oxygen species
SGL2 | Sodium-glucose co-transporter-2
SHP-1 | Src homology-2 domain-containing phosphatase-1
SOD | superoxide dismutase
T1D | type 1 diabetes
T2D | type 2 diabetes
<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tbody>
<tr>
<td>TCA</td>
<td>trichloroacetic acid</td>
</tr>
<tr>
<td>TGF-β</td>
<td>transforming growth factor beta</td>
</tr>
<tr>
<td>TNB</td>
<td>thio-2-nitrobenzoic acid</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumor necrosis factor-alpha</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>UDP-GlcNAc</td>
<td>uridine diphosphate N-acetylglucosamine</td>
</tr>
<tr>
<td>USF</td>
<td>upstream stimulatory factors</td>
</tr>
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<td>WHO</td>
<td>world health organisation</td>
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1.1 Background of study

The word Diabetes was derived from a Greek word around 1500 BC and means to siphon since it was earlier associated with excessive wasting of essential minerals of the body through urination (Lakhtakia, 2013). This urine was discovered to have a sweet taste which then led to the Latin name diabetes which means honey sweet (Zajac et al., 2010). Diabetes mellitus is a complicated metabolic disease which is brought about by high blood glucose levels (hyperglycaemia) due to defective insulin signalling and function (De Felice and Ferreira, 2014). Hyperglycaemia produces symptoms such as excessive thirst (polydipsia), habitual urination (polyuria), unexplained weight loss and frequent eating (polyphagia). Insulin is a hormone synthesized in the pancreatic β-cells and is required to effectively use glucose from digested food as an energy source (Chase et al., 1989). A spike in glucose levels due to postprandial digestion of food triggers insulin to transport the glucose to the needed peripheral organs for utilization (Geijselaers et al., 2017; Nolan et al., 2015). Insulin functions effectively by increasing glycogenesis and transport of glucose to the liver, muscle and adipose tissues. Insulin resistance leads to chronic hyperglycaemia generating anomalies in complex sugars, protein and fatty acid metabolism (Nolan et al., 2015).

Diabetes can be controlled by different types of treatment modalities the most common of which is the use of synthetic drugs. These drugs though sometimes effective when used singly or in combination with other drugs, are not without side effects. Examples of these drugs are: metformin (a biguanide), insulin, rosiglitazone (a thiazolidinedione) with side effects such as nausea, weight gain/lost, high cost and lactic acidosis amongst others (Chaudhury et al., 2017). Interestingly traditional medicine could be effective in the treatment and control of diverse diseases. This is because plants contain phytochemicals which could render synergistic functions in the treatment of diseases such as diabetes. Several researches have mentioned the efficacy of medicinal plants in the management of diabetes (Choudhury et al., 2017; Mohammed et al., 2014). The vast array of medicinal plants gives more opportunities for researchers to carry out more studies with a view to harness the spread of diseases using medicinal plants. Many of these medicinal plants are currently being used by traditional healers for the treatment of diabetes but scientific evidence behind the use...
of many of these medicinal plants is still unknown despite the rapidly growing prevalence of diabetes globally.

1.2 Prevalence of diabetes

Presently, over 422 million cases of diabetes have been recorded, which means 1 in 11 people in the world has diabetes and there will be an estimated increase by 2030 to 640 million cases i.e. 1 in 10 people will have diabetes (Conway et al., 2018; WHO, 2016). This increase can be linked to factors such as change in diet, urbanization, sedentary lifestyles, aging and increasing population. There has been a global rise of diabetic cases as compared to the year 1980 where only 108 million cases were recorded as diabetic (IDF, 2015). This increase can be noticeable mostly in those in the age range of 20-65 years (Conway et al., 2018).

An increase in the number of deaths due to complications of diabetes has also been recorded: 3.96 million deaths were recorded amongst adults aged 20 - 79 years in 2010. This number has risen to 5 million in 2015; signifying a 26% increase within the 5-year period (Zheng et al., 2018). Statistics also show that, as at 2017, 3.2 million deaths have been recorded for the age range of 60 - 99 years (Cho et al., 2018) (Table 1.1). The rise in diabetes has a high impact on the economy globally. Factors like high cost of drugs, loss of productivity and premature deaths negatively impacts on a nation’s Gross Domestic Product (GDP) (Bouguerra et al., 2007; Edelman and Polonsky, 2017).

| Table 1.1: Global estimates of adults above 60 living with diabetes (IDF, 2017). |
|-----------------|-----------------|-----------------|
|                 | 2017            | 2045            |
| Number of adults (65-99 years) | 652.1 million | 1.42 billion |
| Incidence (65-99 years)       | 9.6%           | 17.9%          |
| Number of people living with diabetes (65-99 years) | 122.8 million | 253.4 million |
| Number of deaths from diabetes (60-99 years)        | 3.2 million   | Not yet determined |
| Total healthcare cost for diabetes (60-99 years)     | 527 billion   | 615 billion    |

Africa has the most recorded number of undiagnosed cases of diabetes in the world, over 69.2% (two-thirds) of people are living with diabetes without knowing (IDF, 2017). Though the rate of diabetes is relatively low in Africa compared to other regions of the world (Figure 1.1), in 2017, the number of people living with diabetes was found to be 16 million, this number is estimated to rise to 41 million by 2045 (IDF, 2017). This shows a 156% increase which is the highest rate of increase
compared to other areas of the world. In South Africa, the prevalence of diabetes increased from 5.5% to 9% within 2000 to 2009, and also showed the second highest mean health care expenditure in the African region as a whole (Manyema et al., 2015).

Figure 1.1: Estimate number of diabetes cases globally in 2017 and projected increase by 2045 (Adapted without permission from IDF Diabetes Atlas, 2017).

1.3 Etiologic categories of diabetes mellitus

Classifying diabetes is broadly based on presumed etiology (risk factors), symptoms and causes. In 1998, the WHO proposed a classification method based on the etiological factors presented, though many patients do not always fit into only a particular type of diabetes. For example, a pregnant woman diagnosed with Type 3 diabetes (gestational diabetes) may still be hyperglycemic after birth and later diagnosed with type 2 diabetes (ADA, 2016). Anyway, diabetes mellitus is mainly classified into two major classes: type 1 diabetes (T1D) or insulin dependent diabetes mellitus (IDDM) and type 2 diabetes (T2D) or non-insulin dependent diabetes mellitus (NIDDM). Gestational diabetes mellitus (GDM) is also very common which normally occurs during pregnancy.
1.3.1 Type 1 diabetes (T1D) or insulin dependent diabetes mellitus (IDDM)

Formally known as juvenile onset diabetes, this type of diabetes is a self-destroying (autoimmune) condition, whereby the body’s immune system attacks the pancreatic β-cells initiating insulin deficiency (Loghmani, 2005; Motala et al., 2003). Islet cell autoantibodies, autoantibodies to insulin and GAD (GAD65) and autoantibodies to the tyrosine phosphatases (1A-2 and 1A-2β) are identified as the markers for autoimmune destruction of the pancreatic β-cells (ADA, 2010). Most cases of T1D have exhibited presence of islet cells antibodies in the blood. These antibodies can be detected in human blood for several years before the onset of T1D. Children and babies are more prone to this disease because β-cell dysfunction occurs faster young age compared to adults. Additionally, it is also suggested that environmental factors such as toxins, diets and enteroviruses can induce the generation of T-cell dependent autoimmunity in genetically susceptible individuals leading to T1D (Devendra et al., 2004). T1D holds the lowest prevalence (about 5%) of all diabetic cases (Fonseca, 2009).

Symptoms of T1D include polydipsia (excessive thirst), polyuria (increased urination), polyphagia (unexplained hunger) and persistent hyperglycaemia. The treatment for T1D usually involves the injection of insulin before complications of ketoacidosis and coma occurs and this usually spans through a lifetime (Atkinson, 2012). Unfortunately, no treatment to date has been able to prevent the onset or progression of T1D in those at risk (Siddiqui et al., 2013).

1.3.2 Type 2 diabetes (T2D) or non-insulin dependent diabetes mellitus (NIDDM)

This category of diabetes accounts for 90-95% of all diabetic cases. It presents in individuals with sustained insulin resistance followed by moderate insulin deficiency. T2D has recently been classified as a progressive disease since the function of pancreatic β-cells reduces over time (Fonseca, 2009). Conditions like increased serum low density lipoprotein (LDL), low serum high density lipoprotein (HDL) concentrations, hypertension and increased risk of cardiovascular diseases usually follow the detection of T2D if left unmanaged (Costacou et al., 2011; Stamler et al., 1993; Van Linthout et al., 2010; von Eckardstein and Widmann, 2014). All of these diseases are defined together as metabolic syndrome (Alshehri, 2010; Ford, 2005). Diet, sedentary life style and urbanization are the main factors that lead to the progression of T2D.
1.3.3 Gestational diabetes mellitus (GDM)

This type of diabetes describes the signs of hyperglycemia experienced during pregnancy which disappear immediately after the termination of pregnancy (Bortolon et al., 2016; Qazi et al., 2016). In GDM, progesterone, chorionic somatomammotropin, stress and growth hormones induce insulin resistance which leads to maternal hyperglycemia and hyperinsulinemia in neonates (Kampmann et al., 2015). The existence of GDM during pregnancy is considered as one of the major risk factors of T2D in later life and about 90% of all pregnancies with GDM are complicated compared to non-diabetic pregnancy (Leon et al., 2016). According to the International Diabetes Federation (IDF), 16% of children born during 2013 had complications due to high blood glucose of their pregnant mothers (IDF, 2017). This number has been proposed to be increased due to the rise of obesity and sedentary lifestyle among adults (Kelley et al., 2015). The risk factors of GDM include, increased mothers’ age, visceral adiposity, polycystic ovarian syndrome, hereditary, hypertenstion and finally the delivery of larger babies.

1.4 The role of insulin in the pathogenesis of T2D

After food is ingested, carbohydrate in the food is broken down to glucose, when the insulin is responsible for carbohydrate homeostasis by inducing glucose transport to the muscle and adipose tissue for energy production and reducing the production of more glucose by the liver (Figure 1.2) (Porte, 2006). Insulin is produced in the β-cells of the pancreas and an anomaly in the secretion and action of this hormone has been implicated in the generation of diabetes (Cerf, 2013).

High levels of glucose in the blood is the first trigger of insulin production. When glucose enters the cell, it is phosphorylated by glucokinase to glucose-6-phospahtase (G6P) to release energy in the form of ATP thereby closing the K⁺-ATP channel (McTaggart et al., 2010).
When the channel is closed, it causes a depolarization and stretching of the cell membrane which leads to an influx of calcium ions. Fusion of the insulin vesicles cell membrane on the outer β-cell membrane thus causes the entry of insulin into the blood stream. Additionally, glucagon like peptide-1 (GLP-1), glucose-dependent insulintropic peptide (GIP) might be able to activate protein kinase-C and phospholipase through activation of adenyl cyclase activity and stimulation of β-cell protein kinase A which could account for the second phase synthesis of insulin (Wilcox, 2005).

1.4.1 **Insulin resistance**

This is a complex metabolic condition that occurs when the organs in the body becomes numb to the action of insulin. Prevailing insulin resistance happens when high blood glucose level does nothing to trigger insulin action. It is one of the two major reasons for the development of T2D (Taylor, 2012; Weyer et al., 2001). Normally, β-cells of the pancreas act on insulin resistance by intensifying the synthesis of insulin to act on the need of glucose by the tissues (Halban et al., 2014).

The rise in blood glucose leads to induction of insulin secretion at the same time reducing the secretion of glucagon. This shows that low levels of blood glucose lead to reduction of insulin secretion and increase in glucagon synthesis. Which indicates that these actions are all dependent on glucose and necessary to sustain glucose homeostasis (Guo, 2014). A number of conditions have been postulated as responsible for insulin resistance as discussed below.
1.4.2 Mechanisms behind insulin resistance

a. Dysfunction of pancreatic β-cell and insulin resistance

The combinatorial action of β-cell dysfunction and insulin resistance results in persistent high glucose level, particularly in type 2 diabetic condition (Lorenzo et al., 2010). The pancreatic β-cell dysfunction causes more problems than insulin resistance due to the lack of insulin for glucose metabolism (Cerf, 2013). The β-cell must be in a healthy state in order to meet the metabolic demand for the production of insulin, when decrease β-cell mass has been reported in T2D (Butler et al., 2003). However, hyperglycaemia has been attributed to the function but not the quantity of the β-cells (Ashcroft and Rorsman, 2012).

b. Impaired insulin-dependent PI3K activation

In a high blood glucose state, the skeletal muscle is the main site for disposal of glucose by insulin hence resistance to the action of insulin in muscle is a key feature of either type 1 or T2D (DeFronzo, 2015; Yki-Järvinen et al., 1990). The glucose transporter 4 (Glut 4) stimulates insulin action to effectively transport excess glucose to the skeletal muscle. There is normal occurrence of the Glut 4 gene (Figure 1.3) in the skeletal muscle of normal subjects and an impairment of glucose uptake might occur as a result of impaired Glut 4 gene expression or impaired insulin signalling pathway (Choi and Kim, 2010; Garvey et al., 1988; Kim et al., 1999,).

The signalling of insulin involves the binding of insulin to its required cell surface receptor through self-phosphorylation and induction of receptor tyrosine kinases which in turn results in tyrosine phosphorylation of insulin receptor substrate (IRS) (1-4), Gab1 and Shc (Choi and Kim, 2010).
Phosphoinositide 3-kinase (PI3K) becomes activated through the binding to the IRS which in turn phosphorylates membranous phospholipids and phosphatidylinositol 4,5-bisphosphate (PIP2). This formed complex activates Akt/protein kinase B (PKB) and serine/threonine kinases C λ and ζ (PKC λ/ζ) through the activation of 3-phosphoinositide dependent protein kinase (Figure 1.3). Movement of insulin-activated GLUT 4 from the intracellular vesicles into the plasma membrane is favoured by the activated Akt phosphorylation (Farese et al., 2005; Sale and Sale, 2008).

The insulin receptor can also be deactivated and dephosphorylated by the members of the protein tyrosine phosphatases and this can cause negative insulin action and glucose metabolism (Harley and Levens, 2003). Indeed, the PI3K pathway (Figure 1.4) is an important part of the insulin signalling cascade which is paramount to the correct action of insulin in transporting glucose to the needed organs. Therefore, an impairment in this pathway can lead to defective insulin signalling, thereby causing insulin resistance.
c. Fatty acid-induced insulin resistance

Consuming food high in dietary fat causes lipid overload and ultimately insulin resistance in organs (muscle and liver). Over time, overexposure of the skeletal muscle to high levels of saturated long chain fatty acids leads to insulin resistance, particularly in overweight and obese individuals. Abdominal obesity has also been implicated in sustained insulin resistance in T2D (Kahn and Flier, 2000; Ye, 2013). This link comes from the theory that insulin stimulates the synthesis of fatty acids and glycogen but in some cases some obese individuals have been seen not to develop persistent hyperglycemia but insulin resistance (Kahn et al., 2006). The high prevalence of T2D among youths can be linked to obesity which is seen in the recent years.

High intake of calories with little output of energy leads to increase in size of adipose tissue depots which results in obesity, availability of these depots varies from one person to another and that is why not all obese individuals are at risk of developing hyperglycemia (Lee et al., 2016). Obesity leads to an increased generation of proinflammatory cytokines, glycerol, leptin and adiponectin hormones which aid in loss of insulin sensitivity (Hardy et al., 2012). Hyperplasia in the adipose tissues induces the formation of inflammatory cytokines which might be involved in the induction of hyperglycemia and insulin resistance. On the other hand, high concentration of adipose tissue leads to increase in circulating non-esterified fatty acids (NEFAs) which in turn causes high fat storage in the muscle and
liver (Al-Goblan et al., 2014). The increase of NEFAs in the serum leads to a associated increase in insulin resistance. Interestingly, the use of antilipolytic agents leads to improved insulin function and glucose homeostasis.

The function of insulin is not only limited to blood glucose regulation, storage and optimum utility, fat metabolism is also taken care of by insulin. This occurs by the inhibition of lipolysis through increased lipogenesis and induction of fatty acid uptake which leads to an abundance of energy in the fasting state (Poloz and Stambolic, 2015).

Insulin exerts its aforementioned functions through the family of tyrosine kinase receptors and insulin growth factor-insulin receptor (IGF-IR). Insulin improves glucose homeostasis by increasing the levels of GLUT4 proteins in the plasma membrane (Furtado et al., 2002). Without the action of insulin, GLUT4 reclines within the plasma membrane and vesicular compartments of the cell. Insulin stimulates the movement of GLUT4 through exocytosis consequently reducing the endocytosis of GLUT4 (Jhun et al., 1992; Rea and James, 1997).

1.5 Complications of diabetes

1.5.1 Inflammation

In 1998, a research proposed that inflammation as a result of persistent immune system activation could aggravate instead of attenuate complications of diseases such as T2D (Pickup and Crook, 1998). Over the years some study has backed up this assertion (Cefalu, 2009; Crook, 2004). Inflammation as a result of insulin resistance and impaired glucose tolerance can be said to be the first pathological feature in complications associated with T2D (King, 2008). The exact mechanism by which inflammation can evoke T2D is still not clear (Navarro and Mora, 2005). Inflammatory markers, pro-inflammatory cytokines, tumour necrosis factor-alpha (TNF-α), interleukin-1 and interleukin-6 (IL-1 and IL-6), immune cells (monocytes chemoattractant protein-1: MCP-1) produced by the adipose tissues are linked to the accumulation of body fat (Mahmoud and Al-Ozairi, 2013; Navarro and Mora, 2005). The pro-inflammatory cytokines are associated with multiple pathways linked to insulin resistance and adipocyte function. The increase of these cytokines in the cell leads to the production of C-reactive proteins (CRP) which are acute phase proteins. Hyperglycemia and high fatty acids can also trigger inflammation by increasing glucose utilization and alterations in oxidative phosphorylation concomitantly (Zhou et al., 2010). Oxidative stress and inflammation can lead to β-cell death (apoptosis) and this will aid in loss of β-cell action and insulin sensitivity (Pollack et al., 2016).
1.5.2 Endothelial/mitochondrial dysfunction

The mitochondria provide an avenue for the generation of ATP in other words energy for the cell hence they are called the power houses of the cell. ATP is necessary for the smooth running of the cell or metabolic processes and dysfunction in the generation of ATP will lead to defects in the metabolic process. Mitochondrial malfunction has been associated with insulin resistance as well as the complications of T2D (Montgomery and Turner, 2015). This malfunction expresses itself by reduction in mitochondrial activity, decrease in mitochondrial oxidative phosphorylation processes or increase in production of reactive oxygen species (ROS) (Montgomery and Turner, 2015). Cells of patients with T2D have shown decreased mitochondrial quality and less ATP and mitochondrial RNA (mRNA) production (Leguisamo et al., 2012). Interestingly, mitochondria are the main site for the initiation of reactive oxygen species (ROS).

1.5.3 ROS formation and oxidative stress in the complication of T2D

Reactive oxygen species (ROS) are formed from the intermediates of oxygen obtained from the metabolism of the food we eat (Choe and Min, 2006). ROS refers to diverse chemicals with reactive properties which are capable of gaining or donating electrons (Tangvarasittichai, 2015). Complex I and III of the electron transport chain in the mitochondria routinely releases superoxide a form of ROS through oxidative phosphorylation (OXPHOS) (Choe and Min, 2006). At physiological pH, the superoxide gets catalysed by superoxide dismutase (SOD) to give hydrogen peroxide (H$_2$O$_2$), which is converted to water and oxygen by another anti-oxidative enzyme, catalase. The superoxide ions later lead to the production of hydroxyl radical through different steps where redox metals such as copper and iron are involved, and pancreatic beta cells are very vulnerable to hydroxyl radicals.

Some other previous studies reported that the polyol is responsible for the generation of ROS followed by the generation of advanced glycation end products (AGE) and high expression of their receptors and ligands, protein kinase C activation and increased activity of the hexosamine pathway (Figure 1.5) (Kashiwagi, 2001; Singh et al., 2014; Tang et al., 2012).
The increased production of ROS through these pathways leads to insulin resistance and β-cell dysfunction.

a. The polyol pathway

Under normal conditions in the cell, the glycolytic pathway is activated by the phosphorylation of glucose to glucose 6-phosphate by hexokinase and a small amount of the un-phosphorylated glucose enters into the polyol pathway (Tang et al., 2012). However, hyperglycemic conditions present with an increased influx of sugars through the polyol pathway of which rate limiting step is the transformation of glucose to sorbitol through the aldose reductase enzyme with reduced nicotinamide adenine dinucleotide (NADPH) as a cofactor (González et al., 1984). The increased utilization of NADPH then leads to an increased redox stress since NADPH is required to also generate GSH, a potent antioxidant. Studies have shown that increased expression of aldose reductase in diabetic mice influenced a higher risk of complications of diabetes by reducing the necessary genes which are responsible for the production of GSH (Chung et al., 2003; Lee and Chung; 1999, Li et al., 2004).
b. Increased formation of AGE

AGEs are formed when the glycation of plasma proteins and collagen occurs through increased concentration of glucose in the cell (Singh et al., 2014). This non-enzymatic alteration of plasma proteins could lead to cell damage since AGEs interact abnormally with mitochondrial matrix receptor, consequently leading to the formation of ROS (Goldin et al., 2006). Free radicals produced from glycation can cause protein fragmentation and nucleic acids and lipid peroxidation (Baynes, 1991). Hyperglycemia can increase the formation of AGEs in animal models and AGEs have also been linked to the formation of diabetic retinopathy (Kandarakis et al., 2014).

c. Activation of PKC isoforms

Another pathway actively involved in the generation of ROS in type 2 diabetic condition is the increased isoforms of activated protein kinase C (PKC). This could be as a result of the interrelationship between AGEs and their cell surface receptors (Derubertis and Craven, 1994). Dihydroxyacetone phosphate a glycolytic intermediate, is reduced to glycerol-3-phosphate which leads to the formation of diacylglycerol (DAG). High levels of DAG promote the activation of protein kinase C. In diabetic retinopathy, high blood glucose causes PKC and p38α mitogen-activated protein kinase (MAPK) induction which leads to the expression of Src homology-2 domain-containing phosphatase-1 (SHP-1) (Geraldes et al., 2009). This cascade leads to a decrease in downstream signalling resulting in cell death and increased risks of diabetic complications like atherosclerosis and heart disease.

d. Increased hexosamine pathway

Sustained hyperglycemia causes an increased influx of fructose 6-phosphate through the hexosamine pathway. Glutamine fructose 6-phosphate amidotransferase (GFAT) is the rate-limiting enzyme for the hexosamine pathway and it is powered by the fructose 6-phosphate shifted from glycolysis (Du et al., 2000). Fructose 6-phosphate is transformed to Glucosamine 6-phosphate by GFAT and then to Uridine Diphosphate N-acetylglucosamine (UDP-GlcNAc) a major end product and a substrate for alteration of target proteins. This alteration is done through post-translational modification of serine and threonine residues located on cytoplasmic and nuclear proteins (Giacco and Brownlee, 2010). It has been reported that overexpression of GFAT causes increased expression of TGF-β and TNF-α by increasing the appearance of upstream stimulatory factors 1 and 2 (USF1 and 2) (Weigert et al., 2004). The serine modification caused by UDP-GlcNAc in hyperglycemic conditions could also endothelial nitric oxide synthase (eNOS) in target cells (Du et al., 2001).
However, these proposed pathways responsible for the generation of ROS are all initiated from one source, mitochondria (Giacco and Brownlee, 2010). The mitochondria drive the overproduction of superoxide by the electron transport chain and this is the foundation of ROS production. This is not farfetched since the superoxide ion is the first oxygen free radical produced by the mitochondria and it is sporadically converted to more deleterious free radicals if left uncontrolled (Wallace, 1992). High blood glucose in diabetic cells causes increase in oxidation of pyruvate in the TCA cycle leading to the flow of NADH and FADH$_2$ (electron donors) into the ETC. This influx then leads to blockage of electron transfer in complex III due to increase in voltage gradient in the mitochondrial membrane. Superoxide is then generated by the donation of electrons by the coenzyme Q to molecular oxygen (Figure 1.6). In cultured endothelial cells, hyperglycemia has been discovered to increase the mitochondrial membrane voltage and ultimately increase the generation of ROS (Korshunov et al., 1997).

Since we have understood the different mechanisms through which ROS can be generated in complications of T2D, it is now time to highlight the different complications seen in prolonged T2D. These complications are divided into short-term and long-term complications depending on the duration of the disease before effective management.
1.6 Short-term complications of T2D

Upon immediate diagnosis of T2D, the complications of diabetes are still mild and can be reversed to normal if managed effectively. These short-term complications are:

1.6.1 Hypoglycemia

This occurs as a result of the blood sugar becoming too low (≤ 63 mg/dL or 3.5 mmol/L), which usually happens after the treatment of T2D with oral hypoglycemic drugs or insulin (Pober, 2010). The physical presentation of hypoglycemia includes sweating, fast heartbeat, anxiety, lack of feelings in fingers, toes and lips, sleepiness, confusion, headaches and babbled speech. Drinking orange juice and eating sugar can be used to manage hypoglycemia. It is usually associated with ‘dead in bed syndrome’.

1.6.2 Hyperosmolar Hyperglycaemic Nonketotic Syndrome (HHNS)

Hyperosmolar hyperglycaemic nonketotic syndrome is a very rare (< 1% of hospital admitted diabetic cases) short-term complication of T2D which occurs as a result of very high blood glucose levels (> 600 mg/dL) and glucosuria (Pasquel and Umpierrez, 2014). In HHNS, there is little or no production of ketone bodies unlike ketoacidosis. If HHNS is left unmanaged it can result in hyperosmolar nonketotic coma in old aged patients.

1.6.3 Diabetic ketoacidosis (DKA)

This occurs due to unmanaged high blood glucose and is also very rare among patients with T2D (Lin et al., 2010). Insulin deficiency is a lead cause of DKA. In the absence of insulin, the body will use energy from ketones produced by breakdown of fats. However, a high level of ketones in the body will lead to DKA which will become a complication of T2D. The outward signs of DKA include vomiting, dehydration and can even lead to coma if left untreated (Welch and Zib, 2004).

1.7 Long-term complications of T2D

These long-term complications last a lifetime in patients with T2D. Those diabetic patients who do not manage their blood glucose level effectively are at high risk of developing long-term diabetic complications (Stratton et al., 2000). These occur due to persistent hyperglycemia which results in thickening of the blood vessels. They are broadly classified into two types: damage to small blood
vessels (microvascular complications) and damage to large blood vessels (macrovascular complications).

1.7.1 Microvascular complications

a. Diabetic retinopathy (DR)

This is possibly the most prevalent microvascular complication of T2D, and in combination with diabetic muscular edema are the prime cause of blindness among working-class populace in the world (López et al., 2017). In South Africa, the prevalence of DR is between 5 to 10% and it is the fourth leading cause of blindness (Thomas et al., 2015). It occurs by the blockage of the small blood vessels of the eye by high blood glucose levels. The damage by diabetic retinopathy can be prevented by early detection and effective management. Albuminuria, obesity and duration of diabetes have been considered as risk factors for DR. Oxidative stress and growth factors have been postulated to play a major role in this regard as well. DR can be characterized by proliferative retinopathy which leads to the formation of thick blood vessels on the retina. If left untreated blindness occurs through separation of traction retina as well as haemorrhage.

b. Diabetic nephropathy

Diabetic nephropathy accounts for more than half of all cases of kidney failure (Gheith et al., 2016). The kidney is a major organ responsible for filtering and removal of waste products from the body and also for maintaining the levels of water and salt in the blood (UKPDS, 1998). In T2D, excess glucose in the blood causes a lot of stress on the kidneys which leads to organ damage. The symptoms of DN include persistent proteinuria (proteins in the urine i.e. > 500 mg in 24 hours). In DN, the kidneys experience changes such as thickness of the glomerular basement membrane, formation of micro aneurysm and Kimmelsteil-Wilson bodies (Gheith et al., 2016).

c. Diabetic neuropathy

Neuropathy is the disease of the nerve (nerve damage) and it occurs in long-term conditions of T2D (Jaiswal et al., 2017). This damage can cause loss of feeling or movement in the legs, feet, hands, arms, chest and stomach. This nerve damage can also make it difficult for a man to sustain an erection. The most common manifestation of diabetic neuropathy in T2D is chronic sensorimotor distal symmetric polyneuropathy (Tesfaye et al., 2013). In this case, patients notice numbness with burning and tingling pain. In the feet this can cause sores and if left untreated can lead to amputation of the feet. A high number of amputations have been done so far as a result of T2D (Rodrigues et al., 2016).
1.7.2 Macrovascular complications

These types of complications affect the large blood vessels and they are:

a. Atherosclerosis

Atherosclerosis occurs from inflammation and damage to the arterial wall in the peripheral or coronary vascular system (Pithová et al., 2016). In response to this, oxidized lipids from low density lipoproteins (LDL) gather in the endothelial wall of the arteries. These accumulated lipids form foam cells through the infiltration of the arterial wall by monocytes. Foam cells stimulate the proliferation of macrophage and T-lymphocyte attraction. Collagen accumulation and proliferation of smooth muscle in the arterial wall is induced by the T-lymphocyte, the result of all these processes is the production of a lipid-rich atherosclerotic region which then leads to complications such as atherosclerosis in T2D (Kalofoutis et al., 2007).

b. Cardiovascular disease (CVD)

CVD is the main cause of death of patients with T2D, accounting for the largest part of health care expenditure in people living with T2D (Ahmed et al., 2010). Obesity is associated with an increased risk of CVD in T2D patients. As explained earlier, a possible link between obesity and the complications of diabetes could be low grade inflammation. Patients with diabetes have greater cardiac mass compared to those without diabetes, which might be as a result of raised release of adipocytes (resistin and leptin) leading to hypertrophic effects on the heart cells (Barouch et al., 2003; Leon and Maddox, 2015).

Africa is suffering from the burden of diabetes due to huge cost and expenditure spent of treating and managing diabetes (Mutyambizi et al., 2018). Thus, the management and control of T2D is of paramount importance to the population at large.

1.8 Management of T2D

Different measures and modalities have been proposed to manage diabetes. These measures aim to control blood glucose levels to tolerable points.

1.8.1 Exercise and physical activity

Exercise is the first advice a physician will give to a diabetic patient. Staying fit is a good way to keep blood glucose level at tolerable levels. During exercise the muscle cells becomes more sensitive to
insulin, and the contraction of the muscles leads to increase uptake of glucose even in the absence of insulin (Riddell and Perkins, 2009). Exercise helps T2D patients avoid the long-term complications of T2D.

1.8.2 Dietary modifications

The power of food over diabetes is amazing; a healthy diet can help normalize blood sugar levels, weight and blood pressure and reduce risks of complications of T2D. Foods such as white flour, table sugar and fruit juice are termed high-glycemic foods as they stimulate increase in blood sugar (Inzucchi et al., 2015). Low glycemic foods such as whole grains, nuts and legumes are high in fibre which helps control appetite (Chiu and Taylor, 2011). Counting carbs and eating a balanced diet are effective ways of reducing blood sugar levels to normoglycemic level.

1.8.3 Pharmacologic management of T2D

Therapeutic drugs are usually employed with the action of exercise and diet to help control high blood sugar levels. The need for better treatment modalities of T2D has led to the discovery and manufacture of different types of anti-diabetic drugs. These drugs target what is known as the ‘ominous octet’ that causes T2D. This section will shed more light on the mechanism of action of anti-diabetic drugs and possible side effects.

a. Insulin secretagogues

Impaired insulin secretion in T2D can be tackled using insulin secretagogues. These drugs trigger the release of insulin by blocking the KATP channel in the pancreatic β-cell which leads to depolarization of the membrane, increase in intracellular Ca\(^{2+}\) and insulin granule exocytosis (Cobb and Dukes, 1998). Examples of this class of drugs includes sulfonylureas which bind to plasma proteins and cannot be used by type 1 diabetic patients or pregnant women. Another class of secretagogue is non-sulfonylurea secretagogue, an example of which is meglitinides (also known as short acting insulin secretagogue). They act on the same channels as sulfonylureas but on different binding sites. The side effects of insulin secretagogues include hypoglycemia and weight gain.

b. GLP-1 receptor agonists

Glucagon like polypeptide-1 (GLP-1), DPP-4 (dipeptidyl peptidase-4) inhibitors and amylin agonist pramlintide are the classes of drugs available for addressing abnormalities in glucagon secretion (Godoy-Matos, 2014). The currently used GLP-1 agonists used are exenatide and liraglutide which
help improve weight loss and metabolic dysfunction. Their adverse effects include gastrointestinal disturbances, nausea and mild hypoglycemia.

c. **Drugs that increase glucose uptake**

Biguanides such as metformin help to reduce hyperglycemia by inhibiting hepatic glucose output and increasing the uptake of glucose by the muscle. Amongst the common antidiabetic drugs, metformin remains the only drug that does not encourage weight gain and it is used as a first line treatment in managing T2D. Thiazolidinediones also improve better use of glucose in the cells by binding to PPARγ (a nuclear protein responsible for transcription of genes involved in glucose and fat utilization) (Jerry and Chisholm, 2004). Insulin injections are also prescribed for the better utilization of glucose in the cells. Apart from cost, the side effects of these classes of drugs include hypoglycemia, and pain associated with needle prick (in the case of insulin injections).

d. **Incretin therapeutics**

Enhancing insulin action in response to oral glucose overload compared to intravenous glucose level is known as the ‘incretin effect’. Incretin is a hormone that has been proposed to be responsible for satiety (i.e. not feeling hungry). The GLP-1 and glucose-dependent insulinotropic polypeptide (GIP) are the peptide hormones in charge of the incretin effect (Pratley, 2010, Cernea, 2011). They act by stimulating the release of insulin from pancreatic β-cells, suppressing glucagon secretion, reducing gastric emptying and regulating appetite (Pratley, 2010). These incretin memetics are widely used in addition to metformin to aid efficient glycemic control and their side effects include nausea and weight loss.

e. **Alpha glucosidase inhibitors**

Alpha glucosidase inhibitors such as acarbose act on hyperglycemia by inhibiting the digestion of carbohydrates in food to glucose by inhibiting carbohydrate digesting enzymes such as alpha-amylase and alpha-glucosidase (McCulloch, 2007). The limitations associated with the use of this drug includes cost, gastrointestinal problems, less effective in reducing the amount of glycated hemoglobin in the blood. Most commonly used alpha-glucosidase inhibitors are acarbose, miglitol etc.

f. **Sodium glucose transporters**

These transporters are located in the kidney and they help to tackle the reabsorption of glucose by the kidney (Kalra, 2013). SGL2 inhibitors such as dapaglifoxin, empaglifoxin and canaglifoxin aid in reducing reabsorption of glucose though this causes glucosuria it reduces hyperglycemia in T2D
patients. The mechanism of action of these drugs highlight their side effects (ketoacidosis, urinary tract infections and hypoglycemia).

g. Drugs that improve neurotransmitter dysfunction

Dopamine is a neurotransmitter in the brain which has been shown to be affected by hyperglycemia. The administration of bromocriptine (a systemic and intracerebral dopamine agonist) to insulin resistant animals caused a reduction in increased ventromedial hypothalamus noradrenergic and serotonergic levels through a reduction in gluconeogenesis, adipose tissue lipolysis and improved insulin sensitivity (DeFronzo, 2011).

Considering the numerous side effects of the different classes of conventional antidiabetic drugs, there is a need to develop alternative therapies for the treatment of T2D, particularly from natural products, which are widely available, low cost and with no or less side effects.

1.8.4 The importance of medicinal plants in drug discovery

Plants have since been successfully used in the treatment of diseases such as diabetes. Over 5000 years ago, Sumerian clay tablets were engraved with herbal prescriptions for diseases (Petrovska, 2012). Traditional healers have found the use of dietary modifications with medicinal plants and minerals to be an effective approach for the management of diseases such as T2D, cancer and others (Farag et al., 2016). In 2600 BC, in the area of Mesopotamia, traditional approach has been employed in the treatment and cure of diseases (Choudhury et al., 2017). The use of medicinal plants is even more common in different low-income areas of Africa. Some patients prefer the use of traditional medicine over synthetic drugs because of the cost, numerous side effects and frequent unavailability of synthetic drugs. The WHO has encouraged African countries to embrace the practice of traditional medicine since Africa is filled with an abundance of different varieties of medicinal plants (Mahomoodally, 2013).

Interestingly, the use of traditional medicine is on the rise even in developed countries like America, Australia, Netherlands and France. Factors responsible for this could be linked to curiosity, prevention of sickness, inability to get well with conventional drugs, and believing that a combination of herbs and conventional drugs will bring about faster healing (van Andel and Carvalheiro, 2013).

In this present era, the use of medicinal plants still prevails as 11% of 252 basic prescription drugs are derived from plant sources (Veeresham, 2012), making plants effective in drug delivery and production. The availability of compounds of natural origin in different stages of clinical development
emphasizes the value of natural products as basis for novel drugs. Apart from natural products in direct use as drugs, some products can be used to model and manufacture new drugs for treating diverse diseases.

A recent study showed that about 50% of cancer drugs approved within 30 years (1981 to 2010) are from plant sources either directly or indirectly (Newman and Cragg, 2012). The world’s first anti-malarial drug quinine was derived from the bark of the native cinchona tree (Achan et al., 2011). The drug aspirin used in the treatment of fever and headaches was discovered from Salix alba known as the willow plant (Mahdi, 2010). Quinine, morphine, digoxin, ergometrine and reserpine are examples of popular orthodox drugs with plant sources (Ulrich-Merzenich, 2014). Metformin, a widely used anti-diabetic drug originates from the plant Galega officinalis commonly known as French lilac (Bailey and Day, 2004). The potential of drugs from natural origin has generated the motive to further identify and investigate novel phytochemicals from plant sources with the sole aim of drug discovery for the management of diseases.

1.9 African medicinal plants in the treatment of T2D

Africa is blessed with a rich diversity of medicinal plants, and this marks our socio-economic and cultural heritage. Africa contains over 45,000 unexploited plant species. In East and Central African region, the use of plants is widespread in the treatment of diseases and the common plants include Azadirachta indica, Morinda citrifolia, Zanthoxylum zanthoxyloides and different species of Cactus (EL-Kamali, 2009).

Research carried out in 2013 explained the medicinal value and distribution of 10 commonly used African medicinal plants. These plants include Momordica charantia, Aloe ferox, Catharanthus roseus, Acacia Senegal, Cyclopa genistoides, Pelargonium sidoides, Artemisia herba-alba, Harpagophytum procumbens, Centella asiatica and Aspalathus linearis. The plants were selected because they have their origin from African roots and are widely used in the management and development of drugs for curing diseases (Mahomoodally, 2013).

Over 400 plants and compounds have been discovered to show antidiabetic activities both in vitro and ex vivo and research is still on going in this regard (Chang et al., 2013). Cinnamon (Cinnamomum verum and C. zeylanicum) commonly used as spice and flavorings in food is traditionally being used in treatments of diarrhea, colds and headaches (Khan et al., 2003). Studies have shown the efficacy of cinnamon as an antidiabetic agent. The blood glucose reducing ability of this plant was reported
and was achieved through lowering insulin resistance and increasing liver glycogenesis (Rao and Gan, 2014, Ulbricht et al., 2011).

1.10 Justification of the research

Discussions above show the importance of medicinal plants in the treatment of different diseases, especially T2D. With the staggering need for the discovery of alternate therapies due to the deficiencies found in synthetic drugs, there is need to conduct more research with plants with the hope of drug discovery, since these plants are widely available.

1.11 Scope and limitations of the study

The focus of this study was to screen some African medicinal plants with a view to identify the anti-diabetic potential of the plant parts.

This study extensively covered the antioxidant and anti-diabetic screening of Alstonia boonei, Bridelia ferruginea and Crassocephalens rubens using various in vitro, ex vivo, in silico and ultimately in vivo experiments. Some bioactive components were also identified from the above-mentioned plants and also from Boerhaavia diffusa and Acalypha wilkesiana using GC-MS and LC-MS analysis.

This study did not investigate the in vivo antidiabetic potential of Boerhaavia diffusa and Acalypha wilkesiana due to unavailability of plant samples. It also did not exhaustively screen the bioactive components from all the plants using HPLC, or HPLC coupled with nuclear magnetic resonance (LC/NMR) techniques.

1.12 Aims and objectives

This study aims to conduct a detailed investigation on the antioxidant, anti-diabetic and anti-obesogenic potentials with possible toxicological effects of five African medicinal plants namely; Alstonia boonei, Acalypha wilkesiana, Bridelia ferruginea, Boerhaavia diffusa and Crassocephalens rubens employing different in vitro, ex vivo and in vivo models with the identification of the possible bioactive anti-T2D agents as well as detecting their mechanism of action.

The specific objectives of this research are:

1. To assess the in vitro and ex vivo antioxidant activities of the aqueous, ethanol and ethyl acetate crude extracts of various parts (stem barks, roots and leaves) of the plants.
2. To examine the abilities of the extracts of these plant parts to inhibit key carbohydrate (α-glucosidase and α-amylase) and lipid (lipase) digesting enzymes in vitro.

3. Based on the above results, the best extract from the plant parts was subjected to solvent based fractionation to yield the fractions. The obtained fractions were further examined for their antidiabetic potentials via using in vitro and ex vivo experimental models.

4. The best fractions from the plants were subjected to study of the in vivo anti-diabetic abilities using a T2D model. The molecular mechanism behind insulin-mediated muscle glucose uptake potential of the most effective plant was also investigated.

5. Possible bioactive agents in the extracts and fractions were identified from each plant using Gas chromatography-mass spectrometry (GC-MS).

1.13 Thesis overview
This thesis is organized into 8 chapters. The general structure of the thesis and the focal point of each chapter is described as follows:

CHAPTER 1: Introduction
A general background of the entire study is given in this chapter. It explains the problem (type 2 diabetes), the prevalence, pathological features, and different treatment modalities of this disease. This chapter also pinpoints the rationale, objectives of the study and structure of the thesis.

CHAPTER 2: Materials and methods
This chapter provides a detailed explanation of all the materials and methods used during the entire study.

CHAPTER 3: Alstonia boonei
This chapter provides a compact background, ethnopharmacological and biological importance of A. boonei. The chapter outlines the in vitro, ex vivo, in silico and in vivo antioxidant and anti-diabetic screening of the different parts of this plant with their extracts or fractions. It also explains the identified bioactive constituents present in the plant extract/fraction using different solvents.

CHAPTER 4: Acalypha wilkesiana
This chapter explains the in vitro anti-oxidant activity of the plant. The effect of extracts from the plant parts on ameliorating iron-induced oxidative pancreatic injury was also investigated ex vivo. This chapter also went further to identify the present bioactive components in the plant parts using GC-MS analysis.
CHAPTER 5: *Crassocephalum rubens*

The *in vitro, ex vivo and in silico* antioxidant and anti-diabetic screening of *C. rubens* was explained in this chapter. The chapter also went further to explain the effect of the aqueous the plant on type 2 diabetes using a potent animal model of type 2 diabetes. The identified phytochemicals mainly phenols, might render synergistic effects to the observable promising anti-diabetic activity.

CHAPTER 6: *Bridelia ferruginea*

This chapter reports the detailed *in vitro* antioxidant and anti-diabetic of the leaves, stem and rootbark extracts of *B. ferruginea*. The effect of the most active fraction on inhibition of glucose absorption in small intestinal tissues were examined *ex vivo* together with the induction of muscle glucose uptake by the muscle tissue. The most active fraction was then used for *in vivo* animal study where the fraction was able to reduce blood sugar levels. The identified bioactive constituents from the extracts and fractions might be responsible for the promising antioxidant and anti-diabetic activity of this plant.

CHAPTER 7: *Boerhavia diffusa*

This chapter reports the *in vitro, ex vivo and in silico* antioxidant and anti-diabetic activity of solvent extracts from the aerial parts of *B. diffusa*.

CHAPTER 8: General conclusions and recommendation

This chapter presents a general summary based on the results of the previous chapters. It also provides suggestions and recommendations for future research. This thesis represents a compilation of manuscripts where each chapter is an individual entity and therefore some repetitions between chapters are unavoidable. The last part of the thesis contains a detailed bibliography of all the cited works in the thesis.
CHAPTER 2
MATERIALS AND METHODS

2.1 Experimental design

Collection of leaves, stem and root bark of *Aistonia boonei*, *Acalypha wilkesiana* and *Bridelia ferruginea* and aerial parts and whole plant of *Boerhavia diffusa*, and *Crassocephalum rubens* respectively

Sequential extraction to yield

- Ethyl acetate, ethanol and aqueous extracts

SubJECTED TO ANTIOXIDANT assays both *in vitro* and *ex vivo*

- *In vitro*: Estimation of total phenol content, total reducing power assay, DPPH radical scavenging assay, hydroxyl radical scavenging assay and nitric oxide scavenging assay
  - Estimation of α-glucosidase, α-amylase and lipase inhibitory activities
- *Ex vivo*: Oxidative injury ameliorative abilities by estimation of catalase, SOD activities and reduced GSH and lipid peroxidation levels in pancreatic and hepatic tissues.

GC-MS/ LC-MS analysis

Fractionation

Measurement of intestinal glucose absorption and muscle glucose uptake using fractions and extracts

*In vivo* anti-diabetic studies using 3 most active fractions/extracts in a type 2 diabetes model of rats

Figure 2.1: Flowchart showing the entire experimental approach
2.1.1 Chemicals and reagents

Absolute ethanol, ascorbic acid, calcium chloride di-hydrate, chloroform, dinitrosalicylic acid (DNS), disodium EDTA, ethyl acetate, ferric chloride, ferrous sulphate heptahydrate, Folin ciocalteau reagent, glucose-6-phosphate dehydrogenase, D-glucose 6-phosphate sodium salt, Griess reagent, hydrogen peroxide, isopropanol, maltose, magnesium chloride, magnesium sulphate, mono-basic potassium phosphate, phenol red, sodium chloride, sodium bicarbonate, sodium citrate, sodium hydroxide, sodium hydrogen carbonate, sodium phosphate, sodium nitroprusside, starch, sulphuric acid, trichloroacetic acid, reduced glutathione, thiobarbituric acid, deuterated chloroform and deuterated methanol were purchased from Merck Chemical Company, Durban, South Africa.

Acarbose, ammonium molybdate tetrahydrate, citric acid, 2 deoxy-D-ribose, di-basic sodium phosphate, diethylenetriaminepentaacetic acid (DETAPAC), 5,5′-dithiobisnitrobenzoic acid (DTNB), 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), gallic acid, 6-hydroxy-2,5,7,8-tetramethylethylchroman-2-carboxylic acid (trolox), 6-hydroxydopamine, p-nitrophenyl-α-D-glucopyranoside (pNPG), 3-(N-morpholino) propanesulfonic acid (MOPS)p-nitrophenol, porcine pancreatic amylase, potassium ferricyanide, sodium acetate, streptozotocin and yeast α-glucosidase, were obtained from Sigma-Aldrich through Capital Lab Supplies, New Germany, South Africa. Glucose and fructose were bought from Associated Chemical Enterprise (ACE), South Africa, while metformin and Novo rapid insulin were bought from a local pharmacy (Pharmed) in Durban, South Africa.

A specific ultra-sensitive rat insulin ELISA kit was procured from Mercodia AB, Uppsala, Sweden. Other reagents used for investigating the remaining serum parameters were purchased from Labtest Diagnostics (Lagoa Santa, Brazil) purchased through Replamed Company Ltd., Centurion, located in South Africa.

2.1.2 Equipment

Buchi Rotavapor II, Buchi, Germany, Shimadzu UV mini 1240 spectrophotometer (Shimadzu Corporation, Kyoto, Japan), Steri-Cult CO₂ incubator (Labotec, South Africa), An Automated Chemistry Analyzer (Labmax Plenno, Labtest Inc., Lagoa Santa, Brazil), Glucometer (Glucoplus Inc., Saint-Laurent, Quebec, Canada), Ultra Turrax Tube Drive Work Station Homogenizer (IKA-Works, Staufenim Breisgau, Germany), Hettich Mikro 200 Microcentrifuge (Hettich Lab Technology, Tuttlingen, Germany), Synergy HTX Multi-mode Reader (BioTek Instruments Inc,
Winooski, USA), Microfuge 20R Centrifuge (Beckman Coulter, Inc., Germany), Eppendorf® 5810R Centrifuge (Fisher Scientific, UK).

2.1.3 Collection and preparation of plant materials

The leaf, stem and root bark samples of *Alstonia boonei*, *Acalypha wilkesiana*, *Bridelia ferruginea*, aerial parts of *Boerhavia diffusa*, and *Crassocephalum rubens* were collected in November 2015 from the city of Ibadan, Oyo state, Nigeria. The plant materials of *A. boonei*, *B. ferruginea* and *B. diffusa* were validated at the herbarium unit of the Department of Pharmacognosy, University of Ibadan, Nigeria where their designated voucher specimen numbers DPHI Nos. 1379, 1380 and 1650 were deposited respectively. The authenticity of *C. rubens* and *A. wilkesiana* plant samples were verified at the herbarium section of the Forest Research Institute of Nigeria (FRIN), where a voucher specimen number FHI. 110605 and FHI. 110606 was preserved, respectively.

Dirt and sand stains were promptly removed from the plant materials followed by shade drying for 3-4 weeks till they reached constant weights. When dried, the samples were then ground to powdery form with a mill blender and packaged as a unit in air-tight bags for transportation to the University of KwaZulu-Natal, Durban, South Africa for further analysis.

2.1.4 Preparation of the crude extracts

Individually, 100 g of the samples from each part of plant was defatted with hexane. Defatted samples were soaked with 200 mL of ethyl acetate, ethanol and water for 48 hours respectively followed by consistent shaking for 2 h at 200 rpm. The samples were then sieved using a Whatmann filter paper (No. 1) and the filtrates were dried in vacuum using a rotary evaporator at 40°C with reduced pressure to acquire the crude extracts. The aqueous extracts were dried at 50°C overnight by using a water bath (Figure 2.2). The weights of the resulting extracts in all cases were determined, then they were preserved in glass vials and stored at 4°C until further analysis.
2.2 Analytical methods

2.2.1 *In vitro* antioxidant activities of the isolated crude extracts

i. Evaluation of total phenol content (TPC)

The estimation of TPC (as equivalent of gallic acid) of the extracts were determined using a previously described method (McDonald *et al.*, 2001) with little modifications. Concisely, a volume of 200 μL of the extract (at a concentration of 240 μg/mL) was mixed with 1000 μL of Folin ciocalteau reagent (diluted 10 times) and 800 μL of 0.7 M Na₂CO₃ and incubated at 25°C for 30 min. A Shimadzu UV mini 1240 spectrophotometer set at 765 nm was used to determine their absorbance. All measurements were reproduced 3 times.

ii. Determination of Ferric (Fe³⁺) reducing antioxidant power (FRAP)

The reducing capacity of these extracts was determined by potassium ferricyanide method. Briefly, 50 μL of extract in increasing concentrations (50-250 μg/mL) was incubated with a 50 μL of 0.2 M sodium phosphate buffer (pH 6.6) with 50 μL of 1% potassium ferricyanide for 30 min at 50°C. Acidification of the reaction was achieved using 50 μL of 10% TCA. Then 50 μL of the acidified mixture was added to 50 μL of distilled water (d.H₂O) and 40 μL of FeCl₃(0.1%) and its absorbance was recorded at 700 nm in a 96 well plate reader in triplicates. High reducing activity of the extracts
is seen in increasing absorbance (Oyaizu, 1986). The result was reported as GAE using this expression:

\[
\text{Ferric reducing antioxidant power (\%)} = \frac{\text{Absorbance of Sample}}{\text{Absorbance of Gallic acid}} \times 100
\]

iii. **Free radical scavenging activity using DPPH**

The antioxidant capacity of the extracts was investigated based on their capacity to mop up the non-transient 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical (Ak and Gülçin, 2008). The standards used were ascorbic acid, trolox and gallic acid and absorbance was read at 517 nm. Briefly, 100 µL of 0.3 mM DPPH in methanol was added to 200 µL of extracts (50-250 µg/mL), this was then mixed and incubated in pitch-dark conditions at room temperature for 30 min. Thereafter, the absorbance was recorded at 517 nm in triplicates, and the result was extracted as a proportion of the blank using this expression:

\[
\% \text{ Inhibition} = \frac{\text{Absorbance of Control} - \text{Absorbance of Sample}}{\text{Absorbance of Control}} \times 100
\]

iv. **Non-site-specific OH radical mediated 2-deoxy-D-ribose degradation assay**

The Fenton reaction made up of Ascorbate–EDTA and H\(_2\)O\(_2\) generate hydroxyl (\(\cdot\)OH) radicals which could degrade deoxyribose and it is measured as a product degradation reaction. The \(\cdot\)OH radical inhibiting ability of the extracts were determined by investigating the tussle for the released hydroxyl radicals within the extracts and deoxyribose as reported by Hinneburg et al. (2006). The experiment was done by adding 200 µL of already mixed 100 µM FeCl\(_3\) and 100 µM EDTA (1:1 v/v) solution, 100 µL of 10 mM H\(_2\)O\(_2\), 360 µL of 10 mM 2-deoxy-D-ribose, 1 mL of varying concentrations of the extract (50–250 µg/mL), 400 µL of phosphate buffer (50 mM, pH 7.4) and 100 µL of 1 mM ascorbic acid in progression. The resulting mixture was quickly incubated for 2 hours at 50°C. Then, 1 mL of TCA (2.8%) and 1 mL of 1.0% thiobarbituric acid (in 25 mM NaOH) were added to the reaction tubes. Further incubation in a H\(_2\)O bath at 50°C for 30 min was required for the samples to develop a pink colour. The absorbance at 532 nm was used to determine the level of oxidation of the samples and the \(\cdot\)OH radical scavenging capacity of the extract was recorded as percent inhibition of deoxyribose using this expression:

\[
\text{Inhibition (\%)} = 1 - \left(\frac{\text{Absorbance of sample}}{\text{Absorbance of control}}\right) \times 100
\]
v. Nitric oxide (NO) radical inhibition assay

The ability of aqueous solution of sodium nitroprusside at physiologic pH to aggressively emit nitric oxide (NO) that might interact with oxygen to generate nitrite ions forms the basis of this assay (Kurian et al., 2010). These nitrite ions could be determined using Griess reagent. The experiment was done by briefly incubating 500 μL of 10 mM sodium nitroprusside in sodium phosphate buffer (pH 7.4) and 500 μL of the extract at different concentrations (50-250 μg/mL) for 2 h at 37°C. A 500 μL of Griess reagent was then added to the reaction mixture. A chromophore produced by the coupling of nitrite with sulphanilamide was determined at 546 nm. The percentage inhibition of the NO emitted was calculated by comparison with the absorbance of a prepared control (10 mM sodium nitroprusside in phosphate buffer). The assay was done three times and the scavenging capacities of the plant was estimated using the expression below:

\[
\text{Scavenging activity (\%) } = 1 - \left( \frac{\text{Absorbance of sample}}{\text{Absorbance of control}} \right) \times 100
\]

2.2.2 In vitro key anti-diabetic and anti-obesogenic enzymes inhibitory assays

a) Inhibitory of α-glucosidase activity

The α-glucosidase inhibitory potential of the extracts was determined based on a slightly modified version of a previously published method (Ademiluyi and Oboh, 2013). Briefly, a 250 μL of acarbose (standard) or extract at varying concentrations (50-250 μg/mL) was incubated with 500 μL of α-glucosidase (1.0 U/mL) in phosphate buffer (with a concentration of 100 mM, pH 6.8) for 15 min at 37°C. Then, a 250 μL of 5 mM \(p\)-Nitrophenyl-\(α\)-D-glucopyranoside (\(p\)NPG) solution mixed in the phosphate buffer was added to the mixture and further incubated for 20 min at 37°C. The substrate was \(p\)NPG and the absorbance of the released \(p\)-nitrophenol was noted at 405 nm. The inhibitory activity was reported as a proportion of the control devoid of inhibitors using this expression:

\[
\text{Inhibition (\%) } = 1 - \left( \frac{\text{Absorbance of sample}}{\text{Absorbance of control}} \right) \times 100
\]

b) α-amylase inhibitory activity

The α-amylase inhibitory potential of the extracts were investigated using a previously reported procedure (Shai et al., 2010) with moderate modifications. Briefly, 250 μL of each extract or standard (acarbose) at varying doses (50-250 μg/mL) was incubated with a 500 μL of porcine pancreatic amylase (2 U/mL) in phosphate buffer (100 mM, pH 6.8) for 20 min at 37°C. Then, a 250 μL of starch
was suspended in 100 mM phosphate buffer (pH 6.8) and was subsequently added to the reaction and incubated for 1 h at 37°C. A dinitrosalicylic acid (DNS) colour reagent (1 mL) was thereafter added and boiling was initiated for 10 min. The absorbance of the resulting mixture was recorded at 540 nm and the inhibitory activity was expressed as proportion of a control lacking inhibitors.

\[
Inhibition (%) = 1 - \left( \frac{\text{Absorbance of sample}}{\text{Absorbance of control}} \right) \times 100
\]

c) Estimation of pancreatic lipase inhibitory activity

Pancreatic lipase inhibitory potential of plant extracts was determined using a slightly modified procedure from its originally published form (Kim et al., 2010), here, the substrate used was p-NPB (p-nitrophenyl butyrate) in dimethyl formamide. The lipase inhibitory activity was extrapolated by recording the hydrolysis of p-NPB to p-nitrophenol by measuring absorbance at a wavelength of 405 nm. The inhibition rate of lipase by the plant extract was calculated using the following expression:

\[
\% \text{ Inhibition} = \left( 1 - \frac{A_s}{A_c} \right) \times 100
\]

As: absorbance in the presence of the sample and Ac: absorbance of the control.

2.2.3 Ex vivo antioxidant assays

A 100 µL of pancreatic and hepatic tissue homogenates from rats was pipetted into a reaction mixture containing 100 µL of each extract with 30 µL of pro-oxidant (0.1 mM FeSO\(_4\)). This was then incubated in 5% CO\(_2\) for 30 min, a reaction containing no extract served as negative control i.e. untreated.

This sample was then immediately assayed for Catalase (Chance and Maehly, 1955), Superoxide Dismutase (SOD) (Kakkar et al., 1984), reduced glutathione (GSH) level (Ellman, 1959), and lipid peroxidation (LPO) level (Chowdhury and Soulsby, 2002). The Folin–phenol method was used in estimating the total protein content of the tissue homogenates (Lowry et al., 1951).

i. Catalase activity

The catalase activity was estimated by a spectrophotometric molybdate procedure (Hadwan and Abed, 2016). Concisely, 100 µL of the incubated homogenate was added to 1 mL of H\(_2\)O\(_2\) (65 µM) in sodium phosphate buffer (6.0 mM, pH 7.4) for 3 min. The reaction was ended by adding 4 mL of ammonium molybdate (32.4 mM) and the alterations in the yellow-coloured molybdate/H\(_2\)O\(_2\)
complex was recorded at a wavelength of 347 nm compared to the blank containing H$_2$O$_2$ alone. The catalase activity was estimated using this expression:

$$\text{CAT(kU)} = \frac{2.303}{t} \times \left[ \log \frac{S^0}{S-M} \right] \times \frac{V_t}{V_s}$$

$t = $ time; $S^0 = $ Abs. of standard tube (containing all reagents except tissue sample); $S = $ Abs. of test samples; $M = $ Abs. of control test sample (contains all reagents except H$_2$O$_2$); $V_t = $ summation of volume of total reagents in test tube; $V_s = $ test sample volume; Abs. = Absorbance at 340 nm

ii. Superoxide dismutase (SOD) enzyme activity

This assay was performed according the procedure of Kakkar et al. (1984) with slight modifications. Concisely, 170 μL of a 0.1 mM diethylenetriaminepentaacetic acid (DETAPAC) and 15 μL of the incubated homogenate were plated in a 96-well plate. Immediately, 15 μL of a 1.6 mM 6-hydroxydopamine (6-HD) was put in the mixture followed by gentle mixing by carefully patting the sides of the 96-well plates. Absorbance was measured at 492 nm for 3 minutes at 1 min interval. The activity of the present SOD enzyme was determined by exploiting this formula:

$$\text{Activity} = \frac{(A1 - Ab) \times Rv \times Df}{\varepsilon_{490} \times Sv}$$

$\varepsilon_{490}$ is the standard molar absorptivity at 490 nm wavelength given as 1.742/mM/cm, A1 and Ab are rates of reaction rate for sample and blank. $Rv$ is the volume of the entire reaction, $Df$ is the used dilution factor and $Sv$ is the volume of the sample.

iii. Reduced glutathione (GSH) assay

This assay relies on the rapid oxidation of GSH by 5,5'-dithio-bis (2-nitrobenzoic acid) to generate 5'-thio-2-nitrobenzoic acid (TNB) a yellow formation measurable at 415 nm. The formed oxidised glutathione (GSSG) can be converted to GSH by glutathione reductase in a reaction containing the cofactor NADPH (Rahman et al., 2006). Briefly, 150 µL of incubated sample was precipitated with equal volume of TCA (10%) and then centrifuged at 2000 rpm for 10 min at 25°C. A 80 µL of the supernatant, with 40 µL of 0.5 mM DTNB and a 200 µL of sodium phosphate buffer (0.2 M, pH 7.8) was plated in a 96-well plate. The mixture was then incubated at 25°C for 15 min and its absorbance measured at 415 nm. A standard curve was plotted using varying GSH concentrations as standard and the GSH concentration of each sample was extrapolated from the standard curve.
iv. **Determination of lipid peroxidation**

This was carried out using a slightly modified previously reported method (Oboh et al., 2012). Lipid peroxidation was initiated by incubating 30 μL of ferrous sulphate (15mM) with 100 μL of varying concentration of the extract (50-250 μg/mL) in water and the pancreatic homogenate for 30 min at 37°C. Then, 200 μL of SDS (8.1%) solution, 750 μL of acetic acid (20%), 2 ml of TBA (0.25%) solution and 850 μL of miliQ H₂O and further boiled with the 200 μL of the incubated samples for one hour. After cooling, the absorbance was recorded at 532nm. The MDA concentrations of the tested samples were extrapolated from the MDA standard curve.

v. **Determination of glutathione reductase activity**

This assay relies on measuring the glutathione reductase activity spectrophotometrically by recording reduction in absorbance of the NADPH at 340 nm (Smith et al., 1988). Briefly, 10 µL of sample or glutathione reductase buffer (at a concentration of 50 mM Tris.HCl and 1 mM EDTA solution, pH 8.0) for blank was mixed in a 96-well plate and a 221 µL of the assay buffer with 38 µL of GSSG solution (8 mM) was added consecutively. Thereafter, 10 µL of NADPH was put in the reaction followed by immediate mixing by gently patting the four sides of the 96-well plate. The linear decrease in absorbance at 340 nm was quickly noted for 8 min at a 2 min interval using a multi-plate reader. The activity of the glutathione reductase enzyme was calculated using this expression:

\[
\text{Activity} = 1000 \times \frac{A1 - Ab}{\varepsilon_{340}} \times 0.5 \text{ μmol/min/μg of protein}
\]

Where: \(\varepsilon_{340}\) is the molar absorptivity at 340 nm which is 6.22 mM/cm, A1 and Ab represents rate of the sample and blank reactions.

vi. **Estimation of glutathione peroxidase activity**

This reaction is based on the oxidation of GSH to GSSG through the glutathione peroxidase enzyme in the presence of \(\text{H}_2\text{O}_2\):an antioxidant. Briefly, sample homogenates and serum samples were diluted to a concentration range of 5-10 mg/mL (based on their protein concentration) using the assay buffer. A 5 µL of the diluted sample or homogenization buffer (blank) was platted in a UV disposable 96-well plate, then a 210 µL of assay buffer, 2.5 µL of GSH solution (100 mM), 2.5 µL of glutathione reductase (0.1 U/mL), 2.5 µL of sodium azide solution (100 mM) (added to diluted hepatic homogenates to inhibit catalase activity) or 5 µL of distilled H₂O (to other samples or blank). Thereafter, 2.5 µL of NADPH (15 mM) was added and the t-BHP-independent NADPH oxidation was recorded at 340 nm (reaction 1) for 8 min at 2-min intervals for both the sample (A1) and blank
(A1b) using a multi-plate reader. The reaction was initiated by adding 25 μL of either 12 mM t-BHP or 1.5 mM H₂O₂ solution (for liver sample homogenates) and the hydroperoxide-dependent linear NADPH oxidation at absorbance of 340 nm (reaction 2) was recorded for 6 min at 2 min intervals for both the samples (A1) and the blank (A2) using a multi-plate reader.

The following expression was used to calculate the activity of glutathione peroxidase enzyme:

\[
\text{Activity} = 1000 \times \frac{(A2 - A1) - (A2b - A1b)}{\epsilon_{340} \times 0.5 \ \mu\text{mol/min/μg of protein}}
\]

\(\epsilon_{340}\) is the Molar absorptivity at 340 nm which is 6.22/mM/cm, A1 and A2 is the rate of reaction 1 and 2 for the samples, A1b and A2b is the rate of reaction for blank reaction 1 and 2.

2.2.4 Gas chromatography-mass spectrometric (GC-MS) analysis of most effective extracts and fractions

Based on the results of the experiments carried out so far, the most active extracts from the plant were put through GC-MS analysis. GC-MS analysis was carried out on an Agilent Technologies 6890 Series GC coupled with an Agilent 5973 mass selective detector equipped with Agilent Chemstation software. A HP-5MS capillary column was used (30 m × 0.25 mm ID, 0.25 μm film thickness, 5% phenylmethylsiloxane). Helium was used as the carrier gas at a flow rate of 1.0 mL min⁻¹ and linear velocity of 37 cm/s. The injector temperature was set at 250°C. The oven temperature was programmed to reach 280°C from initial 60°C at a rate of 10°C/min. A 1 μL injection was made in the split mode with a split ratio of 20:1. The MS was operated in the electron ionization mode at 70 eV and electron multiplier voltage at 1859 V. The operating parameters for the instrument were: ion source temp. 230°C, quadrupole temp. 150°C, solvent delay 4 min and scan range 50-70 amu. Compounds were identified using the NIST library by direct comparison to the mass spectral data of known compounds.

2.2.5 Liquid Chromatography-Mass Spectroscopy (LC-MS) profiling

The aqueous extract and fractions were subjected to LC-MS analysis (Shimadzu LCMS-2020 Single Quadrupole) by direct injection into the machine via a loop, with the stop time set at 4 mins. The operating parameters were:

**Photodiode Array (PDA) sampling frequency:** 1.5625 Hz; **Operating mode:** low pressure gradient; **Pump A:** LC-2030 Pump; **Mobile Phase A and B:** water and methanol respectively; **Flow rate:** 0.200 ml/min; **Start and End wavelengths:** 190 and 800 nm respectively; **Cell Temp:**
40°C; **Start and End time**: 0.00 and 4.00 mins respectively; **Acquisition mode**: Scan; **Scan Speed**: 1667 u/s; **Polarity**: Positive; **Event Time**: 1.00 s; **Detector Voltage**: +1.00 kV; **Threshold**: 0; **Start and End m/z**: 50.00 and 1700.00 respectively. Compounds were identified directly by comparing their mass spectral data with those in the NIST library online and Food Database [FooDB Version 1.0] (FoodDB, 2017).

### 2.2.6 Fourier transform infrared spectroscopy (FT-IR) analysis

The chemical functional groups of the extracts and fractions were determined by direct scanning at a spectral range of 380 – 4000 cm\(^{-1}\) on a FT infrared spectrophotometer. The functional groups were determined by comparing the peak frequencies to an IR spectroscopy correlation table.

### 2.2.7 Molecular docking

The docking studies for antioxidant and anti-diabetic enzymes were performed using AutoDock Vina software (Trott and Olson, 2010). The crystal structure of the antioxidant enzymes was downloaded from the protein data bank (PDB). The human SOD (PDB ID: 2C9V, resolution 1.07 Å) (Strange et al., 2006), human erythrocyte catalase (PDB ID: 1QQW, resolution 2.75 Å) (Ko et al., 2000) and human glutathione reductase (PDB ID: 1XAN, resolution 2 Å) (Savvides and Karplus, 1996) were selected as suitable structures for docking of catalase, SOD and glutathione reductase respectively. PDB structures with access code: 1UOK (Watanabe et al., 1997) (resolution 2.4 Å), 1LPB (Egloff et al., 1995) (resolution 2.46 Å) was used for α-glucosidase and lipase respectively. The active binding site of catalase, SOD and glutathione reductase was defined using AutoDock tools (Sanner, 1999) within the grid size of (54x48x46), (64x62x60), (28x26x24) respectively while 44x42x40 dimensions was used for α-glucosidase and lipase with 1.00 Å as the grid spacing. AutoDock tools graphical user interface by MGL Tools (Morris et al., 2009) was applied to add Gasteiger charges. All the ligand structures were retrieved from PubChem compound database and optimized using Gaussian 09, prior to docking to obtain the global minimal structures (Makatini et al., 2013, Oyebode et al., 2018).

### 2.3 *In vitro* and *ex vivo* antioxidant and anti-diabetic activities of solvent fractions derived from the crude extracts

#### 2.3.1 Solvent fractionation of the most active crude extracts from each plant

From the results of the preliminary antioxidant assays (earlier described), the most promising crude extract from each plant was picked for further fractionation (Figure 2.3). The selection was made within crude extracts obtained from the same plant.
The preferred extracts for fractionation were the ethanol extract of leaves (*A. boonei* and *B. ferruginea*). Ten g of these crude extracts was dissolved totally in 100 mL of distilled water: methanol (1:9) and then partitioned successively with hexane (200 mL twice), dichloromethane (200 mL twice), ethyl acetate (200 mL twice) and n-butanol (200 mL twice) (Figure 2.3). All the resulting fractions were evaporated to dryness at 40°C under reduced pressure and the remaining aqueous fraction was dried on a water bath. The fractions were then weighed, put in glass vials and refrigerated at 4°C until needed.

All the fractions were investigated for *in vitro*, *ex vivo* antioxidant and anti-diabetic activities using the procedures earlier described.

2.4 *Ex vivo* glucose absorption and glucose uptake study

a. Animals

Five male Sprague-Dawley rats weighing 180 ± 10.12 g (average body weight) were received from the Biomedical Resource Unit (BRU) located in the Westville Campus of the University of KwaZulu-Natal, Durban, South Africa. The animals were momentarily (12 h) denied food but not water and
euthanized using isofor anaesthesia. Then the entire gastrointestinal tract (GIT) and parts of the psoas muscle were harvested after dissection and instantly used for small intestinal glucose absorption and muscle glucose uptake study, respectively. The animals were handled in cognisance to the rules and regulations of the Animal Research Ethics Committee of the University of KwaZulu-Natal, Durban, South Africa (Ethical approval number: AREC/003/017D).

b. Determination of glucose absorption in isolated rat small intestine

The effects of the varying concentrations (100-250 mg/dL) of extracts/fractions on small intestinal glucose absorption was measured in harvested rat jejunum. In this regard, the concentrations of glucose level in an incubation mixture consisting the pre-cut rat jejunum and test samples was recorded according to a previously published method (Hassan et al., 2010) as modified by Chukwuma et al. (2017). Briefly, jejunal segments of the harvested small intestine were sliced into smaller pieces of 5 cm and the inner jejunal lumen was cleaned with 2 mL of Krebs buffer (118 mM NaCl, 5 mM KCl, 1.328 mM CaCl₂·2H₂O, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄ and 25 mM NaHCO₃) by an antiseptic syringe. The pieces were initially inverted to show the villi, and then placed in an incubation tube with 8 mL of Krebs buffer and 11.1 mM glucose and varying concentrations of extract when glucose with Krebs buffer was used as a control. Then 1 mL mixture was taken from each incubation tube before and after the 2 h incubation time in a Steri-Cult CO₂ incubator (Labotec, South Africa) with 5% CO₂, 95% oxygen and 37°C settings. The amount of glucose in the samples was determined using an Automated Chemistry Analyzer (Labmax Plenno, Labtest Inc., Costa Brava, Brazil). The small intestinal absorption of glucose was extrapolated as the quantity of glucose absorbed per centimetre of rat jejunum using following formula:

Abdominal glucose absorption = \( \frac{GC_1 - GC_2}{\text{length of jejunum (cm)}} \)

Where GC1 & GC2 are the glucose concentrations (mg/dL) before and after the incubation respectively.

Percentage inhibition of glucose intake from the jejunum of small intestine was recorded as per following expression:

\[ \% \text{Inhibition} = \left( \frac{\Delta GC \text{\ control} - \Delta GC \text{\ sample}}{\Delta GC \text{\ control}} \right) \times 100 \]

Where \( \Delta GC \) = Change in glucose concentration before and after incubation
c. Determination of glucose uptake in harvested rat psoas muscle

The effect of the different concentrations (100-250 mg/dL) of the plant extracts/fractions on muscle glucose uptake was measured in harvested rat psoas muscle according to the procedure explained in a previous study (Hassan et al., 2010) as modified by Chukwuma et al. (2017). The harvested psoas muscle was briefly cleaned with Krebs buffer and diced into small chunks of the same weight (500 mg). Each chunk was then incubated in 8 ml of Krebs buffer, pre-mixed with 11.1 mM glucose (control) and varying concentration of the extract. A 0.5 mg/mL metformin solution was used as a positive control. The incubation time lasted for an hour in a CO2 incubator (as mentioned earlier) with 5% CO2, 95% oxygen and 37°C temperature settings. Before and after the incubation period, 1 mL sample was collected from each incubation tube and the glucose concentration was measured using a Chemistry Analyzer as mentioned earlier. The uptake of glucose by the muscle was extrapolated as the quantity of glucose (mg) used up by each gram of muscle tissue using following formula:

\[
\text{Muscle glucose} = \frac{(GC_1 - GC_2)}{\text{Weight of muscle tissue (g)}}
\]

Where GC1 and GC2 are glucose concentrations before and after incubation respectively.

2.5 In vivo antidiabetic activity of the most active fractions in a type 2 diabetes rat model

From the results of all the experiments carried out, the best fraction/extract from each plant was chosen for the in vivo studies. The aqueous fraction of B. ferruginea leaves, the butanol fraction of A. boonei leaves and the aqueous crude extract of C. rubens were selected for in vivo study since they showed the best activities in the antioxidant and antidiabetic models used during in vitro and ex vivo studies.

a. Animals

Seven-week-old male Sprague-Dawley rats were procured from the Biomedical Resource Centre located at the Westville Campus of the University of KwaZulu-Natal, Durban, South Africa. Animals were randomly grouped into seven (7) groups as follows, with five (5) rats each in the normal or non-diabetic groups and seven (7) rats each in the diabetic groups:

Normal Control (NC) = Normal male SD rats (non-diabetic/not treated)
Non-diabetic fraction control (NFC) = non-diabetic rats administered 300 mg/kg body weight fraction
Diabetic Control (DC) = diabetic and untreated rats
Diabetic & fraction Low dose (DFL) = diabetic rats administered 150 mg/kg body weight fraction
Diabetic & fraction High dose (DFH) = diabetic rats administered 300 mg/kg body weight fraction

Diabetic & Standard drug; Metformin and glibenclamide (DBM & DBG) = diabetic rats administered 300 mg/kg body weight metformin and 5 mg/kg body weight glibenclamide.

The same arrangement was used for the other two fraction and extract used for the *in vivo* studies (Figure 2.4).
Figure 2.4: Flow diagram describing comprehensive in vivo experiment

The animals were bred in a maximum of seven-in-one large poly carbonate cages and in a temperature and humidity-controlled room with a 12-hour light-dark cycle. The entire animals were fed with a commercial rat pellet diet ad libitum all through the experimental period. Animals were maintained according to the rules and regulations of the Experimental Animal Ethics Committee of the University of KwaZulu-Natal, South Africa (Animal ethics approval number: AREC/003/017D).
b. Induction of diabetes

The animals were left to acclimatize for one week. During the first 2 weeks of the experiment, the animals in the diabetic groups were supplied with a 10% fructose solution to induce insulin resistance (Wilson and Islam, 2012) while the animals in the normal or non-diabetic groups were supplied with normal drinking water. Thereafter, animals in the diabetic groups were intraperitoneally injected with a low dose of streptozotocin (40 mg/kg body weight) dissolved in a citrate buffer (pH 4.5) to induce partial pancreatic β-cell dysfunction, while animals in the normal or non-diabetic groups were injected with the same volume of citrate buffer alone. A week after the streptozotocin injection, the Non-fasting blood glucose levels of all animals were measured using the whole blood extracted from the tail vein tested with a handy glucometer, and animals with a NFBG level > 200 mg/dL were considered as diabetic. However, animals with a NFBG level < 200 mg/dL were removed from the experiment.

c. Intervention trial

After induction of diabetes, all animals had unlimited access to drinking water and commercial rat chow and the animals in plant fraction/extract groups were given a single oral dose of respective plant fraction/extract (150 or 300 mg/kg body weight) then the animals in the DBM and DBG groups were administered with a single oral dose of metformin (300 mg/kg body weight), glibenclamide (5 mg/kg body weight), respectively. The animals in the NC and DBC groups were supplied with equivalent volume of normal drinking water via the same route.

d. Oral Glucose Tolerance Test (OGTT)

The OGTT was done in the last week of the 5-week intervention period. This was done after an overnight fast, then glucose was orally administered to each animal at a dose of 2.0 g/kg body weight and blood samples were collected from the tail vein at 0 (just before glucose ingestion), 30, 60, 90 and 120 min. Glucose levels were estimated by using a portable Glucometer (Glucoplus, Quebec, Canada).

e. Determination of food and fluid intake, body weight and sampling

All through the study, the body weights of rats were measured weekly and the food and fluid intake was measured daily. The study was concluded with an overnight fasting (12 h) of all animals and consequently anesthetizing using isofor. The blood was collected via cardiac puncture and organs (liver, kidney, heart, muscle and pancreas) were collected, rinsed, weighed and preserved at a temperature of -30°C for subsequent analysis. Sterile centrifuge tubes were used for blood collection and the samples were stored on ice for 2-3 h. Separation of the serum was done by centrifugation at 3000 rpm for 15 min and stored at
-30°C for further usage. A small part of the pancreas from each rat was cut and submerged into a 10% neutral buffered formalin and then stored at room temperature for histopathological analysis.

2.6 Serum analysis

Serum insulin concentration was measured using an enzyme-linked immunosorbent assay (ELISA) procedure done with an ultrasensitive rat insulin ELISA kit (purchased through Mercodia, Uppsala, Sweden) with absorbance read in a multi-plate reader. The total cholesterol, HDL- and LDL-cholesterols, with triglycerides, fructosamine, urea, and uric acid concentrations including liver function enzymes; aspartate and alanine aminotransferases (AST and ALT) and alkaline phosphatase (ALP) were determined with an Automated Chemistry Analyzer (Labmax Plenno, Labtest Co. Ltd., Lagoa Santa, Brazil) using standard assay kits for these analyses purchased from the same production company. Homeostatic model assessment (HOMA-IR and HOMA-β) were estimated using serum insulin levels and FBG concentrations taken at the end of the experiment using the following expression:

\[
\text{HOMA – IR} = \frac{\text{Serum insulin (U/L) \times Blood glucose (mg/dL)}}{22.5}
\]

\[
\text{HOMA – β cell function} = \frac{20 \times \text{Serum insulin in U/L}}{\text{Blood glucose in mg/dL} – 3.5}
\]

The conversion factor for insulin = 1 U/L = 7.174 pmol/L

2.7 Hepatic glycogen content

Estimation of the levels of hepatic glycogen concentrations was done by using a phenol-sulfuric acid method (Lo et al., 1970). One gram of the excised liver was digested with 1.5 mL of 30% KOH saturated in Na₂SO₄. This was put in a test tube completely immersed in ice, later it was boiled for 30 mins. Then 2 mL of 95% ethanol was added to the sample followed by cold centrifugation for 30 mins at 840 g twice to properly precipitate the glycogen content. Thereafter the supernatant was aspirated, and the precipitate dissolved in 3 mL of distilled water. A 1 mL of 5% phenol was added to the dissolved glycogen and 5 mL of concentrated H₂SO₄ was rapidly and carefully added. The mixture was mixed thoroughly and boiled for 20 mins, followed by cooling and the absorbance was read using a 96-well plate at 600 nm. The glycogen content of the samples was extrapolated from a standard glycogen curve and reported in mg/g glycogen.
2.8 **Histopathology analysis of the excised pancreas**

The formalin-preserved pancreatic tissue was subjected to standard laboratory procedures for paraffin fixing/embedding. Tiny sections were cut to a size of approximately 4 μm, followed by deparaffination of the slides using p-xylene. This was consequently rehydrated using ethanol at different concentrations (100%, 80%, 70%, 50%) and cleansed with running water. Subsequently, the hematoxylin was used to stain the slides for 5 mins and then cleansed with running water. Next was counterstaining using eosin. Finally, the stained slides were arranged and mounted in DPX, with a sterile cover-slip and viewed with Leica slide scanner (SCN 4000, Leica Bio-systems, Germany).

2.9 **In vivo antioxidant studies**

To fully understand the likely role of antioxidant activity in combination to the observed anti-diabetic activity, the *in vivo* antioxidant potentials of the fractions/extract were investigated in the serum and tissues of the animals using six *in vivo* oxidative stress markers namely; reduced glutathione (GSH), thiobarbituric acid reactive substances (TBARS) levels, catalase, superoxide dismutase (SOD), glutathione reductase (GR) and glutathione peroxidase (GPx) assays.

To perform these assays, centrifuged homogenates of the excised tissues (liver, kidney, heart and pancreas) were initially prepared by homogenising 0.5 g of the organ tissue sample in 4000 μL of homogenization buffer (50 mM sodium phosphate buffer with triton X-100, pH 7.5) with an Ultra Turrax Tube Drive Work Station Homogenizer (IKA-Works, Staufen Breisgau, Germany). Soon after, the solution was transferred to a microtube and centrifuged for 15 min at 15000 rpm in a Hettich Mikro 200 Microcentrifuge (Hettich Lab Technology, Tuttingen, Germany). The supernatant was then poured in another micro tube and kept at -20°C for subsequent analysis.
CHAPTER 3
THE ANTIOXIDANT AND ANTIDIABETIC ACTIVITIES OF
ALSTONIA BOONEI IN VITRO AND IN VIVO

3.1 Background

Alstonia boonei is a large deciduous tree, which grows up to 35 meters high (Figure 3.1) and is widely distributed in Africa: Cameroon, Nigeria, Egypt, Ghana, Code D’Ivoire and Central African Republic. It belongs to the Apocynaceae family and its species are scattered all over the African continent (Adotey et al., 2012). At the nodes, the leaves of A. boonei are in whorls of 5-8 oblanceolate parts with the lateral vein prominent at right angles to the midrib. The bark of the tree contains an abundant latex supply. The fruits are joined to thin follicles as long as 16 cm with brown floss at each end (Olajide et al., 2000).

Figure 3.1: Alstnia boonei (Apocynaceae); Common names: cheese wood (English), emien (French), Awun (Yoruba, Nigeria), Egbu (Igbo, Nigeria), Ekok (Ewondo, Cameroon). Adapted without permission from (Ileke, 2014, WAP, 2018).
3.1.1 Ethnobotanical uses

The stem bark remains the most commonly used part for therapeutic purposes; though more effective when used fresh, the dried part is also frequently used (Olajide et al., 2000). The bark has been found to contain anti-inflammatory, analgesic, antipyretic, antidiabetic, anti-helminthic and antimicrobial activities (Akinmoladun et al., 2007; Gupta et al., 2005; Okoye et al., 2014; Opoku and Akoto, 2015). A decoction of the stem bark is usually mixed in honey and taken four times a day as painkiller for dysmenorrhea, lower abdominal pain and pains as a result of malaria (Majekodunmi et al., 2008). Cold infusion from the stem bark (fresh or dry) is effective as a hypoglycaemic agent. This is also effective as a good deworming liquid (Adotey et al., 2012; Olajide et al., 2000). A mixture of the decoction of the stem bark of *A. boonei* with other plants can be used in treatment of asthma, fractures, jaundice and breastmilk induction. The leaves and latex are also used in the treatment of rheumatism, hypertension and muscular pain (Iniaghe et al., 2012).

3.1.2 Biological activities

Much attention has been given to the analgesic and anti-malaria effect of *A. boonei* and until recently little has been known on its anti-diabetic effects.

The analgesic property of the methanol leaf extract was investigated, and it was discovered that the analgesic effects of *A. boonei* could be mediated through both central and peripheral mechanisms (Iniaghe et al., 2012). In order to validate the anti-microbial activity of *A. boonei*, the ethanol and aqueous extract of the root was assayed for microbial susceptibility using the agar well diffusion procedure (Opoku and Akoto, 2015). Some gram-positive and gram-negative bacteria such as *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli* and *Pseudomonas aeruginosa* and a fungus (*Candida albicans*) were used for this study and the result revealed that the ethanol extract exhibited better antimicrobial ability than the aqueous counterpart. An isolate obtained from the petroleum ether fraction of *A. boonei* lupeol acetate was investigated for its anti-arthritic activity in an arthritic animal model. Lupeol acetate reversed the increase in spleen weight and serum alkaline phosphatase which were comparable to nonarthritic control values (Kweifio-Okai and Carroll, 1993).
3.2 Phytochemical constituents, antioxidant and antidiabetic activities of different extracts of the leaves, stem and root barks of *Alstonia boonei*: An *in vitro* and *in silico* study

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Preface: This article reports in detail the *in vitro* antioxidant and anti-diabetic studies of the crude extracts from *A. boonei*; it is currently under review in *Journal of Natural Products Research*.

3.2.1 Abstract

This study investigated the antioxidant activities of extracts from three parts of *Alstonia boonei*. Sequential extraction was done on the leaves, stem and root bark samples with solvents of increasing polarity and these extracts were assayed for total phenolic content and *in vitro* antioxidant and antidiabetic activities using different experimental models. The electron donating, 1,1-diphenyl-2-picryl-hydrazyl (DPPH) and nitric oxide radical scavenging capacity of all the extracts was investigated in this study. The ability of these extracts to inhibit α-glucosidase and α-amylase were also examined *in vitro*. The ethanol extract of the leaves showed the highest total phenolic content compared to other extracts. All extracts showed free radical scavenging abilities in the different models. The ethanol and aqueous extracts significantly (*p* < 0.05) inhibited the key anti-diabetic enzymes tested. GC-MS analysis of the different plant parts revealed the presence of sterols, aromatics, aliphatic acids and esters, which when docked with α-glucosidase and α-amylase enzymes revealed a high binding affinity. Data from this study suggest that the different parts of *A. boonei*, especially the ethanol and aqueous extracts, possess potent anti-oxidant activities that points to further studies to identify the active agents.

**Key words:** Medicinal plant, oxidative stress, hyperglycaemia, *A. boonei*
3.2.2 Introduction

Medicinal plants have been widely accepted in Africa in the treatment and management of diverse ailments. Fortunately, Africa is a land flourishing with a diversity of plant species. According to World Health Organization (WHO), between 65% and 80% of the population of developing countries depend on medicinal plants (Palhares et al., 2015). Development of drugs from natural sources is well encouraged because only 15% of this has been fully exploited to determine their therapeutic potentials out of over 300 000 species of plants in the world (De Luca et al., 2012). Since the last century, the sporadic change in life styles and eating habits of the humans has led to unfolding of different types of chronic diseases such as diabetes (Eddouks et al., 2012).

Diabetes is characterized under the category of non-communicable diseases (NCD) and is rapidly emerging as a global health challenge (Arredondo et al., 2018). The global prevalence of adults over 18 years with diabetes has doubled over the past 20 years (Cho et al., 2018). Data from the International Diabetes Federation (IDF) suggest that over 425 million people are currently diagnosed to be living with diabetes and this number has been proposed to increase by over 50% in 2040 (IDF, 2017). Among all cases of diabetes, the most prevalent of all still remains type 2 diabetes (T2D) which is predominant in 95% of all diabetic cases (Fuchsberger et al., 2016).

T2D is characterised by insulin resistance, partial β-cell dysfunction, which leads to and consequently if left unmanaged hyperglycaemia (Fuchsberger et al., 2016). Persistent hyperglycaemia has been implicated in the generation of free radicals through diverse pathways caused by auto-oxidation of the excess glucose in the blood stream (Asmat et al., 2016). The continuous build-up of free radicals which limits the antioxidant supply in the body leads to a condition known as oxidative stress (Asmat et al., 2016). Oxidative stress has been linked to T2D-associated macro- and micro-vascular complications (Matough et al., 2012).

The available synthetic drugs (such as metformin, glibenclamide, thiazolidinedione and others) have not been able to fully manage these T2D associated complications since their efficacy reduces slowly while they have some short- and long-term side effects. These side effects range from nausea, hypoglycaemia, heart problems, and body weight gain. Apart from these, the high cost and availability of these drugs also pose some drawbacks which leads to the need for alternate therapies from medicinal plants among which is Alstonia boonei.

Alstonia boonei De Wild (family: Apocynaceae) is a towering tree which has species scattered all over Africa. In some parts of Africa, it is referred to as “sacred tree” and is worshiped in these areas.
Traditionally, decoctions from the stem bark is used to treat ailments such as fever, inflammations and joint pains (Akinnawo et al., 2017; Majekodunmi et al., 2008). The latex obtained from the stem bark is also used as an analgesic in some parts of Africa (Adotey et al., 2012). Formulations from the stem bark is currently being used in some parts of Nigeria in the effective treatment of malaria (Chime et al., 2013). The crude ethanol leaves extract of *A. boonei* has been shown to have potent high blood glucose reducing activity in an alloxan-induced rabbit model of diabetes (Akinloye et al., 2013; Osadolor et al., 2015). However, the detailed antidiabetic activity of the various parts of this plant are investigated yet and the mechanisms behind its antidiabetic activity are still unknown.

This study was conducted to examine the detailed antidiabetic activity as well as mechanisms behind the antidiabetic effects of the different parts of this plant and thereafter, identify potential bioactive compounds which could render synergistic effects to its activity. *In silico* molecular docking was also performed with the compounds to estimate binding affinities of the identified compounds to α-glucosidase and α-amylase which are potent carbohydrate hydrolyzing enzymes.

### 3.2.3 Materials and methods

Please refer to Chapter 2 for the detailed methods used for *A. boonei*.

### 3.2.4 Results

The ethanol extract of the leaves of *A. boonei* contained a significantly (*p* < 0.05) greater amount of total phenol compared to all the other extracts from the different parts of this plant.

The total reducing power (reported as percentage gallic acid equivalent) of the different extracts of *A. boonei* in comparison to ascorbic acid and Trolox is shown in Figure 3.2.1. The result showed that the total reducing power of all extracts was increased with increasing concentrations. However, the ethanol and aqueous extracts of the leaves, stem- and root barks showed significantly (*p* < 0.05) higher Fe$^{3+}$ to Fe$^{2+}$ reducing capacity than the other extracts.
Table 3.2.1: Total phenolic content of various extracts of *A. boonei* parts

<table>
<thead>
<tr>
<th><em>A. boonei</em> parts</th>
<th>Total polyphenols (mg/g GAE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaves</td>
<td></td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>50.04 ± 0.54&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ethanol</td>
<td>58.14 ± 1.66&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Aqueous</td>
<td>26.71 ± 1.51&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Stem bark</td>
<td></td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>42.79 ± 0.057&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ethanol</td>
<td>31.9 ± 0.384&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Aqueous</td>
<td>30.82 ± 0.85&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>Root bark</td>
<td></td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>33.00 ± 0.86&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ethanol</td>
<td>34.09 ± 1.02&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>Aqueous</td>
<td>34.82 ± 0.73&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Results are reported as mean ± SD of three replicas. <sup>a-d</sup> Different superscripts alphabets within a column are significantly different from one another (Tukey’s-HSD multiple range post hoc test, *p* < 0.05).

**Figure 3.2.2** shows the DPPH radical scavenging activities of *A. boonei* leaves, stem bark and root bark extracts. All extracts were able to scavenge the DPPH free radical in a dose-dependent manner however, the ethanol extract of the leaves and the aqueous extract of the stem- and root barks demonstrated a more potent DPPH inhibiting activity which was almost similar to the standard antioxidants (Trolox and Ascorbic acid) used. The IC<sub>50</sub> values on Table 3.2.2 also for the ethanol extract of the leaves and stem bark and the aqueous extract of the root bark.

The nitric oxide (NO) radical scavenging activities of the different extracts of *A. boonei* is shown in **Figure 3.2.3**. Compared to other parts, only the stem bark extracts showed a concentration-dependent inhibition of nitric oxide. The aqueous extract of the stem bark showed the lowest IC<sub>50</sub> values (**Table 3.2.1**) even lower than the IC<sub>50</sub> values of the tested standard antioxidants.
Figure 3.2.1: Total reducing power (relative to gallic acid) of leaves, stem bark and root bark extracts of *A. boonei*. Results are reported as mean ± SD of three replicas. *a*-Different alphabets presented for a given concentration for each extract indicate significant difference (Tukey’s-HSD multiple range *post hoc* test, *p* < 0.05) EtAc: Ethyl acetate, EtOH: Ethanol, AA: Ascorbic acid.
Figure 3.2.2: DPPH radical scavenging activity of leaves, stem bark and root bark extracts of *A. boonei*. Results are reported as mean ±SD of three replicas. *Different alphabets over the bars for a given concentration for each extract indicate significance of difference (Tukey’s-HSD multiple range post hoc test, *p* < 0.05). EtAc: Ethyl acetate, EtOH: Ethanol, Tx: Trolox, AA: Ascorbic acid.*
Figure 3.2.3: Percentage of nitric oxide scavenging activities of extracts from leaves, stem bark and root bark of *A. boonei*. Results are reported as mean ±SD of three replicas. Different alphabets over the bars for a given concentration for each extract indicate significance of difference (Tukey’s-HSD multiple range post hoc test, \( p < 0.05 \)). EtAc: Ethyl acetate, EtOH: Ethanol, GA: Gallic acid, AA: Ascorbic acid.

The \( \alpha \)-glucosidase and \( \alpha \)-amylase inhibitory activities of the extracts are shown in Figures 3.2.4 and 3.2.5 when their respective IC\textsubscript{50} values are represented in Table 3.2.2. The ethanol extracts demonstrated significantly higher (\( p < 0.05 \)) \( \alpha \)-glucosidase inhibitory activity than the other extracts.
which was similar to the standard drug (acarbose) used. Pancreatic α-amylase activity was also inhibited in vitro by all the extracts in a dose dependent fashion (Figure 3.2.5) and again the ethanol extract showed a significantly higher ($p < 0.05$) α-amylase inhibitory effect than other extracts.

**Figure 3.2.4:** The α-glucosidase inhibitory effects of a) leaves, b) stem bark and c) root bark extracts of *A. boonei*. The results are reported as mean ±SD of three replicas. *Different alphabets over the bars for a given concentration of each fraction indicate significance of difference (Tukey’s-HSD multiple range post hoc test, $p < 0.05$). EtAc: Ethyl acetate.*
Figure 3.2.5: α-amylase inhibitory effects of leaves, stem bark and root bark extracts of *A. boonei*. The results are reported as mean ± SD of three replicas. *a*-Different alphabets over the bars for a given concentration of each fraction indicate significance of difference (Tukey’s-HSD multiple range post hoc test, *p* < 0.05). EtAc: Ethyl acetate, EtOH: Ethanol.
**Table 3.2.2:** The IC\textsubscript{50} values of the different extracts of *A. boonei* in different antioxidant and anti-diabetic models

<table>
<thead>
<tr>
<th></th>
<th>DPPH (µg/mL)</th>
<th>NO (µM)</th>
<th>α-glucosidase (µg/mL)</th>
<th>α-amylase (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Leaves</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>7.54±0.55\textsuperscript{c}</td>
<td>21.16±1.76\textsuperscript{d}</td>
<td>495.65±1.62\textsuperscript{d}</td>
<td>259.21±5.72\textsuperscript{e}</td>
</tr>
<tr>
<td>Ethanol</td>
<td>8.61±0.09\textsuperscript{c}</td>
<td>8.49±1.07\textsuperscript{b}</td>
<td>87.05±0.87\textsuperscript{c}</td>
<td>102.93±9.76\textsuperscript{c}</td>
</tr>
<tr>
<td>Aqueous</td>
<td>13.03±0.71\textsuperscript{f}</td>
<td>59.57±5.69\textsuperscript{f}</td>
<td>&gt;1000</td>
<td>684.59±98.62\textsuperscript{f}</td>
</tr>
<tr>
<td><strong>Stem bark</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>6.24±0.11\textsuperscript{d}</td>
<td>9.44±1.21\textsuperscript{b}</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>Ethanol</td>
<td>3.73±0.08\textsuperscript{b}</td>
<td>17.68±2.64\textsuperscript{c}</td>
<td>26.95±1.72\textsuperscript{a}</td>
<td>16.78±1.02\textsuperscript{a}</td>
</tr>
<tr>
<td>Aqueous</td>
<td>6.95±0.71\textsuperscript{d}</td>
<td>2.92±1.23\textsuperscript{a}</td>
<td>423.61±9.27\textsuperscript{d}</td>
<td>256.91±2.94\textsuperscript{e}</td>
</tr>
<tr>
<td><strong>Root bark</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>7.46±0.46\textsuperscript{d}</td>
<td>23.59±3.71\textsuperscript{d}</td>
<td>&gt;1000</td>
<td>154.83±23.81\textsuperscript{d}</td>
</tr>
<tr>
<td>Ethanol</td>
<td>5.29±0.08\textsuperscript{c}</td>
<td>34.32±4.28\textsuperscript{e}</td>
<td>594.71±9.87\textsuperscript{e}</td>
<td>129.06±3.21\textsuperscript{d}</td>
</tr>
<tr>
<td>Aqueous</td>
<td>2.34±0.10\textsuperscript{a}</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>734.52±3.20\textsuperscript{f}</td>
</tr>
<tr>
<td><strong>Standards</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gallic acid</td>
<td>ND</td>
<td>365.25±14.82\textsuperscript{h}</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>2.19±0.05\textsuperscript{a}</td>
<td>4.13±2.92\textsuperscript{c}</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Trolox</td>
<td>5.79±0.54\textsuperscript{c}</td>
<td>156.83±3.62\textsuperscript{e}</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Acarbose</td>
<td>ND</td>
<td>ND</td>
<td>36.70±5.43\textsuperscript{b}</td>
<td>87.21±7.43\textsuperscript{b}</td>
</tr>
</tbody>
</table>

The results are reported as mean ±SD of three replicas. \textsuperscript{a-b}Different alphabets presented in a column for a given parameter for each fraction are significantly different from one another (Tukey’s-HSD multiple range post hoc test, *p* < 0.05). IC\textsubscript{50}: Concentration for 50% inhibition.

Based on the above-mentioned results, the ethanol and aqueous extracts of the stem bark and leaves were subjected to GC-MS analysis. The results revealed the presence of aliphatic acids, esters, sterols, sterols precursors and aromatic compounds (**Figures 3.2.6 and 3.2.7, Table 3.2.3**).
<table>
<thead>
<tr>
<th>Compounds</th>
<th>Molecular formula</th>
<th>RT (min)</th>
<th>Molecular mass</th>
<th>Relative abundance (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ethanol extract of the leaves</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Megastigmatrienone</td>
<td>C_{13}H_{16}O</td>
<td>13.65</td>
<td>190</td>
<td>0.41</td>
</tr>
<tr>
<td>Tetradecanoic acid</td>
<td>C_{14}H_{20}O_{2}</td>
<td>15.58</td>
<td>228</td>
<td>0.41</td>
</tr>
<tr>
<td>Phytol acetate</td>
<td>C_{22}H_{42}O_{2}</td>
<td>16.43</td>
<td>338</td>
<td>2.96</td>
</tr>
<tr>
<td>Phytol</td>
<td>C_{20}H_{40}O</td>
<td>19.11</td>
<td>296</td>
<td>2.00</td>
</tr>
<tr>
<td>Octadecanoic acid</td>
<td>C_{18}H_{36}O_{2}</td>
<td>19.56</td>
<td>284</td>
<td>2.56</td>
</tr>
<tr>
<td>4-Phenylbenzophenone</td>
<td>C_{19}H_{14}O</td>
<td>20.07</td>
<td>258</td>
<td>1.09</td>
</tr>
<tr>
<td>Stigmasterol-3,4-dedihydro-3-acetate</td>
<td>C_{13}H_{16}O</td>
<td>20.63</td>
<td>452</td>
<td>0.40</td>
</tr>
<tr>
<td>9-(4-Methoxyphenyl) xanthene</td>
<td>C_{20}H_{18}O_{2}</td>
<td>22.40</td>
<td>288</td>
<td>2.89</td>
</tr>
<tr>
<td>Acridin-9-yl-(4-methoxy-phenyl)-amine</td>
<td>C_{20}H_{12}N_{2}O</td>
<td>24.33</td>
<td>300</td>
<td>0.38</td>
</tr>
<tr>
<td>Squalene</td>
<td>C_{30}H_{50}</td>
<td>24.75</td>
<td>410</td>
<td>0.47</td>
</tr>
<tr>
<td>Oleane-11,13(18)-diene</td>
<td>C_{30}H_{46}</td>
<td>27.22</td>
<td>408</td>
<td>1.48</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>C_{20}H_{50}O_{2}</td>
<td>27.38</td>
<td>430</td>
<td>1.16</td>
</tr>
<tr>
<td>Stigmasterol</td>
<td>C_{20}H_{48}O</td>
<td>28.97</td>
<td>412</td>
<td>2.32</td>
</tr>
<tr>
<td>Sitosterol</td>
<td>C_{20}H_{50}O</td>
<td>29.77</td>
<td>414</td>
<td>1.81</td>
</tr>
<tr>
<td><strong>Aqueous extract of the leaves</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Xanthone</td>
<td>C_{13}H_{16}O</td>
<td>16.87</td>
<td>196</td>
<td>1.23</td>
</tr>
<tr>
<td>Hexadecanoic acid methyl ester</td>
<td>C_{17}H_{30}O_{2}</td>
<td>17.25</td>
<td>270</td>
<td>3.27</td>
</tr>
<tr>
<td>Octadecanoic acid methyl ester</td>
<td>C_{19}H_{30}O_{2}</td>
<td>19.18</td>
<td>298</td>
<td>2.08</td>
</tr>
<tr>
<td>Octadecanoic acid</td>
<td>C_{18}H_{36}O_{2}</td>
<td>19.54</td>
<td>284</td>
<td>7.37</td>
</tr>
<tr>
<td>Stigmasta-3,5-diene</td>
<td>C_{20}H_{48}</td>
<td>26.83</td>
<td>396</td>
<td>4.78</td>
</tr>
<tr>
<td><strong>Ethanol extract of the stem bark</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phytol acetate</td>
<td>C_{22}H_{42}O_{2}</td>
<td>16.41</td>
<td>338</td>
<td>0.43</td>
</tr>
<tr>
<td>Xanthone</td>
<td>C_{13}H_{16}O</td>
<td>16.98</td>
<td>196</td>
<td>0.33</td>
</tr>
<tr>
<td>Hexadecanoic acid methyl ester</td>
<td>C_{17}H_{30}O_{2}</td>
<td>17.30</td>
<td>270</td>
<td>0.41</td>
</tr>
<tr>
<td>n-Hexadecanoic acid</td>
<td>C_{16}H_{32}O_{2}</td>
<td>17.65</td>
<td>256</td>
<td>4.85</td>
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<tr>
<td>Hexadecanoic acid ethyl ester</td>
<td>C_{18}H_{36}O_{2}</td>
<td>17.98</td>
<td>284</td>
<td>2.85</td>
</tr>
<tr>
<td>4-Phenylbenzophenone</td>
<td>C_{19}H_{14}O</td>
<td>20.06</td>
<td>258</td>
<td>0.51</td>
</tr>
<tr>
<td>9-(4-Methoxyphenyl) xanthene</td>
<td>C_{20}H_{18}O_{2}</td>
<td>22.40</td>
<td>288</td>
<td>3.56</td>
</tr>
<tr>
<td>Stigmast-4-en-3-one</td>
<td>C_{29}H_{40}O</td>
<td>23.25</td>
<td>412</td>
<td>0.85</td>
</tr>
<tr>
<td>Squalene</td>
<td>C_{30}H_{50}</td>
<td>24.75</td>
<td>410</td>
<td>0.48</td>
</tr>
<tr>
<td>Ergost-5-en-3β-ol</td>
<td>C_{28}H_{40}O</td>
<td>28.65</td>
<td>400</td>
<td>2.30</td>
</tr>
<tr>
<td>Stigmasterol</td>
<td>C_{29}H_{40}O</td>
<td>28.96</td>
<td>412</td>
<td>3.43</td>
</tr>
<tr>
<td>Sitosterol</td>
<td>C_{29}H_{50}O</td>
<td>29.77</td>
<td>414</td>
<td>2.67</td>
</tr>
</tbody>
</table>

The compounds presented in the table are those which matched similar compounds in the NIST library software.
Figure 3.2.6: Aliphatic acids and esters isolated from *Alstonia boonei*

- Tetradecanoic acid
- Octadecanoic acid
- Octadecanoic acid methyl ester
- Hexadecanoic acid methyl ester
- Hexadecanoic acid ethyl ester
- n-hexadecanoic acid

Figure 3.2.7: Sterols, sterol precursors and aromatics from *Alstonia boonei*

- Xanthone
- 9-(4-Methoxyphenyl)xanthene
- 9,19-9,19-Dimethoxy-19-methyl-19-norxanthene
- Megastigmatrienone
- Vitamin E (\(\alpha\)-tocopherol)
- Olea-11,13(18)-dien-3-one
- 4-phenylbenzophenone
- Stigmasterol
- Stigmasterol-3,4-dihydro-3-acetate
- Stigmaster-4-en-3-one
- Stigmast-3,5-diene
- Sitosterol
- Ergost-5-en-3\(\beta\)-ol
- Squalene
- Phytol
- Phytol acetate

*Figure 3.2.6: Aliphatic acids and esters isolated from *Alstonia boonei***

*Figure 3.2.7: Sterols, sterol precursors and aromatics from *Alstonia boonei***
*In silico* analysis (molecular docking) was carried out to determine the inhibition potentials of the extracts. *Table 3.2.3* revealed that α-amylase complex with Vitamin E from ethanol extract of leaves showed the highest inhibition potential with binding energy of 11.66 kcal/mol compared to other compounds while stigmasterol had the strongest binding affinities of 9.98 kcal/mol for α-glucosidase. *Figure 3.2.8* and *3.2.9* showed the hydrogen bonding interactions between the atoms of the compounds with the highest binding energies and the amino acid residues around the active sites. The high binding energy associated with Vitamin E and stigmasterol may be due to the presence of methoxy and hydroxyl groups in their structures, respectively. The binding energies analysis and the hydrogen bonding interactions with active site residues gives further insight about the catalytic mechanism.

*Table 3.2.4*: Highest binding energies (ΔG) of compounds from the different extracts of *Alstonia boonei* obtained after molecular docking simulation.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>ΔG(^a) (kcal/mol)</th>
<th>ΔG(^b) (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ethanol extract of the leaves</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vitamin E</td>
<td>11.66</td>
<td>9.82</td>
</tr>
<tr>
<td><strong>Aqueous extract of the leaves</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>stigmasta-3,5-diene</td>
<td>8.88</td>
<td>-</td>
</tr>
<tr>
<td>Xanthone</td>
<td>-</td>
<td>8.11</td>
</tr>
<tr>
<td><strong>Ethanol extract of the stem bark</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ergost-5-en-3β-ol</td>
<td>9.68</td>
<td>-</td>
</tr>
<tr>
<td>Stigmasterol</td>
<td>-</td>
<td>9.98</td>
</tr>
</tbody>
</table>

\(^a\) = α-glucosidase, \(^b\) = α-amylase
Figure 3.2.8: The 3D representation of (a) hydrogen bonding interaction and (b) complex with active site residues of α-Glucosidase complexed with Vitamin E (highest binding energy).

Figure 3.2.9: The 3D representation of (a) hydrogen bonding interaction and (b) complex with active site residues of α-amylase complexed with Stigmasterol (highest binding energy).
3.2.5 Discussion

Oxidative stress still remains a deleterious process for the generation of maladies such as T2D (Francisqueti et al., 2017). The antioxidant properties of medicinal plants can be measured through their reducing capacity, free radical scavenging ability, hydrogen ion donating and singlet oxygen quenching powers (Kasote et al., 2015, Seifu et al., 2012). The presence of total phenols in all investigated plant parts of A. boonei as shown in Figure 3.2.1 were mainly concentrated in the leaves which showed the antioxidant potential of A. boonei. The method used to determine the total phenol content is based on reducing capacity of A. boonei relative to gallic acid as compared to just reporting the amount of phenols present (Singleton et al., 1999). Compounds of phenolic derivatives are key antioxidants which exhibit free radical scavenging abilities and are interestingly widely distributed in plants (Maqsood et al., 2014). Some studies have reported that plants with an abundance of polyphenols can act as good antioxidants (Hossain and Shah; 2015; Noreen et al., 2017). The effectiveness of plant phenolics as antioxidants is also backed up by the ability of the different extracts to reduce Fe$^{3+}$ to Fe$^{2+}$ (Figure 3.2.2) and to inhibit the free radical DPPH (Figure 3.2.3). From our results, the ethanol extract of the leaves especially showed higher total phenol content and better reducing capacities which may suggest that the antioxidant capacity of A. boonei is mainly due to its more polar constituents as reported previously for the root bark of A. boonei (Obiagwu et al., 2014).

Nitric oxide (NO) (low levels) under normal physiological conditions, acts as a regulator in physiological functions such as immune response, blood pressure and neural communication (Moncada et al., 1991). However, an overabundance of NO can lead to tissue damage and it is also associated with the cardiovascular complications of T2D (atherosclerosis and hypertension) (Adela et al., 2015; Pacher et al., 2007). Therefore, there has been focus on discovering antioxidants which may act as potential inhibitors of NO production (Chen et al., 2017). Our results showed that only ethanol and aqueous extracts of the stem bark were able to inhibit NO in a dose-dependent manner (Figure 3.2.3) which could be linked to the ability of A. boonei to inhibit inducible nitic oxide synthase (iNOS) as reported in a previous anti-diabetic study (Kehinde et al., 2016).

Inhibition of α-glucosidase and α-amylase still remains a potent strategy in the development of antidiabetic drugs (Khan et al., 2016). These two enzymes present in the digestive tract or pancreas are responsible for the hydrolysis of disaccharides and starch to glucose and breakdown of long chain carbohydrates to smaller carbohydrate moieties (Kawamura-Konishi et al., 2012). Natural products which are phenolic in nature have been reported to inhibit the activity of these enzymes (Ramkumar
et al., 2010). In this study, the different extracts from the parts of A. boonei showed potent $\alpha$-glucosidase (Figure 3.2.4) and $\alpha$-amylase (Figure 3.2.5) inhibitory activities. The in-silico results obtained from this study also suggest the ability of some compounds identified in A. boonei to bind effectively to the active and allosteric sites of the target enzymes, $\alpha$-glucosidase and $\alpha$-amylase (Figure 3.2.8 and 3.2.9). These findings further suggest that the plant extracts might reduce the hydrolysis of disaccharides and polysaccharides as well as aid small intestinal absorption of glucose and thereby reduce blood glucose levels.

GC-MS analysis of the leaves, stem bark and root bark revealed the presence of various bioactive compounds (Figures 3.2.6 and 3.2.7). Studies have investigated the characteristics of phenols in relation to their roles as antioxidants and it has been shown that this role can be due to their high capacity of hydrogen atom or single electron transfer potential to a free radical (Craft et al., 2012; Espinosa et al., 2015). Compounds which act best as phenolics have an electron donor group attached to an aromatic ring (antioxidant activity increases with a high number of rings) (Cheng et al., 2003). A total of 14 compounds (most of the phenols) were identified from the ethanol extract of the leaves alone (Table 3.2.2). Phenolics have also been proposed to be responsible for $\alpha$-glucosidase and $\alpha$-amylase inhibitory activities (Ali Asgar, 2013) and this occurs through hydrogen bond formation in the active site of the enzyme between the $\text{-COO}$ groups of Asp 197 and Glu233 with the hydroxyl groups in the phenols.

Phytol and phytol acetate were also identified by GC-MS in the ethanol extract of the leaves and stem bark respectively. Some studies have proven the antioxidant and anti-diabetic effects of phytol and its derivatives (Ali Asgar, 2013; Santos et al., 2013). Our study showed a relative abundance (%) of phytol and phytol acetate in the ethanol extract of the leaves to be 2.00 and 2.96, respectively (Table 3.2.2). Phytosterols are bioactive natural products present in plants with similar structure to cholesterol but have no effect on human cholesterol (Wang et al., 2017). Our study revealed the presence of stigmasterol, a member of the phytosterol family in the ethanol extract of the leaves and stem bark. Interestingly, phytosterols have gained recent attention in their role as antioxidants by preventing angiogenesis through inhibition of reactive oxygen species production and oxidative stress. The antioxidant activities of the plant earlier explained could be due to the synergistic effects of the identified phytosterols. Antidiabetic effects of phytosterols have also been demonstrated in a recent study (Kushwaha et al., 2015), which further gives credence to the efficacy of A. boonei as an antidiabetic agent. However, the observed antioxidant and antidiabetic effects of the different parts, especially the ethanol extract of the leaves and stem bark could be due to the synergistic effects of
the identified bioactive compounds. This assumption was further explored by using in silico molecular docking simulation to investigate the binding affinity of some of the compounds to the active site of the $\alpha$-glucosidase and $\alpha$-amylase enzymes. The highest binding energy of the compounds was exhibited by a phytol precursor (vitamin E) and stigmasterol from the ethanol extract of the leaves and stem bark, respectively (Table 3.2.3).

In conclusion, this study suggests that the ethanol extract of the leaves and stem bark of A. boonei contains potent antioxidant agents coupled with $\alpha$-glucosidase and $\alpha$-amylase inhibitors which could be further exploited for the development of a therapeutic strategy for the management and control of post meal blood glucose levels, T2D and its macrovascular complications. Future work will entail solvent based fractionation of the crude extracts and a detailed study of the active fraction in a T2D model of rats.

Postscript: From the above studies, the ethanol extract of the leaves showed the best antioxidant and antidiabetic activity and will therefore be selected for solvent based fractionation.
3.3 Fractions of *Alstonia boonei* leaves ameliorate oxidative stress and modulate key hypoglycemic processes

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Preface: This chapter reports the detailed *in vitro* and *ex vivo* anti-diabetic effects of the fractions obtained from the ethanol extract of the leaves. It is currently under review in Indian Journal of Experimental Biology.

3.3.1 Abstract

The *in vitro* and *ex vivo* antioxidant, inhibitory effect on α-glucosidase and α-amylase activities as well as glucose absorption in rat jejunum, with concomitant enhanced glucose uptake in rat psoas muscle by different solvent fractions derived from the crude ethanol extract of the leaves of *Alstonia boonei* were investigated. There was marked increase in catalase, SOD activities and GSH levels, as well as reduced MDA levels which signified an improved pancreatic endogenous antioxidant activity. The butanol and aqueous fractions showed a high (*p* < 0.05) inhibitory activity on α-glucosidase (*IC*$_{50}$ = 26.98 and 6.49 µg/mL respectively) even higher than that of the standard drug, acarbose. The aqueous fraction caused a significant (*p* < 0.05) reduction in glucose absorption in isolated rat jejunum and enhanced glucose uptake in isolated rat psoas muscle. GC-MS analysis on the fractions revealed the presence of alkyl esters and alcohols which may be responsible for the antidiabetic potentials of the plant. Data from this study suggest that the butanol and aqueous fractions could possess potent antioxidant agents and inhibitors of conditions linked to sustained hyperglycaemia. This can further be exploited for developing alternate treatments for type 2 diabetes and its accompanying complications.

**Keywords:** *Alstonia boonei*, oxidative stress, type 2 diabetes, glucose absorption, glucose uptake.

3.3.2 Introduction

Globally, diabetes is on the rise with over 451 million people currently living with the disease (IDF, 2017). This number is a lot more than that of the year 1980 (IDF, 2015) and is projected to increase
to 693 million by the year 2045 (IDF, 2017). Shockingly, about 49.7% of people living with diabetes remain undiagnosed and Africa seems to hold the largest population of undiagnosed cases (Cho et al., 2018).

The three common types of diabetes are type 1 diabetes (T1D), type 2 diabetes (T2D) and gestational diabetes (Baynest, 2015). T2D holds the largest percentage (90%) of all the diabetic cases seen in the world today (Baynest, 2015).

The prevalence of T2D is rapidly increasing due to changes in diet and sedentary lifestyle noticeable amongst the population today. Sadly this also arises due to an increase in the number of obese individuals (Kahn et al., 2014). This disease presents with persistent hyperglycaemia which occurs when there is a malfunction in the action of the hormone insulin responsible for transport of glucose to peripheral tissue and this phenomenon is call insulin resistance ((Halban et al., 2014). This hormone is produced in the pancreatic β-cells of the islet of Langerhans (Fu et al., 2013) and insulin resistance encourages continuous build-up of glucose in the blood stream which leads to major complications of T2D (Soumya and Srilatha, 2011). These complications include nephropathy, cardiomyopathy, neuropathy, retinopathy, skin and limbs problems (Soumya and Srilatha, 2011). Complications arising from the deleterious effects of hyperglycaemia could also be pronounced by the actions of reactive oxygen species (ROS) (Kaneto et al., 2010; Volpe et al., 2018). The destructive effects of ROS are usually controlled by the action of the natural endogenous antioxidants present in the body (Sarangarajan et al., 2017). However, in conditions such as oxidative stress, the effect of the ROS overwhelms the action of the antioxidant defence systems. Oxidative stress has been implicated to play a part in the progression of the above-mentioned complications of T2D (Asmat et al., 2016).

Different treatment modalities are currently in place for the management of T2D and their mechanisms of action ranges from inhibition of hepatic glucose output, improvement and secretion of insulin (Meneses et al., 2015). Unfortunately, the affordability, availability and undesirable side effects of these drugs warrant the development of an alternative therapy (Pandey et al., 2011). Since plants are natural carriers of antioxidants, so it is wise for them to be exploited. They are widely available and can be used to boost socio-economic status of humans. Fortunately, Africa has a diverse flora of plants which offers opportunities in the discovery of medicinal plant rich in nutrients and bioactive compounds that can prevent and treat diseases such as T2D.

Alstonia boonei is a large deciduous tree towering up to 45 m in height, which belongs to the dogbane family (Apocynaceae) and is native to tropical West Africa (Okoye et al., 2014). The shape of the
leaves is oblanceolate, with the prominent lateral veins almost at right angles to the midrib. Traditionally, the plant parts of *A. boonei* are used for its aphrodisiac, antimalarial, antipyretic and antimicrobial activities (Adotey *et al.*, 2012). The antioxidant properties of the fractions from the roots of this plant has been demonstrated (Obiagwu *et al.*, 2014). Few research groups have investigated the antidiabetic effects of the plant parts of *A. boonei* (Akinloye *et al.*, 2013; Enechi *et al.*, 2014; Kehinde *et al.*, 2016; Nkono *et al.*, 2014; Zajac *et al.*, 2010), most of these researchers have worked on the stem bark extract, but the other parts of the plant have been neglected. The possible mechanisms behind the antidiabetic effects have also not been investigated. Based on the diverse medicinal values of *A. boonei*, this study was carried out not only to understand the antidiabetic effects of all parts of the plants but also to understand the mechanisms behind its antidiabetic effects.

### 3.3.3 Materials and methods

Please refer to Chapter 2 for the detailed materials and methods used for *A. boonei*.

### 3.3.4 Results

*Table 3.3.1* shows the total phenolic contents of the different fractions obtained from the ethanol extract of *A. boonei* leaves by solvent-based fractionation. The aqueous and butanol fractions showed the highest total phenol content compared to the remaining fractions.

All fractions obtained from the ethanol extract of the leaves were found to inhibit the DPPH radical in a dose-dependent manner (*Figure 3.3.1*) but lowest IC$_{50}$ value was exhibited by the aqueous fraction (*Table 3.3.1*), which was even lower than the IC$_{50}$ of ascorbic acid (standard).
Figure 3.3.1: DPPH radical scavenging activities of different solvent fractions of ethanol extract of A. boonei leaves. The results are reported as mean ± SD of three replicas. *Different alphabets over the bars for a given concentration indicate significant difference (Tukey’s-HSD multiple range post hoc test, p < 0.05). DCM: Dichloromethane, EtAc: Ethyl acetate, AA: Ascorbic acid.

All fractions demonstrated impressive ferric reducing potential. However, the total reducing power of the aqueous fraction was significantly (p < 0.05) higher than that of the other fractions and even than that of the standard antioxidant (Trolox) used (Figure 3.3.2).

Figure 3.3.2: Total reducing power (relative to gallic acid) of different solvent fractions of ethanol extract of A. boonei leaves. The results are reported as mean ± SD of three replicas. *Different alphabets presented over the bars for a given concentration indicate significance of difference (Tukey’s-HSD multiple range post hoc test, p < 0.05). DCM: Dichloromethane, EtAc: Ethyl acetate.

Upon induction of oxidative stress with Fe²⁺ in the liver homogenate, there was a reduction in the levels of the catalase, SOD activity and GSH level when the MDA level was increased in the untreated
groups (Figure 3.3.3 a & b) however; treatment with different concentrations of the fractions significantly ($p < 0.05$) increased the endogenous antioxidant enzyme activity and GSH level and deceased MDA levels almost back to normal levels. The butanol and DCM fractions showed highest ability to induce catalase activities (Figure 3.3.3 a) in the treated cells. The aqueous fraction showed the highest SOD-activity inducing ability compared to other fractions (Figure 3.3.3 b). The butanol and aqueous fractions showed highest GSH level (Figure 3.3.3 c) inducing activities with concomitant reduction in MDA levels (Figure 3.3.3 d).

**Figure 3.3.3:** Effect of fractions of *A. boonei* leaves on a) catalase activity, b) SOD activity, c) GSH level and d) lipid peroxidation in oxidative hepatic injury. Data are presented as mean ± SD of three replicas.

*Significantly different from untreated sample and #Significantly different from normal sample ($p < 0.05$, Tukey’s-HSD-multiple range post-hoc test, IBM SPSS for Windows). Normal = normal tissues, Untreated = oxidative stress injured tissues but not treated. DCM: Dichloromethane, AA: Ascorbic acid, U/L: Unit/Litre.
Figure 3.3.4 shows the effect of the different fractions of ethanol extract of *A. boonei* leaves on α-glucosidase and α-amylase activities. All fractions showed potent α-glucosidase and α-amylase inhibitory activity with the butanol and aqueous fractions giving significantly (*p < 0.05*) higher enzyme-inhibitory activities compared to other fractions, and even higher than that of the standard drug (acarbose) tested.

![Figure 3.3.4](image)

**Figure 3.3.4:** α-glucosidase inhibitory effects of the fractions from ethanol extract of *A. boonei* leaves. The results are reported as mean ±SD of three replicas. *Different alphabets presented over the bars for a given concentration indicate the significance of difference (Tukey’s-HSD multiple range *post hoc* test, *p < 0.05*). DCM: Dichloromethane, EtAc: Ethyl acetate.
Table 3.3.1: IC$_{50}$ values of the different solvent fractions of ethanol extract of A. boonei leaves in different antioxidant and antidiabetic models.

<table>
<thead>
<tr>
<th>TPC (mg/g GAE)</th>
<th>TPC</th>
<th>IC$_{50}$ value (µg/mL)</th>
<th>DPPH</th>
<th>α-glucosidase</th>
<th>α-amylase</th>
<th>Catalase</th>
<th>SOD</th>
<th>GSH</th>
<th>LPO</th>
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<td>DCM</td>
<td>51.4±1.4$^a$</td>
<td>8.6±1.8$^c$</td>
<td>110.1±4.7$^d$</td>
<td>230.1±23.2$^d$</td>
<td>11.3±2.4$^c$</td>
<td>15.4±1.3</td>
<td>8.3±1.4$^b$</td>
<td>17.1±2.2$^d$</td>
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<tr>
<td>EtAc</td>
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<td>11.4±0.5$^d$</td>
<td>122.9±2.1$^e$</td>
<td>145.6±15.1$^c$</td>
<td>8.5±1.5$^b$</td>
<td>26.8±2.3$^c$</td>
<td>13.3±1.0$^e$</td>
<td>21.3±3.3$^e$</td>
<td></td>
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<tr>
<td>Butanol</td>
<td>122.7±0.8$^c$</td>
<td>11.3±1.3$^d$</td>
<td>26.9±1.4$^b$</td>
<td>65.2±9.2$^b$</td>
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</tr>
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<td>Aqueous</td>
<td>126.8±1.8$^d$</td>
<td>2.1±0.2$^a$</td>
<td>6.5±1.0$^a$</td>
<td>53.1±10.2$^a$</td>
<td>6.4±1.1$^b$</td>
<td>5.4±1.1$^b$</td>
<td>2.2±0.1$^a$</td>
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</tr>
</tbody>
</table>

**Standards**

<table>
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<th></th>
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<th>α-glucosidase</th>
<th>α-amylase</th>
<th>Catalase</th>
<th>SOD</th>
<th>GSH</th>
<th>LPO</th>
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<tbody>
<tr>
<td>AA</td>
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<td>2.2±0.4$^a$</td>
<td>ND</td>
<td>ND</td>
<td>1.3±0.4$^a$</td>
<td>1.1±0.04$^a$</td>
<td>7.4±1.1$^b$</td>
<td>9.5±1.7$^c$</td>
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</tr>
<tr>
<td>Trolox</td>
<td>ND</td>
<td>5.8±1.3$^b$</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Acarbose</td>
<td>ND</td>
<td>36.3±4.3$^c$</td>
<td>65.1±2.4$^b$</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

DPPH-1,1-diphenyl-2-picrylhydrazyl radical, ND-not determined, EtAc, Ethyl acetate fraction, TPC, Total Phenol Content, AA, Ascorbic acid, SOD, Superoxide dismutase, GSH, Reduced glutathione, LPO, Lipid peroxidation. Data are presented as mean ± SD values of three replicas. $^a$D$^b$Different letters stand for significantly different values from one another within a column (Tukey’s-HSD multiple range post hoc test, $p < 0.05$); the same letters stand for non-significant difference.

The effect of the fractions from the ethanol extract of A. boonei leaves on glucose absorption in isolated rat intestinal jejunum is depicted in Figure 3.3.5 (a). All fractions inhibited glucose absorption by the intestine in a dose-dependent manner but the aqueous fraction showed the best inhibition.

All fractions also induced glucose uptake by the rat psoas muscle in a dose-dependent manner, but the best activity was observed for the aqueous fraction (Figure 3.3.5 b).
Figure 3.3.5: Effect of the fractions from the ethanol extract of *A. boonei* leaves on (a) glucose absorption in isolated rat jejunum and (b) glucose uptake in rat psoas muscle. Data are presented as mean ± SD of three replicas. a-c Different letters over the bars for a given concentration are significantly different from one another (*p* < 0.05, Tukey’s-HSD-multiple range *post-hoc* test, IBM SPSS for Windows). DCM: Dichloromethane.

From the GC-MS result, the aqueous and butanol fractions of *A. boonei* were found to contain mainly alkyl esters and alcohols (**Figure 3.3.6**). Phytol, phytol acetate and squalene are common natural products; squalene is the precursor to triterpenoids and sterols. The dichloromethane extract also contained these compounds; however, five sterols were also present, ergost-5-en-3β-ol, stigmasterol, sitosterol, olean-12-en-3-one and urs-12-en-3β–acetate (**Figure 3.3.7**). These are common sterols found in most plants. The list of compounds identified in the different extracts are contained in **Table 3.3.2**.
Figure 3.3.6: Long chain hydrocarbons, alkyl esters and alcohols from fractions of A. boonei leaves.

Figure 3.3.7: Sterols and aromatics from fractions of A. boonei leaves.
Table 3.3.2: Identified compounds in the fractions of *A. boonei* by GC-MS

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Molecular formula</th>
<th>RT (min)</th>
<th>Molecular mass</th>
<th>Relative abundance (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Aqueous fraction</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-hydroxy-1-(hydroxymethyl) ethyl hexadecanoate</td>
<td>C₁₉H₃₈O₄</td>
<td>23.58</td>
<td>330.50</td>
<td>41.67</td>
</tr>
<tr>
<td>2,3-dihydroxypropyl octadecanoate</td>
<td>C₂₁H₄₂O₄</td>
<td>25.14</td>
<td>358.56</td>
<td>22.47</td>
</tr>
<tr>
<td>Squalene</td>
<td>C₃₀H₅₀</td>
<td>25.77</td>
<td>410.72</td>
<td>1.25</td>
</tr>
<tr>
<td><strong>Butanol fraction</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4-((1E)-3-hydroxy-1-propenyl)-2-methoxyphenol</td>
<td>C₁₀H₁₂O₃</td>
<td>16.55</td>
<td>180.20</td>
<td>0.27</td>
</tr>
<tr>
<td>Phytol acetate</td>
<td>C₂₂H₄₂O₂</td>
<td>17.42</td>
<td>338.57</td>
<td>0.54</td>
</tr>
<tr>
<td>2-hydroxy-1-(hydroxymethyl) ethyl hexadecanoate</td>
<td>C₁₉H₃₈O₄</td>
<td>23.60</td>
<td>330.50</td>
<td>31.36</td>
</tr>
<tr>
<td>2,3-dihydroxypropyl octadecanoate</td>
<td>C₂₁H₄₂O₄</td>
<td>25.15</td>
<td>358.56</td>
<td>17.37</td>
</tr>
<tr>
<td>Squalene</td>
<td>C₃₀H₅₀</td>
<td>25.76</td>
<td>410.72</td>
<td>0.38</td>
</tr>
<tr>
<td><strong>Dichloromethane fraction</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phytol acetate</td>
<td>C₂₂H₄₂O₂</td>
<td>17.42</td>
<td>338.57</td>
<td>2.04</td>
</tr>
<tr>
<td>Ethyl eicosanoate</td>
<td>C₂₂H₄₄O₂</td>
<td>18.98</td>
<td>340.58</td>
<td>0.82</td>
</tr>
<tr>
<td>Phytol</td>
<td>C₂₀H₄₀O</td>
<td>20.12</td>
<td>296.31</td>
<td>2.19</td>
</tr>
<tr>
<td>Ethyl 14-methylhexadecanoate</td>
<td>C₁₉H₃₈O₂</td>
<td>20.85</td>
<td>298.50</td>
<td>0.51</td>
</tr>
<tr>
<td>2-hydroxy-1-(hydroxymethyl) ethyl hexadecanoate</td>
<td>C₁₉H₃₈O₄</td>
<td>23.57</td>
<td>330.50</td>
<td>13.18</td>
</tr>
<tr>
<td>2,3-dihydroxypropyl octadecanoate</td>
<td>C₂₁H₄₂O₄</td>
<td>25.13</td>
<td>358.56</td>
<td>5.78</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>C₂₀H₅₀O₂</td>
<td>28.04</td>
<td>430.70</td>
<td>0.88</td>
</tr>
<tr>
<td>Ergost-5-en-3β-ol</td>
<td>C₂₈H₄₈O</td>
<td>29.06</td>
<td>400.68</td>
<td>0.70</td>
</tr>
<tr>
<td>Stigmasterol</td>
<td>C₂₈H₄₈O</td>
<td>28.30</td>
<td>412.69</td>
<td>0.72</td>
</tr>
<tr>
<td>Sitosterol</td>
<td>C₂₉H₅₀O</td>
<td>29.91</td>
<td>414.71</td>
<td>0.97</td>
</tr>
<tr>
<td>Olean-12-en-3-one</td>
<td>C₃₀H₅₈O</td>
<td>31.42</td>
<td>424.70</td>
<td>2.96</td>
</tr>
<tr>
<td>Urs-12-en-3β-acetate</td>
<td>C₃₂H₅₂O₂</td>
<td>32.05</td>
<td>468.40</td>
<td>13.76</td>
</tr>
</tbody>
</table>

The compounds presented in the table are those which matched similar compounds in the NIST library software.
3.3.5 Discussion

Antioxidants of natural origin are being used successfully to combat oxidative stress which aids in curbing the alarming increase of complications associated with diseases such as diabetes, cancer and others (Vijay and Vimukta, 2014). This study demonstrated that the fractions of the ethanol extract of *A. boonei* leaves possess significant antioxidant and anti-diabetic activity. The presence of polyphenols in the fractions could suggest the antioxidant activity of this plant. The total phenol content was determined by Folin-Ciocalteu method which gives an estimation of the total phenolic compounds present in a sample (Kopjar *et al.*, 2009). Polyphenols have been reported to be responsible for the antioxidant activity of some medicinal plants due to their free radical scavenging activity (Kopjar *et al.*, 2009). The phenolics present in this plant may delay the onset of lipid oxidation and decomposition of hydroperoxides in tissues by neutralizing free radicals, quenching singlet oxygen through redox reactions. This could make the plant useful in ameliorating oxidative stress in T2D.

DPPH is a free radical that becomes a stable diamagnetic molecule by attracting electrons (hydrogen) from stable molecules (Kedare and Singh, 2011). The odd nitrogen atom in DPPH (violet colour) is reduced to yellow colour by receiving a hydrogen atom from antioxidants to generate a stable hydrazine complex. The observed DPPH radical scavenging activity of fractions of *A. boonei* may be linked to the presence of polyphenols in the plant. Table 3.3.1 shows that the aqueous fraction gave the lowest IC$_{50}$ value and also the highest total phenol content. It was reported that plants with DPPH scavenging activity portray antioxidant activity by transferring hydrogen to free radicals, specifically hydroperoxide radicals which are major initiators of lipid autooxidation (Bamforth *et al.*, 1993; Sowndhararajan and Kang, 2013). Interestingly, our results also reported the ability of these fractions to inhibit lipid peroxidation Figure 3.3.3 d.

FRAP assay is widely used in the evaluation of antioxidant potentials of medicinal plants (Gil *et al.*, 2002). Iron is actively involved in the propagation of free radicals through the Fenton reaction by gaining or loosing electrons (Sudan *et al.*, 2014). The aqueous fraction in this study showed a significant ($p < 0.05$) ferric reducing capacity which serves as an indicator of antioxidant ability Figure 3.3.2. This activity was also seen to be dose-dependent which indicates that with increasing doses there was an elevated activity. Although iron is an important trace element needed for sustaining physiological homeostasis, an overload of this element can lead to toxicity and cell death through formation of free radicals and lipid peroxidation (Imam *et al.*, 2017) therefore, FeSO$_4$ was used to induce oxidative stress in this study.
Incubation of the pancreatic homogenate with FeSO₄ caused a reduction in both enzymatic (catalase and SOD) and non-enzymatic GSH system which signals the initiation of oxidative stress (Figure 3.3.3 a-d).

Catalase and SOD are cellular antioxidant enzymes that work closely to convert superoxide radicals to less harmful H₂O₂ and then finally to water and oxygen molecules. If no antioxidants are present, the H₂O₂ dismutation by SOD from the superoxide ion can react with ferric ions which then elevate MDA levels thereby triggering lipid peroxidation (Erukainure et al., 2017b). Interestingly, the improvement of catalase and SOD activities and consequently inhibition of lipid peroxidation by the fractions of A. boonei signifies the antioxidant potential of this plant.

GSH is an important cellular non-enzymatic antioxidant biomolecule that prevents oxidative damage by alleviating free radical induced lipid peroxidation. Low GSH levels and inactive GSH metabolism have been reported to facilitate the progression of complications seen in T2D (Kalkan and Suher, 2013). Our results showed the ability of the fractions (especially the butanol and aqueous fraction) to significantly (p < 0.05) increase the GSH levels even more than the activity of the standard tested (ascorbic acid) (Figure 3.3.3 c). This could further justify the proposed antioxidant potential of this plant.

An effective way of managing T2D is reduction of postprandial hyperglycaemia which can be accomplished by inhibiting enzymes that hydrolyse carbohydrate (α-glucosidase and α-amylase) in the stomach (Erukainure et al., 2017b, Kalita et al., 2018). Acarbose, a synthetic drug which inhibits carbohydrate metabolism in the gastrointestinal tract presents with side effects such as nausea, blotting, itching, diarrhoea, and easy bruising (Telagari and Hullatti, 2015). Fortunately, medicinal plants have been known to exhibit α-glucosidase inhibiting activities which sheds more light on their hypoglycaemic tendencies (Yin et al., 2014). The ability of the fractions, especially the butanol and aqueous fractions, to inhibit these key carbohydrate hydrolysing enzymes in a dose-dependent manner substantiates the antidiabetic potential of A. boonei (Figure 3.3.4). The ability of this plant to inhibit α-glucosidase and α-amylase might be useful in minimising side effects such as nausea and flatulence associated with anti-diabetic synthetic drugs.

Since the aqueous, butanol and DCM fractions showed inhibition of key carbohydrate digesting enzymes it was important to investigate their ability to inhibit glucose absorption in normal rat jejunum. Excess glucose absorption by the intestine is one key pathological dysfunction in T2D and this, together with ineffective glucose uptake by peripheral organs such as the muscle, leads to
sustained hyperglycaemia (Szablewski, 2011). The rate of absorption of glucose varies from one intestinal segment to the other with highest absorption seen at the joining of the duodenum to the jejunum (proximal to mid small intestine) (Oyebode et al., 2018; Rider et al., 1967). This justified the use of this part of the intestine for our ex vivo study. From our result (Figure 3.3.5 a), without the fractions there was a high peak value absorption of glucose at 13.5±1.02 mg/cm jejunum which was seen to reduce with increasing doses of the different fractions. However, this value was significantly ($p < 0.05$) reduced to 1.13±0.24 mg/cm jejunum in the presence of the highest dose of the aqueous fraction, which suggests the inhibitory effect of the fraction on intestinal glucose absorption. In normal conditions, glucose homeostasis is regulated by insulin through glucose transporters (GLUT4) in effectively transporting excess glucose to peripheral organs where it is needed (Alvim et al., 2015). Insulin resistance in the skeletal muscle occurs as a precursor to beta cell failure and persistent hyperglycaemia (DeFronzo and Tripathy, 2009). In our study, the fractions at increasing doses were able to stimulate induction of glucose uptake in the rat psoas muscle with the highest induction exhibited by the aqueous fraction. This results in collaboration with the $\alpha$-glucosidase and $\alpha$-amylase inhibitory potential further justifies the anti-diabetic activity of $A. boonei$.

Possible bioactive compounds in the aqueous, butanol and DCM fractions of $A. boonei$ were detected using GC-MS analysis. The aqueous fraction showed a high relative abundance of 2-hydroxy-1-(hydroxymethyl) ethyl hexadecanoate (41.67%); this compound has been reported to possess antioxidant and hypocholesterolemic activities (Jegadeeswari et al., 2012; Tyagia and Argawak, 2017). The high abundance of this compound in the aqueous and butanol fractions (Table 3.3.2) could be responsible for the observed antioxidant and anti-diabetic activities of the fractions. Furthermore, the high presence of total phenols in this plant could also account for the presence of the sterols identified in the fractions. Interestingly, the observed anti-diabetic activity of this plant could be due to the likely synergistic actions of the compounds detected through GC-MS analysis.

In summary, this study showed that the fractions of $A. boonei$ leaves (especially the aqueous fraction) contains polyphenolic compounds with free radical scavenging ability and also key anti-diabetic enzymes ($\alpha$-glucosidase and $\alpha$-amylase) inhibitory potential. Furthermore, the identified bioactive agents in this study might be responsible for its antioxidant and anti-diabetic ability which can be further exploited in future studies.

**Postscript:** The butanol fraction was picked for in vivo experiment since it had high antioxidant and antidiabetic activity in the tested models.
3.4 Effects of the butanol fraction of *Alstonia boonei* leaves on pancreatic histology, glucose homeostasis and insulin secretion in diabetic rats

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**Preface:** This article reports the detailed *in vivo* anti-diabetic studies of the butanol fraction and it is currently under revision in the *Journal of Ethnopharmacology*.

3.4.1 Abstract

The potential use of *Alstonia boonei* for the management of symptoms related to type 2 diabetes (T2D) has gained more attention in recent times. However, only a few published evidences explained the roles of *A. boonei* in protecting pancreatic β-cells. This study was conducted to assess changes in polyphagia, polydipsia, body weight, glucose level, insulin secretion, β-cell function (HOMA-β), insulin resistance (HOMA-IR), serum fructosamine, liver glycogen, serum lipids, biomarkers of hepatic and renal function and histological changes in the pancreas of streptozotocin (STZ)-induced diabetic rats following treatment with butanol fraction of *A. boonei* leaves (ABBF). ABBF was given to six-weeks old STZ-induced diabetic rats for a 5-week intervention period. The glucose tolerance was assessed by oral glucose (2 g/kg) tolerance test (OGTT) and subsequently, insulin resistance and pancreatic β-cell function was assessed by calculating homeostatic model assessment (HOMA-IR and HOMA-β). The histological changes in the pancreas were then observed by hematoxylin-eosin (H&E) staining. ABBF treatment at 150 mg/kg bw, significantly (*p* < 0.05) lowered blood glucose level, improved oral glucose tolerance ability, stimulated insulin secretion and hepatic glycogen synthesis and promoted islet regeneration. These results suggest that ABBF could be exploited as a therapeutic agent for treating T2D.

**Keywords:** Type 2 diabetes, *Alstonia boonei*, pancreatic β-cells, histopathology, serum insulin

3.4.2 Introduction

The prevalence of diabetes has seen a steep rise, the IDF reported that in 2017 over 451 million (age 18-99 years) people are living with diabetes and this figure is expected to increase to 693 million by the year 2045 (IDF, 2017). This staggering statistic is being further aggravated as almost half of the
global diabetic population (49.7%) remains undiagnosed (Ogurtsova et al., 2017). In 2017 alone, over 5 million fatalities were attributed to diabetes in the 20-99 age range, and the global expenditure on diabetes rakes USD 850 million (Cho et al., 2018). Over 95% of all diabetic cases are type 2 diabetes which makes T2D the most common form of diabetes (IDF, 2017). T2D is a disease characterised by insulin resistance and partial pancreatic β-cell dysfunction which leads to persistent hyperglycemia (Kohei, 2010). Pancreatic β-cell dysfunction has been proposed to occur not only due to the loss of pancreatic β-cell mass (as a result of exhaustion) but also due to oxidative stress subsequently β-cell failure (Swisa et al., 2017). Replenishing insulin-producing pancreatic β-cells could be a crucial approach to treat T2D and its numerous complications (Aguayo-Mazzucato and Bonner-Weir, 2017). Despite different treatment modalities of T2D, glucose fluctuation in type 2 diabetic patients still remains a challenge (Li et al., 2016). Hence, there is an urgent need to find better alternatives from medicinal plants with glucose lowering abilities as well as fewer side effects.

*Alstonia boonei* is a large deciduous tree which belongs to the Apocynaceae family. *A. boonei* is being used for different purposes due to its high antioxidant power and there is a large volume of published data described that the stem bark of *A. boonei* is rich in polyphenols, tannins and flavonoids which are potent in the treatment of disease such as diabetes (Nkono et al., 2014, Kehinde et al., 2016, Akinloye et al., 2013). In this study, the butanol fraction of the leaves of *A. boonei* was subjected to a detailed anti-diabetic study in a T2D rat model.

### 3.4.3 Materials and methods

Please refer to Chapter 2 for the detailed materials and methods used for *A. boonei*.

### 3.4.4 Results

The data in Figure 3.4.1 represents the mean of daily food and fluid intake during the five-week intervention period. The DBC group showed significantly \((p < 0.05)\) higher food and fluid intake compared to the normal groups (NC and NAB). Although the food consumption in the ABBF treated groups was not statistically significant compared to the DBC group, the fluid intake in the ABBF treated groups was significantly lower than the DBC group, with no significant difference with metformin and glibenclamide treated groups.
Figure 3.4.1: The effects of oral treatment of butanol fraction of *A. boonei* leaves on food and fluid intake in different animal groups during the experimental period. Data are presented as mean ± SD of seven animals. *p*-Values with different letters over the bars for a given parameter are significantly different from one another (Tukey’s-HSD multiple range *post hoc* test, *p* < 0.05). NC, Normal control, DBC, Diabetic control, DABL, Diabetic *A. boonei* low dose, DABH, Diabetic *A. boonei* high dose, DBM, Diabetic metformin, DBG, Diabetic glibenclamide, NAB, Normal *A. boonei* high dose (toxicological control).

The data of weekly body weight changes are presented in Figure 3.4.2. In the initial 2 weeks of the experiment, there was no significant difference in the body weight amongst the different animal groups. However, the diabetic groups showed lower body weight gain compared to the normal groups during the rest of the period of the study (Figure 3.4.2).

Figure 3.4.2: The effects of oral treatment of butanol fraction of *A. boonei* leaves on mean body weight gain for all groups of experimental animals over the seven weeks experimental period. Data are presented as the mean ± SD of seven animals. *p*-Values with different letters for a given week are significantly different from one another (Tukey’s-HSD multiple range *post hoc* test, *p* < 0.05). NC, Normal control, DBC, Diabetic control, DABL, Diabetic *A. boonei* low dose, DABH, Diabetic *A. boonei* high dose, DBM, Diabetic metformin, DBG, Diabetic glibenclamide, NAB, Normal *A. boonei* high dose (toxicological control), STZ, Streptozotocin.
After STZ injection, the diabetic groups showed significantly higher blood glucose concentrations when compared to the normal groups. However, as soon as treatment of the animals started the DABL and the DABH groups portrayed a significantly lower \( p < 0.05 \) blood glucose levels than the untreated group (DBC) throughout the entire intervention period (Figure 3.4.3). The low dose (DABL) showed relatively lower blood glucose levels than the high dose (DABH) (Figure 3.4.3). The DABL group showed lower blood glucose level in both non-fasting (week 1-4) and fasting (week 5) levels compared to the DABH group.

![Figure 3.4.3](image)

**Figure 3.4.3:** The effects of oral treatment of butanol fraction of *A. boonei* leaves on weekly blood glucose concentrations in different animal groups during the intervention period. Data are presented as the mean ± SD of seven animals. *a-d* Values with different letters for a given week are significantly different from one another (Tukey's-HSD multiple range post hoc test, \( p < 0.05 \)). NC, Normal control, DBC, Diabetic control, DABL, Diabetic *A. boonei* low dose, DABH, Diabetic *A. boonei* high dose, DBM, Diabetic metformin, DBG, Diabetic glibenclamide, NAB, Normal *A. boonei* high dose (toxicological control), STZ, Streptozotocin, NFBG, Non-fasting blood glucose, FBG, Fasting blood glucose.

The data for OGTT and the corresponding area under the curve (AUC) are shown in Figure 3.4.4. The glucose tolerance of DABL and DABH groups were significantly \( p < 0.05 \) better than the DBC group and even comparable to the metformin group (DBM). A better glucose tolerance ability was observed in the DABL group at 120 min mark which was manifested all through the OGTT. The total area under the curve (AUC) of DABL and DBM groups were significantly lower than the other diabetic groups (DBC, DABH, and DBG) (Figure 3.4.4).
Figure 3.4.4: The effects of oral treatment of butanol fraction of *A. boonei* leaves on oral glucose tolerance test (OGTT) and corresponding area under the curve (AUC) of different animal groups in the last week of the intervention period. Data are presented as the mean ± SD of seven animals. Values with different letters for a given time are significantly different from one another (Tukey’s-HSD multiple range post hoc test, $p < 0.05$). NC, Normal control, DBC, Diabetic control, DABL, Diabetic *A. boonei* low dose, DABH, Diabetic *A. boonei* high dose, DBM, Diabetic metformin, DBG, Diabetic glibenclamide, NAB, Normal *A. boonei* high dose (toxicological control), AUC, Area under the curve.

The liver weight, relative liver weight and liver glycogen level of different animal groups are shown in Table 3.4.1. There was no significant difference in the liver weight of all animal groups; however, the relative liver weight of the normal groups (NC and NAB) were significantly ($p < 0.05$) lower than that of the diabetic groups. The DABL group showed a significantly ($p < 0.05$) lower relative liver weight compared to the DBC group. The treatment with ABBF increased the liver glycogen level compared to the DBC group (Table 3.4.1).
Table 3.4.1: The effects of different doses of ABBF on liver weight, relative liver weight and liver glycogen levels in the different animal groups at the end of the intervention period

<table>
<thead>
<tr>
<th></th>
<th>NC</th>
<th>DBC</th>
<th>DABL</th>
<th>DABH</th>
<th>DBM</th>
<th>DBG</th>
<th>NAB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver weights (g)</td>
<td>10.7±0.8</td>
<td>8.7±0.9</td>
<td>9.6±0.5</td>
<td>10.9±1.1</td>
<td>9.6±0.7</td>
<td>9.9±0.9</td>
<td>9.7±0.9</td>
</tr>
<tr>
<td>Rel. liver weights (%)</td>
<td>2.9±0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.8±0.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.9±0.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.0±0.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.7±0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.4±0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.7±0.1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Liver glycogen (mg/g tissue)</td>
<td>36.0±0.9&lt;sup&gt;c&lt;/sup&gt;</td>
<td>11.8±1.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>31.3±1.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>29.0±0.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>31.4±1.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>21.8±0.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>34.9±0.6&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Results are reported as mean ± SD (n=7). Different superscript letters for a given parameter along a row indicate the significance of difference (Tukey's-HSD multiple range post hoc test, p < 0.05).

Figure 3.4.5 shows the data for the serum lipid profile of all animal groups at the end of the experimental period. The DBC group showed significantly (p < 0.05) higher total and LDL cholesterol than the normal groups (NC and NAB) however, a reduced level of these cholesterols was noticed in the DABL, DABH and DBM groups. On the other hand, the DBC group showed a significantly (p < 0.05) lower level of HDL-cholesterol which was increased in the ABBF and standard drug-treated groups. Induction of T2D also caused significant (p < 0.05) increase in serum triglycerides level which was reduced in the ABBF treated groups.
Data of other serum indices of diabetic complications investigated are shown on Table 3.4.2. Serum ALT level was not affected neither by the induction of T2D nor by the treatment with plant fraction and standard anti-diabetic drugs. Levels of ALP, AST and urea in the serum were elevated in the DBC group compared to the NC and NAB groups. Interestingly, ABBF treatment significantly ameliorated the ALP, AST, urea and creatinine levels in the respective groups.

Table 3.4.2: Serum biochemical parameters in different animal groups at the end of the experimental period.

<table>
<thead>
<tr>
<th></th>
<th>NC</th>
<th>DBC</th>
<th>DABL</th>
<th>DABH</th>
<th>DBM</th>
<th>DBG</th>
<th>NAB</th>
</tr>
</thead>
<tbody>
<tr>
<td>AST (U/L)</td>
<td>57±5.3b</td>
<td>119±11.4d</td>
<td>45.7±3.4a</td>
<td>86±8.2d</td>
<td>102.5±7.1c</td>
<td>85±7.3d</td>
<td>66.4±3.5c</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>87.8±1.3</td>
<td>87.3±0.7</td>
<td>89.5±1.1</td>
<td>85±2.4</td>
<td>85.5±1.41</td>
<td>86.3±1.6</td>
<td>86.3±1.5</td>
</tr>
<tr>
<td>ALP (U/L)</td>
<td>72.3±2.5a</td>
<td>308.3±45.3c</td>
<td>294.5±12.4d</td>
<td>492.3±9.3f</td>
<td>206.8±30.1c</td>
<td>341.5±45.9c</td>
<td>89.3±2.7b</td>
</tr>
<tr>
<td>Urea (mg/dL)</td>
<td>48±2.3a</td>
<td>77±1.7d</td>
<td>51±2.6c</td>
<td>58±3.1c</td>
<td>51±2.3c</td>
<td>52±2.1b</td>
<td>47±3.6a</td>
</tr>
<tr>
<td>UA (mg/dL)</td>
<td>0.78±0.43a</td>
<td>3.5±0.9d</td>
<td>1.5±0.7bd</td>
<td>2.4±0.3c</td>
<td>1.3±0.5b</td>
<td>1.6±0.7bd</td>
<td>1.5±0.3bd</td>
</tr>
<tr>
<td>CK-MB (U/L)</td>
<td>118.9±15.2c</td>
<td>293.2±21.1d</td>
<td>274.1±15.1d</td>
<td>382.5±19.6e</td>
<td>113.9±11.5e</td>
<td>175.3±18.5c</td>
<td>138.1±15.3b</td>
</tr>
</tbody>
</table>

Data are reported as mean ± SD (n=7). Different superscript letters along a row for a given parameter indicate significance of difference (Tukey’s-HSD multiple range post hoc test, p < 0.05). NC, Normal control, DBC, Diabetic control, DABL, Diabetic A. boonei low dose, DABH, Diabetic A. boonei high dose, DBM, Diabetic metformin, DBG, Diabetic glibenclamide, NAB, Normal A. boonei high dose (toxicological control), AST, Aspartate aminotransferase; ALT, Alanine aminotransferase; ALP, Alanine phosphatase; UA, Uric acid, CK-MB, Creatinine kinase-myocardial band.

The DBC group showed a significantly (p < 0.05) lower serum insulin concentration compared to the normal groups (NC and NAB) however, the ABBF treated groups showed significantly (p < 0.05) higher insulin concentration than the untreated group (DBC) (Table 3.4.4). In the DBC group, the HOMA-IR score was significantly (p < 0.05) higher while HOMA-β score was lower compared to the normal groups. Interestingly, the DABL treatment significantly (p < 0.05) improved the HOMA-IR and HOMA-β scores at the end of the treatment period.

Table 3.4.3: Serum insulin and fructosamine concentrations as well as calculated HOMA-IR and HOMA-β scores of different animal groups at the end of the experimental period.

<table>
<thead>
<tr>
<th></th>
<th>NC</th>
<th>DBC</th>
<th>DABL</th>
<th>DABH</th>
<th>DBM</th>
<th>DBG</th>
<th>NAB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum Insulin (pmol/L)</td>
<td>138.7±19.3c</td>
<td>23.6±3.3a</td>
<td>41.5±2.6b</td>
<td>25.2±1.1a</td>
<td>59.9±8.3c</td>
<td>41.5±5.8b</td>
<td>74.3±3.5cd</td>
</tr>
<tr>
<td>Fructosamine (µmol/L)</td>
<td>495.8±17.0a</td>
<td>956.3±206.7c</td>
<td>827.1±137.1b</td>
<td>1016±309.7d</td>
<td>897.5±160.8b</td>
<td>858.5±138.6b</td>
<td>505.3±104.3a</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>4.1±1.4b</td>
<td>14±2.1d</td>
<td>4.4±0.9b</td>
<td>3.35±1.1b</td>
<td>6.51±0.8b</td>
<td>5.2±0.7bc</td>
<td>2.3±0.6a</td>
</tr>
<tr>
<td>HOMA-β</td>
<td>100.3±1.3d</td>
<td>2.7±0.2a</td>
<td>25.9±0.5a</td>
<td>9.2±0.3a</td>
<td>11.9±1.3a</td>
<td>7.0±0.4a</td>
<td>130.5±5.3b</td>
</tr>
</tbody>
</table>

Results are reported as mean ± SD (n=7). Different superscript letters for a given parameter along a row indicate the significance of difference (Tukey’s-HSD multiple range post hoc test, p < 0.05). NC, Normal control, DBC, Diabetic control, DABL, Diabetic A. boonei low dose, DABH, Diabetic A. boonei high dose, DBM, Diabetic metformin, DBG, Diabetic glibenclamide, NAB, Normal A. boonei high dose (toxicological control).
glibenclamide, NAB, Normal A. boonei high dose (toxicological control), HOMA-IR and HOMA-β; Homeostatic model assessment for IR (insulin resistance) and β (β-cell function).

Histopathological examination of the pancreas is shown in Figure 3.4.6. Microscopic examination of the normal (NC) pancreas sections showed the normal appearance of the pancreatic islets which looked lightly stained than the surrounding acinar cells (black arrow). The acinar cells are generated from pyramidal cells with basal nuclei and apical acidophilic cytoplasm. Induction of T2D caused a severe destruction to both the exocrine and endocrine components of the pancreatic islets in the DBC group (red arrow). The acinar cells appear swollen and vacuoles were seen in almost all acinar cells compared to the pancreas of NC group. The β-cells of pancreatic islets are almost entirely lost in STZ-injected rats (red arrow). Similar findings were seen in the DBG group (green arrow). However, lower dose (grey arrow) of ABBF treatment rendered protection and regeneration abilities to the T2D-associated destruction of β-cells because atrophic alterations of the acinar cells were less severe with larger islets. while higher dose (yellow arrow) of ABBF could not show any such protection of pancreatic β-cells (Figure 3.4.6).

Figure 3.4.6: Histopathological images of the pancreas of different animal groups at the end of the experiment. NC, Normal control, DBC, Diabetic control, DABL, Diabetic A. boonei low dose, DABH, Diabetic A. boonei high dose, DBM, Diabetic metformin, DBG, Diabetic glibenclamide, NAB, Normal A. boonei high dose (toxicological control).
3.4.5 Discussion

Medicinal plants have been used for many years for the treatment of diseases such as type 2 diabetes (T2D). The goal of most treatment modalities of T2D is to maintain near normal levels of blood glucose both in the fasting and non-fasting states. Although extensive researches have been done on the efficacy of medicinal plants as a source of anti-diabetic agents, some plants are yet to be explored and *Alstonia boonei* is one of them.

Frequent thirst (polydipsia) and constant hunger (polyphagia) with subsequent weight loss are major symptoms of T2D (Pan et al., 2017). The ability of ABBF to ameliorate these symptoms particularly fluid intake is noteworthy (Figure 3.4.1). The short time frame for the intervention period (5 weeks) might be responsible for the insignificant alteration of food intake and body weight gain of the experimental animals as shown in Figure 3.4.2.

A common pathogenesis of T2D is sustained hyperglycemia, shown by high fasting (> 7 mmol/L) or non-fasting (> 8.5 mmol/L) blood glucose levels. Insulin resistance together with partial pancreatic β-cell dysfunction has been implicated in favouring the progression of hyperglycemia (Cerf, 2013). Maintaining normal blood glucose levels in diabetic patients is a key factor in eliminating the various subsequent complications associated with this disease (Asif, 2014). Some medicinal plants have been reported to show their anti-diabetic activities through stimulating glucose uptake in peripheral tissues (Oyebode et al., 2018), insulin release (Islam and Choi, 2008; Patel et al., 2012), or β-cell protecting activities (Oh, 2015). In this study, the low dose of the fraction of *A. boonei* (150 mg/kg body weight) showed better anti-hyperglycemic ability than the high dose (300 mg/kg body weight). It has been noticed from other serum parameters and histopathological study of pancreas that the higher dose (300 mg/kg body weight) of ABBF was more detrimental or toxic rather than hypoglycemic and anti-diabetic in this study. However, ABBF did not show any hypoglycemic tendencies since the normal toxicological group (NAB) remained normal all through the experimental period (Figure 3.4.3), however the effects of the higher dose of ABBF in normal and diabetic animals may not be the same.

Additionally, treatment with ABBF led to improved glucose intolerance (Figure 3.4.4), which might be due to increased secretion of insulin or reduced insulin resistance that increased the uptake of glucose in peripheral tissues. In addition to the glucose tolerance improving activity, the lower dose of the fraction reduced HOMA-IR score (insulin resistance) and improved HOMA-β scores (β-cell functions) (Table 3.4.3) and pancreatic β-cell regeneration (Figure 3.4.6). These results suggest the hypoglycemic activity of *A. boonei* may not only be via reducing insulin resistance (HOMA-IR) but
also by improving pancreatic $\beta$-cell dysfunction (HOMA-\(\beta\)). In addition to these two mechanisms, ABBF also increased glucose mobilization by stimulating the synthesis of hepatic glycogen as shown by the increased liver glycogen content (Table 3.4.1).

Reduction of liver glycogen levels as a result of sporadic glycogen phosphorylation or reduced glycogen synthesis is another pathogenic feature of STZ-induced T2D (Islam, 2011). Medicinal plants have been reported to possess hepatic glycogen synthetic abilities and this has been suggested to be responsible for their anti-diabetic activity (Ezeani et al., 2017; Patel et al., 2012). The observed hepatic glycogen stimulatory activity of the fraction could suggest that the fraction might enhance glucose uptake by the liver and skeletal muscles.

Lipids are the key players in the pathogenesis of many complications of T2D. Elevated serum total cholesterol and depletion of serum HDL-cholesterol in diabetes can lead to atherosclerosis and coronary heart diseases, which are some of the fatal macrovascular complications of T2D if left untreated (Farook et al., 2011). In this study, the significant reduction of serum total cholesterol, triglycerides and LDL-cholesterol with concomitant increase of serum HDL-cholesterol after treatment with the lower dose of A. boonei fraction render cardiac protective effect A. boonei in type 2 diabetic condition (Figure 3.4.5). Plants elicit harmful cholesterol reduction and high HDL/LDL ratio by the activation of LDL receptors in liver cells to encourage effective LDL-cholesterol excretion (Kapur et al., 2008).

Other markers of diabetic complications, ALT, AST for hepatocellular injury and ALP for cholestasis were also explored in this study. Induction of T2D caused significant increase in serum levels of AST and ALP suggesting impaired liver function, while increased serum urea levels denotes impaired kidney function (Table 3.4.2). However, treatment of diabetic rats with low dose of ABBF reduced the levels of these enzymes indicating the amelioration of T2D-related liver and kidney damages.

Elevated serum uric acid levels and CK-MB (creatine kinase-myocardial band) causes an accumulation of uric acid in the joints and soft tissues triggering arthritis, gout, insulin resistance, hypertension, cardiovascular and renal diseases (Jalal et al., 2011; Momeni, 2012). The ability of the fractions of A. boonei to reduce serum uric acid levels (Table 3.4.2) could suggest the potency of the fractions in ameliorating insulin resistance and also render cardio and renal protection in type 2 diabetic patients, thereby limiting the risks of diabetic complications.

It is interesting to note that an earlier research reported the toxicity of fractions/extracts of A. boonei at a dose of 200-400 mg/kg bw (Olanlokun and Olorunsogo, 2018). This might be responsible for the
observed low antidiabetic activities and eminent β-cell toxicity as observed in our study with the high dose of ABBF (Figure 3.4.6). From the data of our study, it is clear that the low dose (150 mg/kg bw) of ABBF is a better choice for controlling hyperglycemia while >200 mg/kg bw may be toxic, as observed in a previous study (Olanlokun and Olorunsogo, 2018). However, our data clearly demonstrated the toxicity of 300 mg/kg bw dose in diabetic condition.

In conclusion, this study shows that the butanol fraction of *Alstonia boonei* leaves (at a dose of 150 mg/kg BW) is hypoglycemic which might be mediated by the modulation of pancreatic β-cell function, stimulation of insulin sensitivity and hepatic glycogen synthesis. In addition, the fraction was also able to ameliorate some T2D-related complications via ameliorating organ-function related parameters.
3.5 Butanol fraction of *Alstonia boonei* leaves ameliorates oxidative stress in a type 2 diabetes model of rats

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**Preface:** This article reports the detailed in vivo antioxidant status of the serum and organs collected from the rats in the above experiment and it is currently awaiting revision before submission to a journal for publication.

3.5.1 **Abstract**

The effects of oral administration of the butanol fraction of *Alstonia boonei* (ABBF) on antioxidative status in the serum, pancreas, liver, kidney and heart of type 2 diabetic rats was examined. The markers of oxidative stress measured in vivo were TBARS (thiobarbituric acid reactive substances), catalase, super oxide dismutase (SOD), reduced glutathione, glutathione reductase and peroxidase. Induction of type 2 diabetes (T2D) using 40 mg/kg BW caused a noticeable reduction of endogenous antioxidant enzymes as well as an increment in MDA levels (a marker of lipid peroxidation) in diabetic rats. However, treatment with ABBF (especially the low dose) significantly ($p < 0.05$) increased reduced glutathione levels and other endogenous antioxidant enzymes in the serum and other organs compared to diabetic controls. Results of this study indicate that ABBF has a strong antioxidant effect in a type 2 diabetic rat model.

**Keywords:** *A. boonei*, oxidative stress, antioxidants, lipid peroxidation, type 2 diabetes

3.5.2 **Introduction**

In type 2 diabetes (T2D), macro and micro vascular complications arise as a result of persistent hyperglycemia (Stolar, 2010). This sustained hyperglycemia tends to favor the production of free radicals through different pathways such as: glucose autooxidation, advanced glycation end products, polyol pathway, protein kinase C activation, and increased flux of the hexosamine pathway (Ahmadinejad *et al.*, 2017). When an imbalance in the ratio of free radicals to antioxidant enzymes favors free radicals it leads to a condition known as oxidative stress (Kurutas, 2015). The duo of oxidative stress and hyperglycemia have combinatorial roles in the generation of multiple complications of T2D (Matough *et al.*, 2012). Therefore, amelioration of in vivo oxidative stress is
an important therapeutic mechanism that could be beneficial to people suffering from T2D and its complications.

The deleterious effects of oxidative stress in normal body function is curbed by the action of both enzymatic and non-enzymatic antioxidant defence mechanism (Birben et al., 2012). Enzymatic antioxidants comprise of superoxide dismutase, catalase and glutathione peroxidase while the non-enzymatic antioxidants comprise of glutathione, vitamin C and E (Birben et al., 2012, Nita and Grzybowski, 2016). However, there are significant alterations in the levels and activities of these antioxidants in diabetic condition (Dal and Sigrist, 2016; Asmat et al., 2016). Oxidative stress can cause low grade inflammation which can lead to the alterations in cardiovascular structure and activity promoting cardiac complications during T2D (Valko et al., 2007). Apart from the heart, many organs are also affected by oxidative stress such as nerves, eyes, kidneys and these promote macro- and micro-vascular complications during T2D (Dal and Sigrist, 2016). Fortunately, antioxidants from plant sources have been found to render protection against the different complications of T2D. Medicinal plants have been credited for their role as antioxidants with blood glucose lowering abilities from time immemorial and an example of such a plant is Alstonia boonei.

*Alstonia boonei* belongs to the Apocynaceae family and is currently used in the management of diabetes mellitus in some parts of Africa (Akinloye et al., 2013; Osadolor et al., 2015). Various preliminary *in vitro* and *ex vivo* studies have been done in our lab in order to verify the antidiabetic and antioxidative activity related claims of this plant. The butanol fraction of the leaves of *A. boonei* was found to show promising antidiabetic activity when subjected to *in vivo* antidiabetic study in a T2D rat model (*see section 3.4 above*). The present study was performed in a bid to examine if the observed antidiabetic activity can be credited to the role of the antioxidant defence mechanism.

### 3.5.3 Materials and methods

Please refer to the Chapter 2 for the detailed materials and methods used for *A. boonei*.

### 3.5.4 Results

The extent of lipid peroxidation in the pancreas was lower than the other organs. The DBC group showed a significantly (*p* < 0.05) higher level of MDA compared to the normal and treated groups (*Figure 3.5.1*). This was also replicated in the serum, liver, heart and kidneys of the animals. The MDA levels was seen to reduce upon treatment with the ABBF, which shows the lipid peroxidation alleviating property of the fraction.
Figure 3.5.1: The effects of oral treatment of ABBF on thiobarbituric acid reactive substances (MDA equivalent) in the serum, liver, pancreas, heart and kidneys of all animal groups at the end of the intervention period. Data are presented as mean ± SD of 7 animals. Values with different letter above the bars for a given sample are significantly different from other group of animals (Tukey’s-HSD multiple range post hoc test, \( p < 0.05 \)).

NC, Normal Control, DBC, Diabetic Control, DABL, Diabetic *A. boonei* low dose, DABH, Diabetic *A. boonei* high dose, DBM, Diabetic Metformin, DBG, Diabetic Glibenclamide, NAB, Normal *A. boonei* high dose, MDA, Malondialdehyde.

The reduced glutathione (GSH) concentrations of the serum and organs of all the groups is presented on Figure 3.5.2. The GSH concentration in the serum, liver, pancreas and heart was significantly \( p < 0.05 \) lower in the DBC group upon induction of diabetes compared to the normal groups. However, the reduction in the kidney was not statistically \( p > 0.05 \) different among the animal groups. Oral administration of the fraction significantly \( p < 0.05 \) increased the GSH levels in the serum and all other organs of the DABL group except kidney.

Figure 3.5.3 shows the data for the catalase activity in serum and organs of all animal groups. The catalase activity in the liver, pancreas, heart and kidneys of DBC group was significantly \( p < 0.05 \) decreased compared to the normal groups. However, ABBF treatment in the diabetic animals significantly \( p < 0.05 \) increased the catalase activity in the organs, while it was not significant in the serum.

The activity of superoxide dismutase (SOD) in the serum and organs of all the animal groups is shown on Figure 3.5.4. The SOD activity in the serum and organs of DBC group was significantly \( p < 0.05 \) decreased. However, oral administration of ABBF for 5 weeks significantly \( p < 0.05 \) increased SOD activity in the DABL groups compared to the DBC group of the serum and organs.
**Figure 3.5.2:** The effects of oral treatment of ABBF on reduced glutathione (GSH) levels in the serum, liver, pancreas, heart and kidneys of all animal groups at the end of the intervention period. Data are presented as mean ± SD of 7 animals. **Values** with different letter above the bars for a given sample are significantly different from one another group of animals (Tukey’s-HSD multiple range post hoc test, \( p < 0.05 \)). NC, Normal Control; DBC, Diabetic Control; DABL, Diabetic *A. boonei* low dose; DABH, Diabetic *A. boonei* high dose; DBM, Diabetic Metformin; DBG, Diabetic Glibenclamide; NAB, Normal *A. boonei* high dose.

**Figure 3.5.3:** The effects of oral treatment of ABBF on catalase activity in the serum, liver, pancreas, heart and kidneys of all animal groups at the end of the intervention period. Data are presented as mean ± SD of 7 animals. **Values** with different letter above the bars for a given sample are significantly different from other group of animals (Tukey’s-HSD multiple range post hoc test, \( p < 0.05 \)). NC, Normal Control; DBC, Diabetic Control; DABL, Diabetic *A. boonei* low dose; DABH, Diabetic *A. boonei* high dose; DBM, Diabetic Metformin; DBG, Diabetic Glibenclamide; NAB, Normal *A. boonei* high dose.
Figure 3.5.4: The effects of oral treatment of ABBF on SOD activity in the serum, liver, pancreas, heart and kidney of all animal groups at the end of the intervention period. Data are presented as mean ± SD of 7 animals. *Values with different letter above the bars for a given sample are significantly different from other group of animals (Tukey’s-HSD multiple range post hoc test, p < 0.05). NC, Normal Control; DBC, Diabetic Control; DABL, Diabetic A. boonei low dose; DABH, Diabetic A. boonei high dose; DBM, Diabetic Metformin; DBG, Diabetic Glibenclamide; NAB, Normal A. boonei high dose.

The results of the glutathione reductase activity in the serum and organs of all the animal groups are shown in Figure 3.5.5. In the kidneys, the glutathione reductase activity was not altered by diabetes induction. However, the activity of this enzyme was significantly (p < 0.05) decreased in the serum, liver, pancreas and heart of the DBC group when compared to the NC and NAB groups. Oral administration of the ABBF improved the enzyme activity in the serum and organs.

Figure 3.5.5: The effects of oral treatment of ABBF on glutathione reductase (GR) activity in the serum, liver, pancreas, heart and kidney of all animal groups at the end of the intervention period. Data are presented as mean ± SD of 7 animals. *Values with different letter above the bars for a given sample are significantly different from other group of animals (Tukey’s-HSD multiple range post hoc test, p < 0.05). NC, Normal Control; DBC, Diabetic Control; DABL, Diabetic A. boonei low dose; DABH, Diabetic A. boonei high dose; DBM, Diabetic Metformin; DBG, Diabetic Glibenclamide; NAB, Normal A. boonei high dose.
Figure 3.5.6 shows the glutathione peroxidase (GPx) activity in the serum and organs. The GPx activity was significantly \((p < 0.05)\) decreased in the serum and organs of DBC group compared to the NC and NAB groups. However, treatment with ABBF significantly \((p < 0.05)\) increased the GPx activity in the diabetic treated groups which was comparable to the DBM and DBG groups.

![Graph showing GPx activity in different organs](image)

**Figure 3.5.6:** The effects of oral treatment of ABBF on glutathione peroxidase (GPx) activity in the serum, liver, pancreas, heart and kidney of all animal groups at the end of the intervention period. Data are presented as mean ± SD of 7 animals. **a-c**Values with different letter above the bars for a given sample are significantly different from other group of animals (Tukey's-HSD multiple range post hoc test, \(p < 0.05\)). NC, Normal Control; DBC, Diabetic Control; DABL, Diabetic A. boonei low dose; DABH, Diabetic A. boonei high dose; DBM, Diabetic Metformin; DBG, Diabetic Glibenclamide; NAB, Normal A. boonei high dose.

### 3.5.5 Discussion

The role of plants in the treatment of diseases such as T2D which arises as a result of the impact of oxidative stress is very important. Medicinal plants contain numerous antioxidants which are able to lend a helping hand in scavenging free radicals that are formed due to autooxidation of glucose in the severe cases of hyperglycemia.

In our previously reported data (Section 3.3), we have shown that the fractions of *Alstonia boonei* leaves contain high total phenol content, which might be responsible for their observed antioxidant activity. The present study went a step further by investigating the ability of the butanol fraction of *A. boonei* to upregulate the endogenous antioxidant system in the blood and various organs of a T2D model of rats. The serum and some major organs (liver, heart, pancreas and kidneys) of the type 2
diabetic rats were examined for both enzymatic (Catalase, SOD, GPx, GR) and non-enzymatic (MDA, GSH) after the treatment with ABBF.

Increased oxidative stress or free radicals in T2D leads to a concomitant increase in MDA levels which is a key index in determining the initiation of lipid peroxidation in the cell membrane (Nita and Grzybowski, 2016). Lipid peroxidation as a result of unmanaged hyperglycemia and hyperlipidemia causes damages to the cell membrane favouring the progression of T2D and its complications. In this study, the DBC groups of the serum and organs showed an elevation of MDA levels compared to the NC group which signified the initiation of lipid peroxidation. Our previous findings showed that (Section 3.4) the increase in cardiac and liver function biomarkers in the diabetic animals could also be related for the observed increase of MDA levels in this study. However, administration of ABBF led to the reduction of MDA levels in the serum and organs with even more promising activity than glibenclamide (Figure 3.5.1). This result confirms the free radical scavenging and lipid peroxidation reducing ability of ABBF. The increase of levels of GSH (Figure 3.5.2) in the serum and organs of the treated diabetic rats was also in line with the MDA results.

In T2D, increased free radical production reduces the activities of endogenous antioxidant enzymes, this was proven in our result (Figure 3.5.2) where the diabetic groups showed a reduction in GSH level compared to the NC group. GSH is an intracellular thiol that promotes free radical eradication process in oxidative stress. Reduced GSH levels in diabetes occurs as a result of the competition between glutathione reductase and aldose reductase for NADPH (a cofactor) (Kalkan and Suher, 2013). Figure 3.5.2 showed the ability of the plant fraction (especially the low dose) to improve GSH levels in the serum and organs (except the kidneys where no GSH activity was noticed). The results of our study showed the higher concentration of reduced glutathione in the serum and organs of the DABL and DABH groups when compared to the DBC group. This result is in line with the reduced MDA levels as recorded earlier.

Catalase and SOD are important endogenous antioxidant enzymes that are capable of transforming harmful superoxide radicals to H$_2$O$_2$ and then to harmless water and oxygen. Figure 3.5.3 showed a reduction of catalase activity in the diabetic control group which might have occurred due to the induction of T2D by a low dose of streptozotocin. The increase of catalase and SOD levels noticed in the DABL and DABH groups highlights the potency of ABBF as an antioxidant agent in managing the complications linked to T2D. The ability of ABBF to reduce lipid peroxidation and increase antioxidant enzymes correlates with an earlier reported study where the ethanol extract of the leaves of A. boonei were used (Enechi et al., 2014). Similar results were obtained from both studies.
GPx and GR are glutathione dependent enzymes and a reduction in their activities can be linked to the reduction of GSH level. GPx catalyses the reduction of lipid peroxides at the expense of GSH, which also favours H$_2$O$_2$ inactivation and encourages the higher consumption of GSH (Kumawat et al., 2013). Thus, lower level of GSH reduces the activity of these antioxidant enzymes and contributes to worsening oxidative stress. However, treatment with ABBF showed an increase in GPx and GR activities in the treated groups even up to normal levels. This shows that the butanol fraction of A. boonei (especially the low dose) has the potential to improve the antioxidant status as well as reduce the complications of T2D.

Studies have shown the presence of potent phytochemicals such as alkaloids, tannins, saponins, flavonoids and cardiac glycosides (Akinmoladun et al., 2007) and bioactive constituents such as 2-hydroxy-1-(hydroxymethyl) ethyl, eugenol, benzenedicarboxylic acid and alpha-amyrin in A. boonei (Babatunde, 2017). These bioactive compounds may be responsible for the antioxidant activities of ABBF.

From the results of this study, we have seen so far, the low dose of the fraction has shown better antioxidant activity than the high dose, this can be related to a previously reported work that showed the toxicity of a high dose (200-400 mg/kg body weight) of A. boonei fractions and also reported a longer time in recovery of subjects from the toxic effects of high dose of the plant (Olanlokun and Olorunsogo, 2018). We have also noticed the similar toxicological effects of the high dose (300 mg/kg bw) which have been evident by increment of several biochemical parameters and pancreatic histopathology in our study.

In conclusion, oral administration of ABBF especially low dose (150 mg/kg bw) showed potent antioxidant activity through the reduction of lipid peroxidation, upregulation of glutathione levels and endogenous antioxidant enzymes in the serum and organs of type 2 diabetic animals. Therefore, ABBF could serve as a good antioxidant agent and could be used in controlling oxidative stress in T2D. Further studies are required to confirm its efficacy in human type 2 diabetic patients.
CHAPTER 4
THE ANTIOXIDANT AND ANTIDIABETIC ACTIVITIES OF
ACALYPHA WILKESIANA IN VITRO AND IN VIVO

4.1 Background

*Acalypha wilkesiana* ‘Java white’ is a member of the family Euphorbiaceae and sub family Acalyphoideae (Figure 4.1), it has great decorative value due to its lovely coloured foliage. It is cultivated widely in tropical and subtropical countries (Bailey and Day, 2004). The Java white variant which is our plant of interest, has leaves with colour ranging from greenish yellow to ivory. Although it is native to the Pacific Islands (Tahiti, Fiji, New Zealand and Australia), it also widely seen in some Asian and African countries. The leaves are oval shaped with toothed and lightly coloured edges. They have tiny flowers which are reddish in colour and are formed in thin hanging spikes known as catkins (Bailey and Day, 2004).

*Figure 4.1: Acalypha wilkesiana* ‘Java white’ also known as Java white copperleaf (English), Mirror bush, Jacob’s coat (English). Photo credit: Oluwaseun Oyebode (2018) Durban, South Africa
4.1.1 Ethnomedical uses

The leaves of this plant are eaten as a vegetable in treatment of hypertension in the southern part of Nigeria (Bailey and Day, 2004). Most Acalypha species are widely exploited for their role in traditional medicine in diverse parts of Africa and some parts of Asia (Duraipandiyan et al., 2006; Sofowora, 1982). A. wilkesiana is used in parts of Nigeria in the treatment of body aches and common cold; and the boiled leaves are also used in bathing babies with skin infection (Adesina et al., 2000).

4.1.2 Biological activities

Research carried out on the leaf extract of A. wilkesiana, showed a high activity against the propagation of gram-positive bacteria while extracts obtained from the seeds showed immunomodulating activities that were able to reduce tumours in inflammatory cells (Büssing et al., 1999). Recently, ethyl acetate extract of A. wilkesiana in direct use with β-, γ- and δ-tocotrienols has been shown to possess potent anticancer activities where it was able to reduce the proliferation of cancer cells in the lungs (A549) and brain (U87MG) cells (Abubakar et al., 2018). The antioxidant activity of isolates from the butanol fraction of the leaves of A. wilkesiana was investigated recently and it was discovered that the isolates possess potent free radical scavenging activity (Oladipo et al., 2016). The antioxidant capacity of this plant cannot be farfetched since phytochemical screening from the crude extract of the plant has indicated the presence of tannins and terpenes (Akinloye et al., 2016; Oladimeji et al., 2012), which have been earlier reported to exhibit antioxidant activities (Amarowicz, 2007, Gonzalez-Burgos and Gomez-Serranillos, 2012).

4.1.3 Phytochemistry

To validate the antidiabetic traditional use of this plant (mentioned earlier), a comprehensive in vitro investigation of the antioxidant activity of this plant was tested. The different solvent crude extracts of this plant were subjected to a systematic and detailed investigation on its antioxidant capacities using different in vitro and ex vivo studies.
4.2 *Acalypha Wilkesiana* ‘Java white’ scavenges free radicals in vitro and abates Fe$^{2+}$-induced oxidative stress in rat pancreatic homogenates

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**Preface:** This article reports the detailed in vitro and ex vivo antioxidant studies on *A. wilkesiana*. The article is currently awaiting revision before submission to a suitable journal for publication.

4.2.1 **Abstract**

The in vitro antioxidant potentials of extracts from the leaves, stem and root bark of *Acalypha wilkesiana* were investigated. The plant parts were extracted sequentially with solvents of increasing polarity where aqueous and ethanol extracts showed higher polyphenol content compared to the ethyl acetate extract. However, the ethanol extract of the root bark showed significantly ($p < 0.05$) higher DPPH (1,1-diphenyl-2-picryl-hydrazyl), hydroxyl radical (OH$^-$) and nitric oxide (NO) scavenging abilities, as well as ferric reducing antioxidant power (FRAP) when compared to other extracts. This was confirmed by lower IC$_{50}$ values and comparable with the activity of positive controls (ascorbic or gallic acid and trolox). Their abilities to activate antioxidant enzymes in Fe$^{2+}$-induced pancreatic oxidative injuries was also investigated in rat pancreatic homogenates. Catalase, SOD and reduced glutathione levels were significantly ($p < 0.05$) increased by the extracts as compared to low levels seen in untreated groups. Data from this study suggest that the different parts of *A. wilkesiana*, especially the ethanol and aqueous extracts possesses potent anti-oxidant potentials that could be further investigated for medicinal benefits.

**Key words:** antioxidants; medicinal plants; *A. wilkesiana*; oxidative stress

4.2.2 **Introduction**

Medicinal plants have long been regarded as better alternatives to pharmaceutical drugs and have gained much interest in the area of drug discovery. This can be attributed to the fact that they contain necessary and substantial level of antioxidants and bioactive agents that have been reported in the management and treatment of diseases such as diabetes (Dewanjee *et al.*, 2011).
A host of plant sources are found to be rich in polyphenols such as herbs, fruits, flowers and these plants are found in abundance everywhere in the world especially in Africa where there is high reliance on medicinal plants for the treatment of diverse ailments (Hamzah et al., 2013).

Plants are rich in polyphenolic agents which are highly beneficial as they can react with reactive oxygen species/reactive nitrogen species (ROS/RNS) to bring about reduction in deleterious effects to cell biomolecules (Gach et al., 2015). Plant antioxidants are capable of attenuating damages to biomolecules (lipids, proteins and DNA), with damage ranging from lipid peroxidation to cell membrane damage through oxidation (Hossain and Shah, 2015).

Oxidative stress occurs as a result of imbalance between free radical-generating enzymes (reactive oxygen species [ROS]) and free radical-scavenging enzymes (antioxidant enzymes) and plays a key role in the progression of diseases (Asmat et al., 2016). This triggers an increased level of free radicals which initiates cellular damage and has been recognized as the main contributing factor to late complications of ailments such as diabetes and cancer (Son, 2012).

*Acalypha wilkesiana* 'Java white' belongs to the genus *Acalypha* of the family *Euphorbiaceae* (spurge family). It has over 570 variants which include *A. amentacea* and *A. tricolor* (Sule et al., 2012). Its common names include copperleaf, fire dragon and java white due to the green and white coloured variant. They are found all over the world most especially in the tropics of Africa and its native to Fiji and nearby islands in the South Pacific. It is also widely spread in the southern part of Nigeria and many parts of South Africa (Ikewuchi, 2013).

In some parts of Africa, *A. wilkesiana* is used in the treatment of bacterial and fungal infections, and the leaves and root bark are used as antidiuretics (Alade and Irobi, 1993; Ikewuchi, 2013). Plants from the *Acalypha* genus are exploited traditionally for the treatment of diseases such as jaundice, diabetes, hypertension, skin and respiratory problems (Seebaluck et al., 2015). Although, a number of studies have been conducted with this plant, the detailed antioxidant and antidiabetic potential of the green leaved species i.e. java white (*A. wilkesiana* ‘godseffiana’) will be explored.

Hence, apart from illustrating a detailed comparison of the *in vitro* antioxidant abilities of the extracts from the different parts of the plant, this study also aims to further examine the antioxidant effects of this plant on some endogenous antioxidant enzymes in rat pancreatic homogenate under conditions of oxidative stress.
4.2.3 Materials and methods

Please refer to Chapter 2 for details of the methods used for *A. wilkesiana*.

4.2.4 Results

Both the ethanol and aqueous extracts from the different parts of the plant were found to contain significant (*p* < 0.05) higher amount of total phenol reported as gallic acid equivalent as seen in Figure 4.2.1, with highest concentration observed in the ethanol extract of stem and root bark followed by the aqueous extract of leaves and root bark.

![Figure 4.2.1: Total phenolic contents of the *A. wilkesiana* extracts. Data are reported as mean ± SD. *a-c* Values with different letter above the bars for a given extract are significantly different from one another (*p* < 0.05, Tukey's-HSD-multiple range post-hoc test, IBM SPSS for Windows).](image)

The results of the DPPH radical scavenging activity of *A. wilkesiana* leaves, stem and root bark are depicted in Figure 4.2.2 and Table 4.2.1.

The results obtained from the different antioxidant models (DPPH, HRS and NO) for the plants parts of *A. wilkesiana* are summarized in Table 4.2.1. They all showed scavenging activity against DPPH radicals. The ethanol extracts of both the leaves and root bark showed significantly (*p* < 0.05) higher activity as well as lower IC₅₀ values than the others even when compared to the standards ascorbic acid and trolox. In addition, all the extracts significantly (*p* < 0.05) scavenged nitric oxide (NO) radicals (Figure 4.2.3). The ethanol extracts of the root bark showed significantly (*p* < 0.05) higher NO scavenging activity (IC₅₀ value of 14.23±0.68 µg/mL) compared to the standards used in this experiment.
Total reducing power analysis showed the extracts could reduce Fe$^{3+}$ to Fe$^{2+}$ in a dose-dependent manner as depicted in Figure 4.2.4 (a-c). The highest level of reduction was exhibited by the ethanol extract of the root bark (Figure 4.2.4 c).

The highest hydroxyl radical scavenging activity (Figure 4.2.4 d-f) was exhibited by the ethanol extract of the root bark (Figure 4.2.4 f). This was justified with a significantly ($p < 0.05$) lower IC$_{50}$ value of 7.25±1.28 µg/mL as compared to the other crude extracts from the different parts of the plant.

Figure 4.2.2: DPPH scavenging activities of A. wilkesiana extracts. Data are reported as mean ± SD. *

*Values with different letter above the bars for a given extract are significantly different from one another ($p < 0.05$, Tukey’s-HSD-multiple range post-hoc test, IBM SPSS for Windows) EtAc: Ethyl acetate, EtOH: ethanol, AA: ascorbic acid.
Figure 4.2.3: Nitric oxide scavenging activities of \textit{A. wilkesiana} extracts. Data are reported as mean ± SD. Values with different letter above the bars for a given extract are significantly different from one another (\( p < 0.05 \), Tukey’s-HSD-multiple range post-hoc test, IBM SPSS for Windows) EtAc: Ethyl acetate, EtOH: ethanol, GA: Gallic acid, AA: Ascorbic acid.
Figure 4.2.4: Total reducing power of a) leaves, b) stem bark and c) root bark extracts and hydroxyl oxide scavenging activities of A. wilkesiana d) leaves, e) stem bark and f) root bark extracts respectively. Data are reported as mean ± SD. *Values with different letter above the bars for a given extract significantly different from one another (p < 0.05, Tukey’s-HSD-multiple range post-hoc test, IBM SPSS for Windows). EtAc: Ethyl acetate, EtOH: Ethanol, Ascorbic Ac, Ascorbic acid, OH: Hydroxyl.
Table 4.2.1: IC<sub>50</sub> values of various extracts of A. wilkesiana parts in different antioxidative models

<table>
<thead>
<tr>
<th>Sample</th>
<th>DPPH</th>
<th>NO</th>
<th>Hydroxyl</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Leaves</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>2.88±0.33&lt;sup&gt;b&lt;/sup&gt;</td>
<td>&gt;1000&lt;sup&gt;i&lt;/sup&gt;</td>
<td>335.46±23.84&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ethanol</td>
<td>1.63±0.39&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&gt;1000&lt;sup&gt;ı&lt;/sup&gt;</td>
<td>82.44±27.12&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Aqueous</td>
<td>2.14±0.10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>32.41±7.94&lt;sup&gt;d&lt;/sup&gt;</td>
<td>87.34±35.91&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Stem bark</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>7.78±0.06&lt;sup&gt;d&lt;/sup&gt;</td>
<td>18.12±3.23&lt;sup&gt;ă&lt;/sup&gt;</td>
<td>156.21±48.26&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ethanol</td>
<td>5.96±0.29&lt;sup&gt;c&lt;/sup&gt;</td>
<td>269.73±52.1&lt;sup&gt;ı&lt;/sup&gt;</td>
<td>19.67±2.03&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Aqueous</td>
<td>9.63±0.31&lt;sup&gt;c&lt;/sup&gt;</td>
<td>53.22±12.39&lt;sup&gt;c&lt;/sup&gt;</td>
<td>58.78±8.62&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Root bark</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>3.73±0.69&lt;sup&gt;b&lt;/sup&gt;</td>
<td>50.52±3.65&lt;sup&gt;c&lt;/sup&gt;</td>
<td>98.47±21.69&lt;sup&gt;de&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ethanol</td>
<td>1.83±0.69&lt;sup&gt;a&lt;/sup&gt;</td>
<td>14.23±0.68&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.25±1.28&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Aqueous</td>
<td>282±35.38&lt;sup&gt;ı&lt;/sup&gt;</td>
<td>304.23±14.83&lt;sup&gt;ı&lt;/sup&gt;</td>
<td>47.79±5.73&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Standard</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gallic acid</td>
<td>ND</td>
<td>108.72±23.05&lt;sup&gt;f&lt;/sup&gt;</td>
<td>ND</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>2.19±0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>19.40±1.45&lt;sup&gt;c&lt;/sup&gt;</td>
<td>ND</td>
</tr>
<tr>
<td>Trolox</td>
<td>5.79±0.43&lt;sup&gt;c&lt;/sup&gt;</td>
<td>196.7±27.03&lt;sup&gt;ı&lt;/sup&gt;</td>
<td>3.54±0.91&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

DPPH-1,1-diphenyl-2-picrylhydrazyl radical, HRS-hydroxyl radical scavenging, NO-nitric oxide radical, ND-not determined. Data are reported as mean ± SD values of three replicas. *Different letters stand for significantly different values from one another within a column (Tukey’s-HSD multiple range post hoc test, p < 0.05): the same letters stand for non-significant difference.

The ability of the extracts, especially the ethanol extract to increase catalase activity in Fe<sup>2+</sup>-induced oxidative pancreatic injury is shown in Figure 4.2.5 with the ethanol extract of the root bark (Figure 4.2.5 c) and aqueous extract of the leaves (Figure 4.2.5 a) showing the highest activity. This was also reflected in their lower IC<sub>50</sub> values (Table 4.2.2).

There was a significant (p < 0.05) reduction in SOD activity upon induction of oxidative injury by Fe<sup>2+</sup> (Figure 4.2.5 d-f). However, treatment with different doses of the extracts led to remarkably increased SOD activities. The ethanol extracts of the stem and root bark, and the aqueous extract of the root bark showed the highest activities.
Figure 4.2.5: Catalase activities of a) leaves, b) stem bark and c) root bark extracts and SOD activities of d) leaves, e) stem bark and f) root bark extracts of *A. wilkesiana* in oxidative pancreatic injury. Data are reported as mean ± SD of three replicas. *Significantly different from untreated sample and †Significantly different from normal sample (p < 0.05, Tukey's-HSD-multiple range post-hoc test, IBM SPSS for Windows). AA: Ascorbic acid (Standard).
The effect of Fe$^{2+}$-induction of oxidative pancreatic injury on GSH level is depicted in Figure 4.2.6 (a-c). A noticeable increase in GSH levels was observed on treatment with the extracts as evident by the low IC$_{50}$ values (Table 4.2.2), the ethanol extract of the leaves showed the lowest IC$_{50}$ values compared to its counterparts and even lower than that of the standard, Ascorbic acid.

**Figure 4.2.6:** Effect of *A. wilkesiana* a) leaves, b) stem bark and c) root bark extracts on reduced glutathione (GSH) level in oxidative pancreatic injury. Data are reported as mean ± SD of three replicas. *Significantly different from untreated sample and §Significantly different from normal sample ($p < 0.05$, Tukey’s-HSD-multiple range post-hoc test, IBM SPSS for Windows). AA: ascorbic acid.
Table 4.2.2: IC<sub>50</sub> values of various extracts of A. wilkesiana parts in different enzymatic <i>ex vivo</i> models

<table>
<thead>
<tr>
<th>Sample</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; Values (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Catalase</td>
</tr>
<tr>
<td>Leaves</td>
<td></td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>5.25±1.09&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ethanol</td>
<td>2.28±0.03&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Aqueous</td>
<td>1.09±0.07&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Stem bark</td>
<td></td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>4.99±0.85&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ethanol</td>
<td>1.61±0.14&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Aqueous</td>
<td>3.66±0.27&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Root bark</td>
<td></td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>17.25±5.83&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ethanol</td>
<td>1.61±0.17&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Aqueous</td>
<td>5.09±0.04&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Standard</td>
<td></td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>19.71±8.76&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data are reported as mean ± SD values of three replicas. <sup>a-e</sup> Different letters stand for significantly different values from one another within a column (Tukey's-HSD multiple range <i>post hoc</i> test, <i>p</i> < 0.05): the same letters stand for non-significant difference. SOD: superoxide dismutase, GSH: reduced glutathione.

4.2.5 Discussion

The role and influence of oxidative stress in the pathogenesis and complications of diseases such as cancer and diabetes has long been validated as essential to understand the impact, trend and possible ways of alleviating their prevalence (Matough <i>et al.</i>, 2012). The complications of these diseases arise because of poor handling of free radicals by the body’s natural defence mechanism (Bajaj and Khan, 2012). Plants have been reported to have antioxidant activities, with polyphenolics playing a major role in this regard (Sowndhararajan and Kang, 2013).

Figure 4.2.1 demonstrates the presence of total phenols in all extracts of the three parts of A. wilkesiana (leaves, stem and root bark) which may be responsible for its potency and continuous use in traditional medicine as an antibacterial, antidiuretic and anti-diabetic agent (Ikewuchi <i>et al.</i>, 2010).
The mechanism of action of polyphenols can be attributed mainly to their structure which comprises a hydroxyl group on an aromatic ring, a key factor to its radical scavenging and metal chelating activities (Scherer and Godoy, 2014).

DPPH is a transient free radical which absorbs light maximally at 517 nm and can become stable by accepting an electron. This odd electron on nitrogen can be reduced by a donation of a hydrogen atom from an antioxidant to form a hydrazine complex. The DPPH assay is a quick and inexpensive method to determine the ability of compounds to act as free radical scavengers or hydrogen donors (Kedare and Singh, 2011). Using this model, the ethanol extracts of *A. wilkesiana* demonstrated valuable DPPH scavenging abilities, as expressed by their low IC$_{50}$ values shown in Table 4.2.1.

The hydroxyl radical is the second most deleterious and most biologically free radical. It is formed because of continuous reduction of oxygen to water. The first step in its formation is the reduction of Fe$^{3+}$ to Fe$^{2+}$ which is then closely followed by the Fenton reaction (Lipinski, 2011). This makes it easy to understand that an accumulation of iron in the body can lead to excessive generation of free radicals and ultimately progressive complications of diseases such as atherosclerosis, age related diseases and diabetes (Altamura and Muckenthaler, 2009; Brewer, 2007; Ramakrishna *et al.*, 2003). Widely known polyphenols such as curcumin, resveratrol and ferulic acid have been shown to have hydroxyl radical scavenging activity and this action is executed through oxidation of aromatic compounds (Kulling and Rawel, 2008; Lipinski, 2011).

The observed potent hydroxyl radical scavenging activities of *A. wilkesiana* (Figure 4.2.4 d-f) can be attributed to the high phenolic content *Figure 4.2.1*. This may also be responsible for the observed high reducing power of the extracts (Figure 4.2.4). This corresponds to reports on the positive correlation of high total phenolic content and high reducing power activities by plant extracts (Anahita *et al.*, 2015; Hossain and Shah, 2015). The highest reducing power of Fe$^{3+}$ was demonstrated by the ethanol extracts and this shows the capability of ethanol in extracting potent free radical scavengers.

NO is a relatively small and lipophilic molecule which acts continuously with superoxide anion radical ($O_2^-$) to produce peroxynitrite (ONOO$^-$) a very strong oxidant capable of attacking a wide range of biological molecules (Nimse and Pal, 2015). When the amount of NO exceeds that of the superoxide anion, it can act as an effector molecule in the defence against cancer cells and bacteria and inhibit lipid peroxidation by the formation of nitrated lipid radical inhibitors thereby protecting the lipid bilayer from peroxides (RapoZZi *et al.*, 2015). However, oxidant metabolites of NO such as ONOO$^-$ are capable of deleterious actions by depleting enzymatic antioxidants such as glutathione.
and ascorbic acid (Bloodsworth et al., 2000). Interestingly, extracts of A. wilkesiana showed significant inhibition of nitric oxide (Table 4.2.1). The ethanol extract of the root bark had the lowest IC$_{50}$ values and showed the highest inhibition. This indicates potent antioxidant and free radical scavenging activity.

The pancreas, liver and other organs play major roles in the activities of some endogenous antioxidant enzymes such as catalase, superoxide dismutase (SOD) and glutathione peroxidase (GPx), which converts the tripeptide glutathione (GSH) into oxidized glutathione (GSSG) and in the process, reduces H$_2$O$_2$ to H$_2$O and lipid hydroperoxides (Bhattacharyya et al., 2014). Catalase converts hydrogen peroxide (H$_2$O$_2$); a major precursor in the formation of free radicals into water and oxygen. SOD is a first line action against free radicals and catalyses the breakdown of superoxide into oxygen and peroxide (Asmat et al., 2016). GSH catalyses the conversion of peroxides to water thereby converting glutathione disulphide back into glutathione (Githens, 1991). Oxidative stress occurs due to the insensitivity or reduced quantity of these enzymes in the organs particularly in the pancreas since it is a major site for metabolism and insulin production. High intracellular levels of these enzymes cushion the effect of oxidative stress (Githens, 1991; Yeh and Yen, 2006). From the results, there was reduction in catalase and SOD activities indicating an occurrence of oxidative stress in the untreated groups (refer to Figure 4.2.5). Treatment with the extracts, especially the ethanol and aqueous extracts of both the leaves and the root bark led to remarkable increase in catalase activity (Figure 4.2.5 a-c), thus indicating the antioxidant potential of this plant. SOD activities were also found to increase upon treatment with different doses of the extracts with the ethanol extracts of both the stem and root barks showing high potentials (Figure 5 d-f). The ethanol extract of the root bark increased the GSH levels as compared with the depleted levels of the untreated groups (Figure 4.2.6). This can be attributed to the high phenolic content and high ferric reducing ability of the ethanol root bark of this plant as seen in Figure 4.2.1 and 4.2.4 (a-c).

### 4.2.6 Conclusion

The existence of free radical scavenging and oxidative stress ameliorating compounds in A. wilkesiana has been established in this study. These results indicate that no part of A. wilkesiana is a waste as all its parts have been demonstrated to possess rich antioxidant activities which gives antecedence to its use in folk medicine. These findings specifically portray the ethanol extract of the root bark as the most capable of modulating oxidative stress. Further research will entail bioassay guided fractionation and in vivo experiments to further establish the potentials of this plant in drug discovery.
4.3 *Acalypha wilkesiana* ‘Java white’: Identification of some bioactive compounds by GC-MS and effects on key enzymes linked to type 2 diabetes

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*Correspondence; islamd@ukzn.ac.za

**Preface:** This article has been published in *Acta Pharmaceutica* journal.

4.3.1 **Abstract**

In this study, we identified bioactive compounds from the ethanol extracts of the leaves, stem and root bark of *Acalypha wilkesiana* through GC-MS analysis and investigated the effects of these extracts on some of the enzymes linked to type 2 diabetes. Plant parts were extracted sequentially with ethyl acetate, ethanol and water. GC-MS analysis revealed the presence of long chain alkyl acids, esters, ketones and alcohols including phytol and phytol acetate along with some secondary metabolites such as xanthone, vitamin E and various types of sterols including stigmasterol, campesterol and sitosterol. Ethanolic extracts of all the parts showed a dose-dependent inhibition of $\alpha$-glucosidase and $\alpha$-amylase activity. The extracts also demonstrated anti-lipase activity. The ethanolic extract of the root bark showed the highest inhibition of enzymes compared to other extracts. The EC$_{50}$ values (concentration for 50% inhibition) of $\alpha$-glucosidase, $\alpha$-amylase and lipase inhibition were 35.75±1.95, 6.25±1.05 and 101.33±5.21 µg/mL, respectively. This study suggests that *A. wilkesiana* ethanolic extracts have the ability to inhibit the activity of enzymes linked to type 2 diabetes. Further studies are needed to confirm the responsible bioactive compounds in this regard.

**Keywords:** *Acalypha wilkesiana*, $\alpha$-glucosidase, $\alpha$-amylase, lipase, type 2 diabetes, ethanolic extract

4.3.2 **Introduction**

Diabetes has gradually become a major health concern in both developed and developing countries, while controlling hyperglycaemia is one of the major challenges in the management of this disease (IDF, 2017). Apart from hyperglycaemia, almost 50% of diabetic patients suffer from at least one or two diabetic complications such as diabetic retinopathy, cardiomyopathy, nephropathy, neuropathy and lower limb amputations, which are more noticeable in elderly patients (Roglic, 2016).
Approximately 108 million people were diagnosed with diabetes in 1980, which has increased to 422 million in 2014, with a projected increase to 642 million by the year of 2030 (Roglic, 2016).

Diabetes is a metabolic disorder caused by deficiency of insulin secretion by the pancreatic beta-cells (type 1), insulin insensitivity, and partial pancreatic beta-cell malfunction (type 2) (Lakshmi et al., 2012). Type 2 diabetes (T2D) is the most prevalent form of diabetes and occurs due to the insensitivity of insulin to postprandial glucose overload which causes persistent hyperglycaemia (Al-Goblan et al., 2014). The spread of this disease has been linked to societal and cultural changes in diet and lifestyle which could lead to obesity, a major risk factor for the rapidly rising prevalence of T2D (Tobias et al., 2014). Obesity as a result of abdominal fat deposit may lead to sustained insulin resistance in obesity related T2D (Levelt et al., 2016). Controlling hyperglycaemia is a major challenge in the management of T2D.

A number of conventional drugs such as sulfonylureas, biguanides and α-glucosidase inhibitors are currently being used for the management of hyperglycaemia as well as T2D. α-glucosidase is linked to the breakdown of disaccharides and starch into glucose and α-amylase breakdowns long-chain carbohydrates into glucose (Kumar et al., 2011). Inhibition of α-glucosidase and α-amylase is one of the key targets in the discovery of potential drugs for the treatment of T2D via reducing small intestinal glucose absorption (Yilmazer-Musa et al., 2012). However, high cost and numerous side-effects of these synthetic drugs has led to the exploration of some alternative therapies in alleviating T2D (Safavi et al., 2013). Additionally, the side-effects including nausea, diarrhoea (commonly seen in patients on metformin), hypoglycaemia, weight gain (sulfonylureas), increased risk of heart failures and bone fractures (thiazolidinediones) (Wu et al., 2016) led to the search for alternative therapies, particularly from medicinal plants. Medicinal plants are found in abundance in all over the world including Africa and have been reported to be rich in bioactive phenolics which can be used to treat diverse diseases (Bahadoran et al., 2013) including T2D.

*Acalypha wilkesiana* 'Java white' is a member of the spurge family (Euphorbiaceae). It is a tropical and subtropical evergreen shrub with greenish yellow to white dotted green leaves mottled with irregular green spots. It grows in various parts of Africa but native to South Pacific islands. In western Nigeria, a boiled decoction of the leaves of *A. wilkesiana* Müll. Arg. is used in the treatment of fungal infections seen in new-borns, and also against hypertension and diabetes mellitus in adults (Olukunle et al., 2016). The leaves of this plant also possess anti-inflammatory, anti-microbial and anti-pyretic activities (Ikewuchi et al., 2011). Although some hypoglycaemic, anti-diabetic and hypolipidemic
effects of *A. wilkesiana* Müll. Arg. of leaves and roots have been reported in some recent studies (Olukunle *et al.*, 2016, Odoh *et al.*, 2014, Mohammed *et al.*, 2014), underlying mechanisms behind these effects and phytochemical constituents of different parts of this plant are not fully understood.

Odoh *et al.* (2014) and Olukunle *et al.* (2016) reported the antidiabetic and hypoglycaemic as well as hypolipidemic activities of root and leaf extracts respectively in alloxan-induced diabetic rats, however, stem bark was not used in their studies and Odoh *et al.* (2014) only qualitatively determined the phytochemical constituents in root extracts. Furthermore, Igwe *et al.* (2016) only analysed leaf ethanol extract to identify its bioactive compounds by using GC-MS, when other parts of the plant have not been analysed. On the other hand, although aqueous and ethanol extracts of the leaves and root bark of this plant have been shown to reduce blood glucose levels in alloxan-induced diabetic animals (Odoh *et al.*, 2014; Olukunle *et al.*, 2016), the mechanisms behind these effects are still unknown.

Hence, this study was designed not only to analyse the phytochemical constituents of the ethanolic extracts of leaf, root bark and stem bark of *A. wilkesiana* “Java white” via GC-MS analysis but also to examine the effects of various extracts from the different parts of the plant on the activity of enzymes linked to T2D such as α-glucosidase, α-amylase and lipase to understand the mechanisms behind their reported hypoglycaemic and antidiabetic effects (Odoh *et al.*, 2014; Olukunle *et al.*, 2016).

4.3.3 Materials and methods

Please refer to Chapter 2 for details of the methods used for *A. wilkesiana*.

4.3.4 Results and Discussion

*GC-MS analysis*

Ethanolic extracts of the leaves, stem bark and root bark of *A. wilkesiana* ‘Java white’ were subjected to GC-MS analysis to identify some major bioactive compounds.

According to the data reported (Figures 4.3.1, 4.3.2, 4.3.3 and Tables 4.3.1, 4.3.2 and 4.3.3), 25 phytochemicals were identified from this plant which included four major classes of compounds: fatty acids and fatty acid esters, phytols, xanthones and sterols, many of which have been reported to have various medicinal effects including α-amylase and α-glucosidase inhibitory activities (Fotie and Bohle, 2006; Ghosh *et al.*, 2015; Olofsson *et al.*, 2014; Takato *et al.*, 2017; Mahendran *et al.*, 2014;
Mahammed et al., 2015; Luo et al., 2014). Additionally, EC50 or the maximum concentration required for 50% inhibition of enzyme activity was also calculated for different extracts from each part of the plant for different enzymes and reported in Table 4.3.4.

**Figure 4.3.1:** Long chain alkyl acids, esters, ketones and alcohols identified from the ethanolic extract of the leaves, stem bark and root bark of *A. wilkesiana.*
Figure 4.3.2: Aromatics, sterols and triterpenes identified in the ethanolic extract of leaves, stem and root bark of *A. wilkesiana*. 
Figure 4.3.3: GC-MS chromatogram of ethanol extract of *A. wilkesiana* leaves a), stem bark b) and root bark c).
**Table 4.3.1:** Compounds identified in the ethanolic extract of the leaves of *A. wilkesiana* by GC-MS

<table>
<thead>
<tr>
<th>Compound</th>
<th>Molecular formula</th>
<th>$t_R$ (min)</th>
<th>$M_r$</th>
<th>Relative abundance (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phytol acetate</td>
<td>C$<em>{22}$H$</em>{42}$O$_2$</td>
<td>16.42</td>
<td>338.57</td>
<td>5.76</td>
</tr>
<tr>
<td>6-Octadecenoic acid methyl ester</td>
<td>C$<em>{19}$H$</em>{36}$O$_2$</td>
<td>19.00</td>
<td>296.49</td>
<td>0.57</td>
</tr>
<tr>
<td>Phytol</td>
<td>C$<em>{20}$H$</em>{40}$O</td>
<td>19.10</td>
<td>296.53</td>
<td>2.00</td>
</tr>
<tr>
<td>Octadecanoic acid</td>
<td>C$<em>{18}$H$</em>{36}$O$_2$</td>
<td>19.55</td>
<td>284.48</td>
<td>2.56</td>
</tr>
<tr>
<td>9-(4-Methoxyphenyl) xanthene</td>
<td>C$<em>{20}$H$</em>{16}$O$_2$</td>
<td>22.39</td>
<td>288.39</td>
<td>2.89</td>
</tr>
<tr>
<td>2-Hydroxy-1-(hydroxymethyl)ethyl hexadecanoate</td>
<td>C$<em>{19}$H$</em>{38}$O$_4$</td>
<td>22.52</td>
<td>330.50</td>
<td>1.76</td>
</tr>
<tr>
<td>Acridin-9-yl-(4-methoxy-phenyl)-amine</td>
<td>C$<em>{20}$H$</em>{16}$N$_2$O</td>
<td>24.33</td>
<td>300.35</td>
<td>0.38</td>
</tr>
<tr>
<td>Oleana-11,13(18)-diene</td>
<td>C$<em>{30}$H$</em>{48}$</td>
<td>27.22</td>
<td>408.70</td>
<td>1.48</td>
</tr>
<tr>
<td>Vitamin E (α-tocopherol)</td>
<td>C$<em>{29}$H$</em>{50}$O$_2$</td>
<td>27.37</td>
<td>430.71</td>
<td>1.16</td>
</tr>
<tr>
<td>Campesterol</td>
<td>C$<em>{28}$H$</em>{48}$O</td>
<td>28.64</td>
<td>400.68</td>
<td>2.00</td>
</tr>
<tr>
<td>Stigmasterol</td>
<td>C$<em>{29}$H$</em>{48}$O</td>
<td>28.96</td>
<td>412.69</td>
<td>2.32</td>
</tr>
<tr>
<td>Sitosterol</td>
<td>C$<em>{29}$H$</em>{50}$O</td>
<td>29.77</td>
<td>414.71</td>
<td>1.81</td>
</tr>
</tbody>
</table>

The compounds reported in the table are those which matched the same compounds in the NIST library software.

**Table 4.3.2:** Compounds identified in the ethanol extract of the stem bark of *A. wilkesiana* by GC-MS

<table>
<thead>
<tr>
<th>Compound</th>
<th>Molecular formula</th>
<th>$t_R$ (min)</th>
<th>$M_r$</th>
<th>Relative abundance (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tetradecanoic acid</td>
<td>C$<em>{14}$H$</em>{28}$O$_2$</td>
<td>15.56</td>
<td>228.37</td>
<td>0.39</td>
</tr>
<tr>
<td>1-Methylethyl tetradecanoate</td>
<td>C$<em>{13}$H$</em>{34}$O$_2$</td>
<td>16.25</td>
<td>270.45</td>
<td>0.31</td>
</tr>
<tr>
<td>Phytol acetate</td>
<td>C$<em>{22}$H$</em>{42}$O$_2$</td>
<td>16.41</td>
<td>338.57</td>
<td>2.00</td>
</tr>
<tr>
<td>Ethyl hexadecanoate</td>
<td>C$<em>{18}$H$</em>{36}$O$_2$</td>
<td>17.97</td>
<td>284.48</td>
<td>2.60</td>
</tr>
<tr>
<td>Methyl cis-13-octadecenoate</td>
<td>C$<em>{19}$H$</em>{38}$O$_2$</td>
<td>18.99</td>
<td>296.43</td>
<td>0.43</td>
</tr>
<tr>
<td>Phytol</td>
<td>C$<em>{20}$H$</em>{40}$O</td>
<td>19.10</td>
<td>296.53</td>
<td>1.05</td>
</tr>
<tr>
<td>9-(4-Methoxyphenyl) xanthene</td>
<td>C$<em>{20}$H$</em>{16}$O$_2$</td>
<td>22.39</td>
<td>288.34</td>
<td>5.01</td>
</tr>
<tr>
<td>Stigmast-4-ene-3-one</td>
<td>C$<em>{20}$H$</em>{48}$O</td>
<td>23.24</td>
<td>412.69</td>
<td>1.82</td>
</tr>
<tr>
<td>Campesterol</td>
<td>C$<em>{29}$H$</em>{48}$O</td>
<td>28.64</td>
<td>400.68</td>
<td>3.08</td>
</tr>
<tr>
<td>Stigmasterol</td>
<td>C$<em>{29}$H$</em>{48}$O</td>
<td>28.96</td>
<td>412.69</td>
<td>1.56</td>
</tr>
<tr>
<td>Sitosterol</td>
<td>C$<em>{29}$H$</em>{50}$O</td>
<td>29.75</td>
<td>414.71</td>
<td>7.57</td>
</tr>
</tbody>
</table>

The compounds reported in the table are those which matched the same compounds in the NIST library software.
Table 4.3.3: Identified compounds in the ethanol extract of the root bark of A. wilkesiana by GC-MS

<table>
<thead>
<tr>
<th>Compound</th>
<th>Molecular formula</th>
<th>$t_R$ (min)</th>
<th>$M_r$</th>
<th>Relative abundance (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-(4-Hydroxy-3-methoxyphenyl)-2-butanone</td>
<td>C$<em>{11}$H$</em>{14}$O$_3$</td>
<td>14.37</td>
<td>194.23</td>
<td>0.63</td>
</tr>
<tr>
<td>Phytol acetate</td>
<td>C$<em>{23}$H$</em>{46}$O</td>
<td>16.41</td>
<td>338.57</td>
<td>0.46</td>
</tr>
<tr>
<td>6,10,14-Trimethyl-2-pentadecanone</td>
<td>C$<em>{18}$H$</em>{36}$O</td>
<td>16.46</td>
<td>268.48</td>
<td>0.89</td>
</tr>
<tr>
<td>Xanthone</td>
<td>C$<em>{13}$H$</em>{30}$O$_2$</td>
<td>16.95</td>
<td>196.20</td>
<td>1.68</td>
</tr>
<tr>
<td>Hexadecanoic acid methyl ester</td>
<td>C$<em>{17}$H$</em>{36}$O$_2$</td>
<td>17.30</td>
<td>270.45</td>
<td>0.43</td>
</tr>
<tr>
<td>Hexadecanoic acid ethyl ester</td>
<td>C$<em>{18}$H$</em>{36}$O$_2$</td>
<td>17.97</td>
<td>284.48</td>
<td>1.54</td>
</tr>
<tr>
<td>8-Octadecenoic acid methyl ester</td>
<td>C$<em>{19}$H$</em>{36}$O$_2$</td>
<td>18.99</td>
<td>296.49</td>
<td>1.91</td>
</tr>
<tr>
<td>(Z,Z)-9,12-octadecadienoic acid</td>
<td>C$<em>{18}$H$</em>{32}$O$_2$</td>
<td>19.28</td>
<td>280.45</td>
<td>0.70</td>
</tr>
<tr>
<td>9-(4-Methoxyphenyl) xanthene</td>
<td>C$<em>{20}$H$</em>{18}$O$_2$</td>
<td>22.38</td>
<td>288.34</td>
<td>21.02</td>
</tr>
<tr>
<td>Acridin-9-yl-(4-methoxy-phenyl)-amine</td>
<td>C$<em>{20}$H$</em>{16}$N$_2$O</td>
<td>24.55</td>
<td>300.35</td>
<td>0.36</td>
</tr>
<tr>
<td>Campesterol</td>
<td>C$<em>{28}$H$</em>{48}$O</td>
<td>28.64</td>
<td>400.68</td>
<td>3.71</td>
</tr>
<tr>
<td>Stigmasterol</td>
<td>C$<em>{29}$H$</em>{48}$O</td>
<td>28.96</td>
<td>412.69</td>
<td>2.08</td>
</tr>
<tr>
<td>Sitosterol</td>
<td>C$<em>{29}$H$</em>{50}$O</td>
<td>29.75</td>
<td>414.71</td>
<td>9.60</td>
</tr>
</tbody>
</table>

The compounds reported in the table are those which matched the same compounds in the NIST library.

Although Odoh et al. (2014) qualitatively determined the presence of alkaloids, terpenoids, flavonoids, saponins, steroids and tannins in root methanol extract and Igwe et al. (2016) identified 12 compounds in leaf ethanol extract, in our study, 25 major bioactive compounds were identified in the ethanol extracts of various parts of the plant (Figures 4.3.1-3 and Tables 4.3.1-3). Igwe et al. (2016) identified predominantly terpenes followed by long chain fatty acids, long chain fatty acyl alcohols, esters and their derivatives when we additionally identified phytol, phytol acetate, vitamin E, and various types of sterols such as campesterol, stigmasterol and sitosterol in our study (Table 4.3.1, Figures 4.3.1-3). Apart from leaf, the ethanol extracts of root bark and stem bark were also analysed by GC-MS in our study although no additional type of compounds were identified (Figures 4.3.1-3 and Tables 4.3.2, 4.3.3) except xanthone and an aromatic amine in root ethanol extract (Figure 4.3.2 and Table 4.3.3). From the results of the GC-MS analysis, although a number of bioactive compounds have been identified in A. wilkesiana extracts, some additional techniques such
as- LC-MS or HPLC-PDA could expand the list of bioactive compounds so further studies are warranted in this regard.

**α-glucosidase and α-amylase inhibitory activity**

The effects of various extracts on two key enzymes, α-glucosidase and α-amylase, implicated in T2D are shown in Figure 4.3.4 (a-f). All the extracts showed significant α-glucosidase ($p < 0.05$) inhibitory activity, in a dose dependent manner. The ethanolic extracts from the stem bark and root barks, however, showed significantly ($p < 0.05$) higher activities than the others. This is also evident by their lower $EC_{50}$ values compared to other extracts (Table 4.3.4). The ethanol extract of the root bark showed the presence of xanthone (Figure 4.3.2, Table 4.3.3). Xanthones are biologically active plant phenols which have received profound interest lately (Fotie and Bohle, 2006). Xanthones have been found to possess anti-inflammatory, anti-cancer, anti-hyperglycemic and anti-hypertensive effects amongst others (Mahendran et al., 2014, Mahammed et al., 2015). A study conducted in 2014 has shown that xanthones extracted from the plant *Swertia mussotii* exert α-glucosidase inhibitory activity (Luo et al., 2014). In a previous study, it has been also reported that long chain unsaturated fatty acids have significant α-glucosidase inhibitory activity (Artanti et al., 2012). This implies that the α-glucosidase inhibitory activity of *A. wilkesiana*, especially the low $EC_{50}$ values of the ethanolic root bark (Table 4.3.4) could be attributed to the xanthones and long-chain unsaturated fatty acids and their derivatives.

In vitro pancreatic α-amylase activity was also inhibited by all extracts of the plant parts, with the most significant ($p < 0.05$) activity exhibited by the ethanol extract of the root bark, with the lowest $EC_{50}$ value of 6.25±1.05 μg/mL.

Ethanolic extracts of the various parts of *A. wilkesiana* have been reported to have various long chain fatty acid and fatty acid esters e.g. tetradecanoic acid, octadecanoic acid, octadecanoic acid methyl ester, phytol, phytol acetate, xanthone and various sterols including stigmasterol (Figures 4.3.1-3). In a number of recent studies, plants extract containing these compounds have been shown to have α-amylase, α-glucosidase and lipase inhibitory activity (Mopuri et al., 2018, Chen et al., 2017, Payghami et al., 2015). Hence, the enzyme inhibitory activity of this plant might be linked to some of these bioactive compounds. We are inclined to believe that the inhibition of these enzymes by *A. wilkesiana* might indicate potential anti-hyperglycemic efficacy, which could corroborate its folkloric use as a medicinal plant for the treatment and management of T2D.
Figure 4.3.4: α-glucosidase and α-amylase inhibitory activities of leaves (a & b), stem bark (c & d) and root bark (e & f) extracts of *A. wilkesiana*. Data are reported as mean ± SD of three replicas. **Different letters over the bars for a given concentration are significantly different from one another (p < 0.05). EtAc: ethyl acetate, EtOH: ethanol."
Figure 4.3.5: Anti-lipase activities of: a) leaves, b) stem bark and c) root bark extracts of *A. wilkesiana*. Data are reported as mean ± SD of three replicas. *a-d* Different letters over the bars for a given concentration are significantly different from one another (*p* < 0.05). EtAc; ethyl acetate, EtOH; ethanol.
Table 4.3.4: EC₅₀ values of various extracts of *A. wilkesiana* parts in inhibiting α-glucosidase, α-amylase and lipase activities

<table>
<thead>
<tr>
<th>Sample</th>
<th>EC₅₀ (µg/mL)</th>
<th>α-glucosidase</th>
<th>α-amylase</th>
<th>Lipase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(µg/mL)</td>
<td>(µg/mL)</td>
<td>(µg/mL)</td>
</tr>
<tr>
<td><strong>Leaves</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>74.63±2.87ᵇ</td>
<td>296.18±37.18ᶠ</td>
<td>384.55±23.84ᶜ</td>
<td></td>
</tr>
<tr>
<td>Ethanol</td>
<td>67.18±7.65ᵇ</td>
<td>75.35±8.25ᶜ</td>
<td>149.70±8.34ᶜ</td>
<td></td>
</tr>
<tr>
<td>Aqueous</td>
<td>88.35±15.37ᶜ</td>
<td>118.9±19.3ᵈᵉ</td>
<td>203.71±1.82ᵈ</td>
<td></td>
</tr>
<tr>
<td><strong>Stem bark</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>242.99±41.83ᶜ</td>
<td>274.55±31.09ᶠ</td>
<td>146.88±19.84ᶜ</td>
<td></td>
</tr>
<tr>
<td>Ethanol</td>
<td>37.10±2.71ᵃ</td>
<td>&gt;1000ᵍ</td>
<td>113.38±18.23ᵇ</td>
<td></td>
</tr>
<tr>
<td>Aqueous</td>
<td>267.94±24.90ᶜ</td>
<td>&gt;1000ᵍ</td>
<td>894.61±36.28ᶠ</td>
<td></td>
</tr>
<tr>
<td><strong>Root bark</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>124.04±16.72ᵈ</td>
<td>98.65±15.11ᵈ</td>
<td>&gt;1000ᶠ</td>
<td></td>
</tr>
<tr>
<td>Ethanol</td>
<td>35.75±1.95ᵃ</td>
<td>6.25±1.05ᵃ</td>
<td>101.33±5.21ᵇ</td>
<td></td>
</tr>
<tr>
<td>Aqueous</td>
<td>127.25±23.60ᵈ</td>
<td>151.04±12.59ᵉ</td>
<td>&gt;1000ᵉ</td>
<td></td>
</tr>
<tr>
<td><strong>Standard</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acarbose</td>
<td>36.27±1.84</td>
<td>53.77±3.95</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Orlistat</td>
<td>ND</td>
<td>ND</td>
<td>0.03</td>
<td></td>
</tr>
</tbody>
</table>

Data are reported as mean ± SD values of three replicas.ᵃᵇ Different superscripts in a column for a given parameter denote significant difference from one another; *p* < 0.05. EC₅₀ - the minimum extract concentration required for 50 % inhibition, ND - not determined. Due to the difference between the extracts (mixture of many compounds) and standard (pure compound) their values cannot be compared with one another.

Pancreatic lipase inhibitory activity

Results given in Figure 4.3.5 (a-c) show the inhibitory potential of the extracts of all parts for pancreatic lipase activity, thus demonstrating potential anti-obesogenic activity, with the best activity exhibited by the ethanol extract of the root bark (EC₅₀: 101.33±5.21 µg mL⁻¹).

Overweight and obesity are responsible for causing insulin resistance, one of the two major pathogenesis ways of T2D (Wing *et al.*, 2011). On the other hand, the most popular screening method in the discovery of anti-obesogenic drugs is to identify potent lipase inhibitors (Ado *et al.*, 2013).
Hence, one of our objectives was to examine the ability of the plant extracts to inhibit lipase. Ethanolic and aqueous extracts of the leaves have been reported to have serum triglycerides lowering abilities (Omage et al., 2018). This might be due to their reducing small intestinal absorption of dietary lipids via inhibiting lipase activity. The pancreatic lipase inhibitory activity displayed by the ethanolic extract of the root bark (Figure 4.3.5 c) with considerably low $EC_{50}$ values (Table IV) could further support the potential of $A. wilkesiana$ for the management of T2D and its associated risk factors such as overweight and obesity.

4.3.5 Conclusions

The presence of potentially bioactive compounds in $A. wilkesiana$ exerting carbohydrates and lipids digesting enzymes inhibitory effects is suggested in this study. These results indicate that no part of $A. wilkesiana$ is a waste since it could contain potent inhibitory activities against some key enzymes linked to diabetes and obesity, which also gives antecedence to its use in folk medicine. These findings specifically point out the ethanolic extract of the stem bark and root bark as the more active extracts compared to the leaf extracts. Although a number of bioactive compounds have been identified in $A. wilkesiana$ extracts by GC-MS analysis, some additional techniques such as LC-MS or HPLC-PDA could expand the list of bioactive compounds, so further studies are warranted in this regard.
5.1 Background

*Crassocephalum rubens* Juss. ex Jacq. is an erect annual aromatic shrub (traditional leafy vegetable) growing up to 1 m in height, it belongs to the family Asteraceae. The plant is cultivated in western tropical Africa as a vegetable it is commonly eaten in parts of Africa like Nigeria and Benin republic and falls under the under-utilized and neglected plants (Dansi *et al.*, 2012). It is usually found in areas with an annual rainfall of 1000-1600 mm and openly distributed in lowlands. The leaves are mucilaginous in nature and they are eaten raw or cooked in some parts of Africa (Cameroon and Nigeria) (Dansi *et al.*, 2008, Yehouenou *et al.*, 2010).

*Figure 5.1: Crassocephalum rubens*  English name: Ragweed, Nigerian names: Ebire, Ebolo, Gbolo  Photo credit: Adjatin *et al.* (2013b).
5.1.1 Ethnobotanical uses

The leaves can be used to treat liver problems as a laxative and provided to women before or during childbirth to ease labour pains (Burkill and Dalziel, 1985). Skin burns can be treated using a poultice made from the leaves. The sap from the leaves has anti-parasitic and analgesic properties. A trace presence of alkaloids has been discovered in the leaves. The powdered root has been used for the treatment of breast cancer externally. It is also used as a nutraceutical as it is believed to have antibiotic, anti-inflammatory, anthelminthic, anti-diabetic, antimalaria and blood purifying properties. The leaves are eaten to manage indigestion, colds and intestinal worms (Dansi et al., 2012).

5.1.2 Biological activities

The fresh leaves of *C. rubens* has been reported to contain essential oils which have antimicrobial properties and inhibitory effects on food borne pathogenic microorganisms (Yehouenou et al., 2010). The phytochemical analysis of the leaves of *C. rubens* showed the presence of coumarins, tannins, mucilage, flavonoids and other reducing compounds (Adjatin et al., 2012). The aqueous extract of the leaves of this plant has also been shown to possess hepatoprotective abilities in a study where co-administration of rifampicin with 300 mg/kg body weight of the extract alleviated oxidative stress in the liver considerably (Omoregie et al., 2015). Another study also reported the hepatoprotective effect of *C. rubens* in carbon tetrachloride-induced hepatic damage (Olusola et al., 2015). The β-cell protective and antidiabetic activity of *C. crepidioides* (also a member of the Asteraceae family) has been reported (Bahar et al., 2017) but there is a dearth of information on the antidiabetic activity of *C. rubens*. 
5.2 Crassocephalum rubens, a leafy vegetable suppresses oxidative pancreatic and hepatic injury, and inhibits key enzymes linked to type 2 diabetes in vitro, ex vivo and in silico

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Preface: This article reports the detailed in vitro antioxidant and anti-diabetic effects of the crude extracts from C. rubens, which is currently under review in the Journal of Food Biochemistry.

5.2.1 Abstract

Crassocephalum rubens is a shrub that falls under the wild, underutilized and under cultivated traditional leafy vegetables (TLV) in Africa. It is eaten by few (old aged people) in the management of symptoms of diabetes mellitus. This study was undertaken to examine the in vitro, ex vivo antioxidant and antidiabetic potential of different extracts of C. rubens. The ferric reducing antioxidant power (FRAP), DPPH and NO (nitric oxide) scavenging activity as well as α-glucosidase and pancreatic lipase inhibitory potentials of the extracts were examined in vitro. Also, the ameliorative effects of the extracts on Fe²⁺-induced oxidative injury was investigated ex vivo together with the effects of the aqueous extract on intestinal glucose absorption and muscle glucose uptake in freshly harvested tissues from normal rats. The aqueous extract was further subjected to Liquid Chromatography-Mass Spectrometry (LC-MS) analysis to identify the possible bioactive compounds. The extracts showed potent free radical scavenging and antioxidant activities, while ethanol and aqueous extracts showed better effects compared to others. The aqueous extract also significantly (p < 0.05) inhibited α-glucosidase and lipase enzymes activity, intestinal glucose absorption and enhanced muscle glucose uptake when compared to controls. The extract significantly (p < 0.05) improved the activity of catalase, superoxide dismutase (SOD) and GSH levels in Fe²⁺-induced oxidative injury as evident in concomitant reduction of lipid peroxidation ex vivo. The results of this study suggest that the aqueous extract of C. rubens possess better antioxidant and anti-diabetic potentials compared to other extracts which could be associated to some of its identified bioactive compounds.
Keywords: *Crassocephalum rubens*, antioxidant, glucose absorption, glucose uptake, enzyme inhibition

5.2.2 Introduction

Globally, the spread of diabetes mellitus cannot be underestimated; it currently affects over 425 million people and this number will experience an increase to over 600 million by the year 2045 (IDF, 2017). More than 16 million from this number are located in the African region (Roglic, 2016). This increase has largely occurred due to a rapid increase in sedentary lifestyle and increased consumption of junk foods which are responsible for the increase of central or visceral obesity, one of the major risk factors of type 2 diabetes (Forouhi and Wareham, 2010).

Diabetes mellitus is a disease which occurs due to prolonged impaired insulin secretion (type 1) or action (type 2) which then leads to hyperglycaemic (Kerner and Brückel, 2014). Type 2 diabetes (T2D) is the most prevalent form (95%) of all diabetes types and is characterized mainly by loss of insulin sensitivity (WHO, 2016), while partial pancreatic beta-cells dysfunction as well as lower insulin secretion is the another major pathogenesis of T2D. These all lead to sustained hyperglycaemic. Sustained hyperglycaemic leads to the generation of reactive oxygen species which overburdens the antioxidant defence mechanism present in the body, resulting in oxidative stress (Yan, 2014). Oxidative stress leads to deleterious consequences which affects the cell macromolecules such as DNA, proteins and lipids. It has long been implicated in the development of multiple complications such as diabetic nephropathy, retinopathy, neuropathy, and cardiomyopathy (Asmat et al., 2016; Vikram et al., 2014).

Different treatment modalities are currently available for the management of T2D, but the shortcomings and limitations of this approach has led to the search for alternative therapies with the aim of reducing hyperglycemia (Kokil et al., 2015, Pandey et al., 2011). Traditional leafy vegetables (TLV) are strongly consumed due to their high nutritional value and are mostly grown in the rural areas. TLVs have recently started gaining recognition for the management and control of various noncommunicable diseases including diabetes. These plants are rich in fibres, micronutrients such as minerals and vitamins which can act as antioxidants (Adjatin et al., 2012; Wais et al., 2012; Yuan et al., 2016). An example of such plant is *Crassocephalum rubens*.

*C. rubens* is an erect herb 30-70 cm in height. It belongs to the family Asteraceae and its common English names include thickhead, fireweed, or regleaf while its local names are ebolo (Yoruba,
Unlike most TLVs that occur seasonally, *C. rubens* is available all year round since it can be harvested when it is blooming, sundried, stored and consumed in dry seasons when it becomes unavailable (Adjatin *et al.*, 2012). This plant is highly recommended because it has a high nutritional value and gives diversity to food by adding flavour (Ahohuendo *et al.*, 2012, Oseni and Olawoye, 2015). The leaves are eaten like a salad or in soups and sauces and it is believed to be fibre-rich, which is good for lowering blood glucose levels in diabetic patients (Adjatin *et al.*, 2012). The plant is also consumed for its antioxidant, anti-helminthic, anti-inflammatory and anti-malarial properties (Iwalewa *et al.*, 2005).

The toxicity of the aqueous extract of the leaves of *C. rubens* has been investigated on experimental animals and up to 1000 mg/kg body weight dosages were found to be non-toxic and even offer protection to some body organs (Adewale *et al.*, 2016, Iwalewa *et al.*, 2005). The phytochemical screening of the leaves of this plant showed the presence of pharmacologically active substances such as tannins, coumarins, flavonoids and all which works to emphasize its role as a nutraceutical (Adjatin *et al.*, 2013b). The details of the hypoglycemic properties of this plant remains unresolved and this has led to the aim of this study.

### 5.2.3 Materials and methods

Please refer to Chapter 2 for the detailed materials and methods used for *C. rubens*.

### 5.2.4 Results

The ethanol extract showed the highest total phenol contents recorded as gallic acid equivalent compared to the other extracts (Figure 5.2.1).
Figure 5.2.1: Total phenolic contents of C. rubens extracts. Data are reported as mean ± SD of three replicas. Values with different letters above the bars for a given extract are significantly different from each other (p < 0.05, Tukey’s HSD-multiple range post-hoc test, IBM SPSS for Windows).

Figure 5.2.2 shows the DPPH free radical scavenging activities of the different extracts of C. rubens. All extracts showed a dose-dependent inhibition of DPPH with the aqueous extract showing the best activity with resultant low EC_{50} values (Table 5.2.1).

The total reducing power of all the extracts is shown in Figure 5.2.3; all extracts showed high Fe^{3+} to Fe^{2+} reducing potential. However, the ethyl acetate extract showed the highest total reducing power amongst the extracts.
Figure 5.2.3: Ferric reducing antioxidant power (FRAP) activity of *C. rubens* extracts. Data are reported as mean ± SD of three replicas. *a-d* Values with different letter above the bars for a given concentration are significantly different from each other (*p* < 0.05, Tukey’s HSD-multiple range post-hoc test, IBM SPSS for Windows). EtAC, Ethyl acetate; EtOH, Ethanol; AA, Ascorbic acid.

Figure 5.2.4 depicts the nitric oxide scavenging activities of the plant. All extracts were able to inhibit nitric oxide effectively in a dose-dependent manner with the ethanol and aqueous extract showing better activities even from their calculated EC_{50} values (Table 5.2.1).

The effect of different doses of the extracts from *C. rubens* on endogenous antioxidant enzymes is shown in Figure 5.2.5 (a-d). Induction of oxidative injury in the pancreatic tissue homogenate is confirmed by the low levels of antioxidant enzymes compared to the respective controls. The extracts were able to increase the activities of catalase (Figure 5.2.5 a) and SOD (Figure 5.2.5 b) with the aqueous extract showing the best activity for both enzymes. Figure 5.2.5 c shows the ability of the extracts to improve GSH levels in oxidative pancreatic injury. The ethanol extract showed the best activity in this experiment. The MDA levels is also depicted in Figure 5.2.5 d as a representation of lipid peroxidation in the pancreatic tissue homogenate. Interestingly, the aqueous extract showed the best lipid peroxidation inhibiting activity which proves the presence of potent endogenous antioxidant defence mechanism. The EC_{50} values of these models are shown on Table 5.2.1.
Figure 5.2.5: Effect of extracts of *C. rubens* on (A) catalase activity, (B) Superoxide dismutase (SOD) activity, (C) Reduced glutathione (GSH) level and (D) lipid peroxidation in oxidative pancreatic injury. Data are reported as mean ± SD of three replicas. *Significantly different from untreated sample and **significantly different from untreated sample (p < 0.05, Tukey's-HSD multiple range post-hoc test, IBM SPSS for Windows). Normal = normal tissues, Untreated = tissues oxidatively injured but not treated. EtAc; Ethyl acetate, EtOH; Ethanol, AA; Ascorbic acid, MDA; Malondialdehyde.

Figure 5.2.6. a) and b) shows the α-glucosidase inhibiting and anti-lipase activity of the plant extracts. The aqueous extracts showed best results for the two enzymes inhibiting assays.
Figure 5.2.6: (A) α-glucosidase, (B) Lipase inhibitory activity of extracts of C. rubens. Data are reported as mean ± SD of three replicas. a-c Different letters over the bars for a given concentration for each extract are significantly different from each other (p < 0.05, Tukey's-HSD multiple range post-hoc test, IBM SPSS for Windows). EtAC, Ethyl acetate; EtOH, Ethanol.

The effect of the aqueous extract (since it showed best results in the tested models) of C. rubens on glucose absorption and glucose uptake in tissues is shown in Figure 5.2.7 a & b. The aqueous extract was able to significantly (p < 0.05) inhibit glucose absorption and induce glucose uptake in the tested tissues. The GU₅₀ value for the glucose uptake is shown in Table 5.2.1.
Figure 5.2.7: Effect of the ethanol extract of *C. rubens* on (A) glucose absorption in isolated rat jejunum and (B) glucose uptake in rat psoas muscle. Data are reported as mean ± SD of three replicas. Different letters over the bars for a given concentration are significantly different from each other \( p < 0.05 \), Tukey's-HSD-multiple range post-hoc test, IBM SPSS for Windows). Metformin: 2 mg/dL.
**Table 5.2.1:** EC$_{50}$ summary table for inhibitory effects of different extracts of *C. rubens*

<table>
<thead>
<tr>
<th>Sample</th>
<th>DPPH (µg/mL)</th>
<th>NO (µg/mL)</th>
<th>Catalase (µg/mL)</th>
<th>SOD (µg/mL)</th>
<th>GSH (µg/mL)</th>
<th>Lipid peroxidation (µg/mL)</th>
<th>α-glucosidase (µg/mL)</th>
<th>Anti-lipase (µg/mL)</th>
<th>GU$_{50}$ (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Aerial parts</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>2.08±0.71$^a$</td>
<td>&gt;1000$^d$</td>
<td>35.03±2.53$^c$</td>
<td>25.82±3.61$^a$</td>
<td>12.51±2.04$^d$</td>
<td>52.68±9.34$^b$</td>
<td>2.26±1.08$^a$</td>
<td>1.69±1.03$^b$</td>
<td>ND</td>
</tr>
<tr>
<td>Ethanol</td>
<td>2.97±0.23$^a$</td>
<td>14.46±3.24$^a$</td>
<td>15.72±1.04$^a$</td>
<td>42.85±5.64$^c$</td>
<td>1.95±0.83$^a$</td>
<td>64.28±7.51$^b$</td>
<td>19.66±3.84$^b$</td>
<td>13.12±6.23$^c$</td>
<td>4.35±0.74</td>
</tr>
<tr>
<td>Aqueous</td>
<td>6.73±0.16$^b$</td>
<td>20.86±2.81$^b$</td>
<td>13.51±1.26$^a$</td>
<td>33.32±4.62$^b$</td>
<td>5.21±1.17$^c$</td>
<td>31.27±3.62$^a$</td>
<td>2.16±1.12$^a$</td>
<td>2.84±1.01$^b$</td>
<td>ND</td>
</tr>
<tr>
<td><strong>Standard</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>2.19±0.05$^a$</td>
<td>196.71±27.4$^5c$</td>
<td>19.71±3.91$^b$</td>
<td>32.01±5.27$^b$</td>
<td>3.05±0.58$^b$</td>
<td>35.15±7.24$^a$</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>ND</td>
<td>191.12±23.0$^5c$</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Trolox</td>
<td>5.79±0.4$^b$</td>
<td>19.4±1.06$^b$</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Acarbose</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Orlistat</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.03±0.12$^a$</td>
<td>ND</td>
</tr>
</tbody>
</table>

DPPH-1,1-diphenyl-2-picrylhydrazyl radical, NO-nitric oxide radical, ND-not determined. Data are reported as mean ± SD values of three replicas. $^a$-$^d$ Different letters stand for significantly different values from each other within a column (Tukey’s-HSD multiple range post hoc test, $p < 0.05$): the same letters stand for non-significant difference.
Figure 5.2.8. shows the structures of the compounds identified by Liquid Chromatography-Mass Spectrometry.

![Structures of compounds](image)

**Figure 5.2.8**: Structures of the compounds isolated from the aqueous extract of *C. rubens* by LC-MS analysis.

Table 5.2.2 shows the binding affinities (kcal/mol) of compounds isolated from the extract with Sanguisorbic acid dilactone showing the highest binding affinity for catalase (-10.5 kcal/mol) and lipase (-10.4 kcal/mol) enzymes.

**Table 5.2.2**: Binding affinities (kcal/mol) of compounds isolated from the aqueous extract with antioxidant and anti-diabetic enzymes.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Antioxidants</th>
<th>Anti-diabetic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Superoxide dismutase</td>
<td>Catalase</td>
</tr>
<tr>
<td>6-hydroxy-2-cyclohexen-one-carboxylate</td>
<td>-4.8</td>
<td>-6.9</td>
</tr>
<tr>
<td>3,4-Methylenedioxybenzoic acid</td>
<td>-5.0</td>
<td>-7.3</td>
</tr>
<tr>
<td>8-Methoxy-6,7-methylenedioxycoumarin</td>
<td>-5.1</td>
<td>-8.3</td>
</tr>
<tr>
<td>Sanguisorbic acid dilactone</td>
<td>-7.9</td>
<td>-10.5</td>
</tr>
</tbody>
</table>
Figure 5.2.9 shows images of 3-D (a) and 2-D (b) interactions of Sanguisorbic acid dilactone isolated from the aqueous extract (the highest binding affinity -10.5 kcal/mol) with amino acid residues of docked complex of catalase enzyme.

![Image of 3-D (a) and 2-D (b) interactions with amino acid residues of docked complex of catalase enzyme isolated from the aqueous extract with the highest binding affinity -10.5 kcal/mol.](image.png)

**Figure 5.2.9:** Illustration of 3-D (a) and 2-D (b) interactions with amino acid residues of docked complex (Sanguisorbic acid dilactone-catalase) isolated from the aqueous extract with the highest binding affinity -10.5 kcal/mol (Highest binding affinity compared to all antioxidant enzyme considered).

Figure 5.2.10 shows images of 3-D (a) and 2-D (b) interactions of Sanguisorbic acid dilactone with amino acid residues of docked complex of lipase. When compared to the binding energy of this compound with the α-glucosidase enzyme, lipase had the highest binding energy.

![Image of 3-D (a) and 2-D (b) interactions with amino acid residues of docked complex of lipase isolated from the aqueous extract with the highest binding affinity -10.4 kcal/mol.](image.png)

**Figure 5.2.10:** Illustration of 3-D (a) and 2-D (b) interactions with amino acid residues of docked complex (Sanguisorbic acid dilactone-lipase) isolated from the aqueous extract with the highest binding affinity -10.4 kcal/mol (Highest binding affinity compared to α-glucosidase; anti-diabetic).
5.2.5 Discussion

The antioxidant activities of traditional leafy vegetables have recently been exploited. These plants contain a wide array of phytochemicals (Kinyi et al., 2016), so it is not surprising that they are enjoyed as a delicacy and used by the locals in the treatment of diseases like T2D (Chauhan et al., 2014). Out of the complex multiple pathogenesis of T2D, oxidative stress remains the precursor of the diverse complications associated with this disease (Folli et al., 2011). Fortunately, alternate therapies can be produced from plants rich in antioxidants, which can serve as free radical scavengers (Heravi et al., 2015).

The presence of total phenols in the different extracts of C. rubens in this study shows its strong antioxidant potentials (Figure 5.2.1). Polyphenols in food and vegetables are antioxidant phytochemicals that contribute to free radical scavenging activities (Zhang et al., 2015).

DPPH assay is a fast and reliable method to estimate free radical scavenging activity (Pavithra and Vadivukkarasi, 2015). DPPH, a stable free radical, which accepts an electron of hydrogen radical from an antioxidant to form a diamagnetic molecule (Jadid et al., 2017). An antioxidant can donate an electron to DPPH, thereby reducing the violet color of DPPH to orange. The DPPH scavenging activity demonstrated by the different extracts of C. rubens especially the aqueous extract (Figure 5.2.2 & Table 5.2.1) suggests that this plant has constituents with hydrogen-donating ability.

Iron has been reported to play a major role in the generation of free radicals through its enzymatic or non-enzymatic reaction with superoxide ion (Haber Weiss reaction) or hydrogen peroxide (Fenton reaction). The reducing power of plants has been linked to their antioxidant capacity (Apak et al., 2016). The reduction of Fe$^{3+}$ is often used as a marker for electron donating ability which signals potent antioxidants (Nabavi et al., 2009). Antioxidants perform their roles by donating a hydrogen atom with the sole intention of breaking a free radical chain (Meir et al., 1995). The extracts showed good reducing power in a dose dependent manner (Figure 5.2.3). The polyphenols present in the extracts of C. rubens caused the reduction of Fe$^{3+}$ to the ferrous form (Fe$^{2+}$) thereby proving its antioxidant potential.

Nitric oxide is a free radical normally produced in mammalian cells and is involved in the regulation of various physiological processes. Unfortunately, high generation of nitric oxide radicals has been linked to the progression of several diseases (Ohkawa et al., 1979). Substances which prevent the production of NO radicals have become an interesting material of research for targeting chronic inflammatory diseases (Phaniendra et al., 2015). This study investigated the inhibitory effects of the
plant extracts on nitric oxide production. The generation of nitric oxide from sodium nitroprusside at physiological pH was inhibited by *C. rubens* (Figure 5.2.4 & Table 5.2.1).

In the mitochondria, the electron transport chain (ETC) causes the formation of reactive oxygen species (ROS), molecular oxygen is reduced to form superoxide anions which can yield more ROS. H$_2$O$_2$ is either scavenged by catalase or it helps in the formation of more ROS. Studies have shown that depletion of endogenous antioxidant enzymes such as superoxide dismutase (SOD), catalase, reduced glutathione (GSH) leads to the progression of lipid peroxidation which can ultimately lead to oxidative stress (Tiwari *et al.*, 2013). In Figure 5.2.5, incubation of the pancreatic homogenate with FeSO$_4$ led to a reduced concentration of SOD, catalase and GSH levels which is indicative of successful induction of pancreatic oxidative injury (Oyebode *et al.*, 2018; Ramakrishnan *et al.*, 2011). There was also a concomitant increase in malondialdehyde (MDA) levels which signalled the induction of lipid peroxidation (Figure 5.2.5 D). The dismutation of superoxide by SOD forms molecular oxygen or hydrogen peroxides (H$_2$O$_2$), which is more stable than the superoxide anion. The H$_2$O$_2$ was efficiently broken down to water and molecular oxygen. Extracts from *C. rubens* were able to effectively improve the activities of these enzymes with simultaneous reduction of lipid peroxidation (Figure 5.2.5). These activities could be due to the presence of polyphenols which could also be responsible for the observed *in vitro* antioxidant ability (Table 5.2.1).

The treatment of diabetes and its related complications can be achieved by inhibiting the action of key enzymes linked to carbohydrate (α-glucosidase) and lipid (lipase) digestion (McCulloch, 2007). Bioactive compounds from plant sources have been reported to be useful in inhibiting α-glucosidase and lipase activities (Mai *et al.*, 2007; Oyebode *et al.*, 2018; Tan *et al.*, 2017). The aqueous and ethanol extract of *C. rubens* demonstrated potent α-glucosidase and lipase inhibitory activities which could be due to the presence of polyphenols in these extracts (Figure 5.2.6).

The high antidiabetic activity exhibited by the aqueous extract prompted the need for more investigation of the effect of this extract on glucose absorption and uptake in jejunal tissues and psoas muscle of normal rats respectively. In T2D, there is sustained and prolonged hyperglycemia as a result of build-up of glucose in the blood stream. Peripheral tissues such as the jejunum engage actively in the uptake of the excess glucose from the blood which then causes complications such as T2D and obesity (Thazhath *et al.*, 2014). This also occurs with concomitant inhibition of glucose uptake by the muscle as a result of defective insulin signalling (Bonadonna *et al.*, 1996; Kelley *et al.*, 1993). *C. rubens* was able to inhibit intestinal glucose absorption in harvested rat jejunum (Figure 5.2.7 A) and
also induce the uptake of glucose in isolated rat psoas muscle (Figure 5.2.7 B). This effect was consistent and dose-dependent in both assays which shows that a higher dose of the aqueous extract of the plant will bring the required anti-diabetic activity.

Liquid chromatography-mass spectrometry analysis on the aqueous extract was carried out to identify bioactive components present in the aqueous extract. This study revealed the presence of four compounds, including a polyphenolic dilactone (Figure 5.2.8). 3,4-Methylenedioxybenzoic acid found in the plant is a phenol and it is also known as piperonylic acid. 3,4-Methylenedioxybenzoic acid has been reported to possess antioxidant properties (Polya, 2003; Tan et al., 2017). The anti-diabetic properties of 8-Methoxy-6,7-methylenedioxycoumarin also found in this study has been reported (Tk, 2012, Venugopala et al., 2013). Sanguisorbic acid dilactone an ellagitaninn found present in the extract (Figure 5.2.8) has been reported to alleviate oxidative stress (García-Villalba et al., 2015). The presence of these compounds in the aqueous extract of C. rubens might render synergistic activities to its anti-diabetic and antioxidant activity of the plant.

Molecular docking results revealed Sanguisorbic acid dilactone have the highest binding potential for catalase when compared to other antioxidant enzymes considered in this study. The docking study for Sanguisorbic acid dilactone showed the formation of hydrogen bonding, Pi interactions and Van der Waals interactions as shown in Figure 5.2.9. The O-H and oxygen group of Sanguisorbic acid dilactone forms hydrogen bonding with the following amino acid residues of catalase (ARG72, ARG112, GLY147). The Van der Waals interactions were with MET350, HIS218, LEU299, SER217, ASN148, PHE 132 TYR358, PHE132, GLY131 ALA133, PHE3334, HIS362, THR361, VAL74 VAL73, PHE161. It was also observed that Sanguisorbic acid dilactone gave the highest docking binding energy for lipase compared to α-glucosidase (Table 5.2.2, Figure 5.2.10). These high binding energies of Sanguisorbic acid dilactone revealed more stable interactions with catalase and lipase compared to other compound identified in this study. This could provide valuable information towards proposing the catalytic mechanism of these compounds.

5.2.6 Conclusions

This study sheds light on the antioxidant and anti-diabetic capacity of C. rubens extracts and also identified the role of the bioactive constituents present in the aqueous extract. The free radical scavenging, inhibiting key enzymes linked to diabetes and intestinal glucose absorption together with the induction of muscle glucose ability of C. rubens was well justified in this study. However further in vivo work should be done to confirm the results obtained in this study.
5.3 *Crassocephalum rubens* improves pancreatic histology, insulin secretion, liver and kidney function parameters in a type 2 diabetic rat model

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**Preface:** This article reports the detailed *in vivo* anti-diabetic effects of the crude extracts from *C. rubens*, it is currently awaiting revision before submission to a journal for publication.

5.3.1 **Abstract**

*Crassocephalum rubens* is a traditional leafy vegetable (TLV) eaten in parts of Africa for the management of diabetes mellitus. This study was conducted to investigate the *in vivo* anti-diabetic activity of aqueous extract of *C. rubens* aerial parts (CRAQ) in a type 2 diabetes model of rats. In this study, type 2 diabetes (T2D) was induced in male Sprague dawley (SD) rats by feeding 10% fructose solution for two weeks followed by a single intraperitoneal injection of streptozotocin (40 mg/kg bw). After confirmation of T2D, animals were treated with a low (150 mg/kg bw) and a high dose (300 mg/kg bw) of the extract for five weeks. During this period, food and fluid intake was measured daily and body weight and blood glucose were measured weekly when oral glucose tolerance test was conducted in the last week of the intervention period. Treatment with the high dose of extract significantly (*p* < 0.05) reduced blood glucose level, improved oral glucose tolerance level and pancreatic β-cell function and histology compared to diabetic control group. The high dose also increased the serum insulin, liver glycogen, and improved cardiac, liver and renal damages significantly compared to the untreated diabetic group. Data from this study suggest that *C. rubens* possesses anti-diabetic activity and could be useful in ameliorating some macrovascular complications associated with T2D, therefore this plant can be exploited in finding new alternate therapies for the treatment of T2D.

**Key words:** *Crassocephalum rubens*, type 2 diabetes, histopathology, liver glycogen, rats.

5.3.2 **Introduction**

Diabetes mellitus is a global world health problem, the prevalence is on the rise and in 2017 over 427 million people were considered diabetic (IDF, 2017). This number represents only the people who
were tested as a large number of people are presently living with diabetes undiagnosed (WHO, 2016). Diabetes is a complex metabolic disease characterised by hyperglycaemia, pancreatic β-cell dysfunction and insulin resistance (Arredondo et al., 2018). Type 2 diabetes (T2D) accounts for over 90% of all recorded diabetic cases and unhealthy eating habits, lack of exercise, urbanisation favours the prevalence of T2D. Hyperglycaemic-induced oxidative stress is responsible for the multiple complications associated with T2D. These complications grouped into microvascular (affecting the small blood vessels) and macrovascular (affecting the large blood vessels) complications range from retinopathy, nephropathy, neuropathy, and cardiovascular problems (Forbes and Cooper, 2013). The effective control of high blood glucose levels is very important for curbing the complications associated with T2D.

Presently, different drugs are available for the treatment and management of T2D. These drugs include but are not limited to sulfonylureas, biguanides, thiazolidinediones, glucagon-like peptide (GLP-1 analogues), incretin memetics and so on. These drugs although quite effective when used alone or in combination with other drugs, possess some unwanted short and long-term side effects such as nausea (biguanides such as metformin), increased risk of heart problems (thiazolidinediones), weight gain (metformin, insulin secretagogues), weight loss (glibenclamide) (Chaudhury et al., 2017).

Despite different treatment modalities currently used for T2D, getting a diabetic patient to normal glucose levels still remains a challenge. Fortunately, nature has provided mankind with medicinal plants which holds the key to wellness and longevity. These plants have since time immemorial been exploited for treating diverse diseases since they are loaded with antioxidants and blood lowering nutrients in abundant supply (Rani and Maheshwari, 2014).

An example of such potent medicinal plant is *Crassocephalum rubens* which is a traditional leafy vegetable (Figure 5.1) and is used in various parts of Africa for the treatment of diseases such as T2D. In previous sections of this chapter, the *in vitro*, *ex vivo* antioxidant and antidiabetic activities of this plant has been reported in detail. This section serves to outline the effect of aqueous extract of aerial parts of *Crassocephalum rubens* (CRAQ) in a type 2 diabetes rat model.

5.3.3 Materials and methods

Please refer to Chapter 2 for the detailed materials and methods used for *C. rubens.*
5.3.4 Results

The data for mean daily food and fluid intake during the five-week intervention period are shown in Figure 5.3.1. The normal groups (NC and NCR) showed significantly \((p < 0.05)\) lower food and fluid intake compared to the diabetic untreated group (DBC). Although, the food intake of the CRAQ groups showed no statistically significant difference compared to the standard drugs (DBM and DBG), it was relatively lower compared to the DBC group. However, there was significantly \((p < 0.05)\) lower fluid consumption seen in the DCRH group compared to the DBC and DCRL group although not as low as the standard drug metformin but statistically the same as glibenclamide.

![Figure 5.3.1](image)

**Figure 5.3.1**: Mean feed and fluid intake in different animal groups during the experimental period. Data are reported as mean ± SD of seven animals. °°Values with different letters over the bars for a given parameter are significantly different from each other (Tukey’s-HSD multiple range post hoc test, \(p < 0.05\)). NC, Normal control, DBC, Diabetic control, DCRL, Diabetic C. rubens low dose, DCRH, Diabetic C. rubens high dose, DBM, Diabetic metformin, DBG, Diabetic glibenclamide, NCR, Normal C. rubensi high dose (toxicological control).

**Figure 5.3.2** shows the mean weekly body weight change over the seven-weeks experimental period. No significant difference was observed in the body weight of all animal groups (both normal and diabetic) before the induction of T2D. However, there was a reduction in the body weight of the animals in the diabetic groups which is very common after streptozotocin injection. Interestingly, in the last 2 weeks of the experiment (weeks 6 and 7), there was a steady increase in the body weight of animals treated with a higher dose of the extract (DCRH group) compared to the DBC group.
Figure 5.3.2: Mean body weight change in all groups over the seven weeks experimental period. Data are reported as the mean ± SD of seven animals. *Values with different letters for a given week are significantly different from each other (Tukey’s-HSD multiple range post hoc test, p < 0.05). NC, Normal control, DBC, Diabetic control, DCRL, Diabetic *C. rubens* low dose, DCRH, Diabetic *C. rubens* high dose, DBM, Diabetic metformin, DBG, Diabetic glibenclamide, NCR, Normal *C. rubens* high dose (toxicological control), STZ, Streptozotocin, T2D, Type 2 diabetes.

All the diabetic groups showed significantly higher blood glucose concentrations compared to the normal groups after injection of STZ. However, treatment of the diabetic animals with CRAQ and the standard drugs showed a reduced blood glucose levels than the untreated groups all through the intervention period (Figure 5.3.3). The high dose (DCRH) showed significantly lower blood glucose level than the low dose which demonstrated a dose-dependent reduction of blood glucose levels by the extract. The DCRH group showed lower blood glucose level in both non-fasting (week 1-4) and fasting (week 5) levels (near normal) compared to the DCRL group.
Figure 5.3.3: Weekly blood glucose concentrations in different animal groups during the intervention period. Data are reported as the mean ± SD of seven animals. Values with different letters for a given week are significantly different from each other (Tukey's-HSD multiple range post hoc test, \( p < 0.05 \)). NC, Normal control, DBC, Diabetic control, DCRL, Diabetic C. rubens low dose, DCRH, Diabetic C. rubens high dose, DBM, Diabetic metformin, DBG, Diabetic glibenclamide, NCR, Normal C. rubens high dose (toxicological control), STZ, Streptozotocin, NFBG, Non-fasting blood glucose, FBG, Fasting blood glucose.

The data for OGTT and the corresponding area under the curve (AUC) are shown in **Figure 5.3.4**. The glucose tolerance of the CRAQ-treated groups was significantly \(( p < 0.05)\) better than the DBC group. A better glucose tolerance ability was observed in the DCRH group at the 60 min mark which was manifested all through the OGTT period. The total area under the curve (AUC) of DCRH group was significantly lower than the other diabetic groups (DBC, DCRL, DBM and DBG), which again confirms its better glucose tolerance ability compared to other diabetic groups **(Figure 5.3.4)**.
Figure 5.3.4: Oral glucose tolerance test (OGTT) and corresponding area under the curve (AUC) of different animal groups in the last week of the intervention period. Data are reported as the mean ± SD of seven animals. Values with different letters for a given time (line graph) or AUC in different groups are significantly different from each other (Tukey’s HSD multiple range post hoc test, p < 0.05). NC, Normal control; DBC, Diabetic control; DCRL, Diabetic C. rubens low dose; DCRH, Diabetic C. rubens high dose; DBM, Diabetic metformin; DBG, Diabetic glibenclamide; NCR, Normal C. rubens high dose (toxicological control).

Table 5.3.1 shows the liver weight, relative liver weight and liver glycogen level of different animal groups at the end of the experimental period. No significant difference in the liver weight was observed in all the animal groups, however, the relative liver weight of the normal groups (NC and NCR) and DCRH group were significantly (p < 0.05) lower than that of the diabetic untreated group (DBC). The treatment with CRAQ increased the liver glycogen level in the DCRH group when compared to the DBC group (Table 5.3.1).

Table 5.3.1: The effects of different doses of CRAQ on liver weight, relative liver weight and liver glycogen levels in the different animal groups at the end of the intervention period.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>NC</th>
<th>DBC</th>
<th>DCRL</th>
<th>DCRH</th>
<th>DBM</th>
<th>DBG</th>
<th>NCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver weights (g)</td>
<td>10.7 ± 0.8</td>
<td>8.7 ± 0.9</td>
<td>9.5 ± 0.5</td>
<td>8.2 ± 0.7</td>
<td>9.6 ± 0.7</td>
<td>9.9 ± 0.9</td>
<td>10.7 ± 1.1</td>
</tr>
<tr>
<td>Rel. liver weights (%)</td>
<td>2.9 ± 0.3a</td>
<td>3.8 ± 0.2b</td>
<td>4.1 ± 0.7b</td>
<td>2.5 ± 0.2a</td>
<td>2.7 ± 0.1a</td>
<td>2.4 ± 0.1a</td>
<td>2.9 ± 0.5a</td>
</tr>
<tr>
<td>Liver glycogen (mg/g tissue)</td>
<td>36.0 ± 0.9c</td>
<td>11.8 ± 1.1a</td>
<td>22.3 ± 1.5b</td>
<td>28.3 ± 0.6c</td>
<td>31.4 ± 1.1c</td>
<td>21.8 ± 0.8b</td>
<td>24 ± 1.6b</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SD (n=7). Different superscript letters for a given parameter along a row indicate the significance of difference (Tukey’s-HSD multiple range post hoc test, p < 0.05).
At the end of the experimental period, the serum lipid profile of all the animals were measured and reported in Figure 5.3.5. The DBC group showed a significantly ($p < 0.05$) higher total- and LDL-cholesterol compared to the normal groups. However, treatment with CRAQ and standard drug (metformin) reduced the levels of these cholesterols as seen in the DCRL, DCRH and DBM groups, respectively, while the DBG and DCRL groups showed similar results compared to the DBC group for total cholesterol and triglycerides. T2D-induction caused a significant ($p < 0.05$) elevation of serum triglycerides level which was reduced by the CRAQ and metformin treatment in the DCRH and DBM groups, respectively.

![Figure 5.3.5](image.png)

**Figure 5.3.5:** Serum lipid profile of different animal groups at the end of the intervention period. Data are reported as the mean ± SD of seven animals. *Values with different letters for a given parameter significantly different from each other (Tukey's-HSD multiple range post hoc test, $p < 0.05$). NC, Normal control, DBC, Diabetic control, DCRL, Diabetic C. rubens low dose, DCRH, Diabetic C. rubens high dose, DBM, Diabetic metformin, DBG, Diabetic glibenclamide, NCR, Normal C. rubens high dose (toxicological control). HDL, High density lipoprotein, LDL, Low density lipoprotein.

**Table 5.3.2** shows the data for other serum parameters of diabetic complications. Serum levels of AST, ALP and urea were elevated in the DBC group compared to the NC and NCR groups. Interestingly, CRAQ significantly ameliorated the ALP, AST and urea with uric acid levels in the respective groups. Serum CK-MB was increased in the DBC group compared to the normal (NC and NCR) and DBM groups. Treatment with DCRH reduced the elevated CK-MB levels significantly.
Table 5.3.2: Serum biochemical parameters in different animal groups at the end of the experimental period.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>NC</th>
<th>DBC</th>
<th>DCRL</th>
<th>DCRH</th>
<th>DBM</th>
<th>DBG</th>
<th>NCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>AST (U/L)</td>
<td>49.5±5.6a</td>
<td>147±16.3a</td>
<td>120±4.2e</td>
<td>74±3.9a</td>
<td>102.5±12.5de</td>
<td>85±8.8ad</td>
<td>57.7±6.9ad</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>87.8±1.1</td>
<td>87.3±2.0</td>
<td>86.3±2.7</td>
<td>87.5±0.5</td>
<td>85.5±2.1</td>
<td>86.3±1.3</td>
<td>85.8±1.1</td>
</tr>
<tr>
<td>ALP (U/L)</td>
<td>72.3±6.2a</td>
<td>308.3±44.8a</td>
<td>353.3±43.9a</td>
<td>233.7±81.1b</td>
<td>206.8±59.5b</td>
<td>395.9±86.2c</td>
<td>76.8±4.7a</td>
</tr>
<tr>
<td>Urea (mg/dL)</td>
<td>52.6±4.8a</td>
<td>73.8±3.4d</td>
<td>62.3±1.9a</td>
<td>43.1±7.3a</td>
<td>51±5.8b</td>
<td>71.5±3.9d</td>
<td>52.5±2.2b</td>
</tr>
<tr>
<td>UA (mg/dL)</td>
<td>0.78±0.43c</td>
<td>3.5±0.9a</td>
<td>3.2±0.5a</td>
<td>2.3±0.3a</td>
<td>1.3±0.5ab</td>
<td>1.6±0.7ab</td>
<td>1.1±0.1ab</td>
</tr>
<tr>
<td>CK-MB (u/L)</td>
<td>118.9±14.7a</td>
<td>305.5±27.8a</td>
<td>249.4±14.1c</td>
<td>146.5±24.6a</td>
<td>113.9±17.7a</td>
<td>185.9±7.6a</td>
<td>120.2±3.2a</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SD (n=7). Different superscript letters for a given parameter along a row indicate the significance of difference (Tukey’s-HSD multiple range post hoc test, p < 0.05). NC, Normal control, DBC, Diabetic control, DCRL, Diabetic C. rubens low dose, DCRH, Diabetic C. rubens high dose, DBM, Diabetic metformin, DBG, Diabetic glibenclamide, NCR, Normal C. rubens high dose (toxicological control). AST, Aspartate aminotransferase, ALT, Alanine aminotransferase, ALP, Alanine phosphatase, UA, Uric acid, CK-MB, Creatinine Kinase-myocardial band.

The NC, NCR and DCRH groups showed significantly (p < 0.05) higher serum insulin levels compared to the DBC group (Table 5.3.3). The HOMA-IR score in the NC group was significantly lower than that of the DBC group and the treatment with CRAQ was able to reduce to HOMA-IR scores. In the other case, the HOMA-β scores of the DBC group was seen to be significantly lower than the NC group and all treated groups showed a significant increase in HOMA-β scores compared to the DBC group. Serum fructosamine level significantly increased in the DBC group compared to normal groups which was significantly improved in the diabetic treated groups compared to the DBC group.

Table 5.3.3: Serum insulin and fructosamine concentrations as well as calculated HOMA-IR and HOMA-β scores in different animal groups at the end of the experimental period.

<table>
<thead>
<tr>
<th></th>
<th>NC</th>
<th>DBC</th>
<th>DCRL</th>
<th>DCRH</th>
<th>DBM</th>
<th>DBG</th>
<th>NCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum Insulin</td>
<td>65.2±1.1c</td>
<td>23.5±1.8a</td>
<td>58.5±1.8c</td>
<td>99.3±7.0a</td>
<td>59.8±6.7c</td>
<td>41.8±2.3b</td>
<td>96.8±2.5d</td>
</tr>
<tr>
<td>(pmol/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fructosamine</td>
<td>495.8±17.9a</td>
<td>990±10.4d</td>
<td>792.4±72.9c</td>
<td>607.3±5.3b</td>
<td>572.5±9.2b</td>
<td>743.6±37.4c</td>
<td>438.8±38.8a</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>2.1±0.3a</td>
<td>14.0±0.3c</td>
<td>8.4±0.3c</td>
<td>6.9±0.5d</td>
<td>6.5±0.7a</td>
<td>5.2±0.3c</td>
<td>3.1±0.1b</td>
</tr>
<tr>
<td>HOMA-β</td>
<td>100.3±1.7c</td>
<td>2.7±0.2a</td>
<td>8.3±0.3ac</td>
<td>35.6±2.5a</td>
<td>11.9±1.3c</td>
<td>7.0±0.4b</td>
<td>169.7±4.3f</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SD (n=7). Different superscript letters for a given parameter along a row indicate the significance of difference (Tukey’s-HSD multiple range post hoc test, p < 0.05). NC, Normal control, DBC, Diabetic control, DCRL, Diabetic C. rubens low dose, DCRH, Diabetic C. rubens high dose, DBM, Diabetic metformin, DBG, Diabetic glibenclamide, NCR, Normal C. rubens high dose (toxicological control). HOMA-IR and HOMA-β; Homeostatic model assessment for IR (insulin resistance) and β (β-cell function).
Histopathological analysis of pancreatic tissues excised from normal as well as extract and drug-treated rats are shown in Figure 5.3.4. The parenchyma of normal rats (green and black arrow) showed compact acinar cells with an abundance of granular eosinophilic cytoplasm and normal interlobular connective tissues with intact islets of Langerhans. Examination of the DBC pancreas on the other hand, showed a degeneration of endocrine cells within the islets (red arrow) indicating necrosis of the pancreatic β-cells. Pancreatic sections from the metformin and extract-treated groups (blue, grey and yellow arrows respectively) showed although not quite as intact as the normal cells but a promising regeneration of the pancreatic islets were achieved. The DBG pancreas (purple arrow) looked almost same as the untreated islets (DBC).

Figure 5.3.6: Histopathological images of the pancreas of different animal groups at the end of the experiment. NC, Normal control, DBC, Diabetic control, DCRL, Diabetic C. rubens low dose, DCRH, Diabetic C. rubens high dose, DBM, Diabetic metformin, DBG, Diabetic glibenclamide, NCR, Normal C. rubens high dose (toxicological control).
5.3.5 Discussion

The T2D animal model used in this study successfully mimicked the two main pathological features of T2D which are insulin resistance and partial pancreatic \(\beta\)-cell dysfunction. Normal rats were first given a 10\% fructose solution for two weeks to induce insulin resistance and then an intraperitoneal injection of streptozotocin (40 mg/kg bw) to induce partial pancreatic \(\beta\)-cell dysfunction (Wilson and Islam, 2012).

Severe complications of T2D include hyperosmolar and hyperglycaemic non-ketotic syndrome (Knentz et al., 1991) while more common symptoms of T2D include polydipsia, polyphagia, lethargy, visual blurriness and weight loss (Afolayan and Sunmonu, 2010). In this study, the increased fluid intake seen in the diabetic groups reflected successful T2D-induction (Figure 5.3.1). Although CRAQ-treatment reduced the fluid intake of the animals, it was not as low as the fluid intake of the normal group which could be as a result of a short intervention time (5 weeks). Body weight loss is another physical symptom of T2D which occurs due to increased protein glycation which is a consequence of persistent hyperglycaemic and leads to the loss of muscle and tissue protein (Mbaka et al., 2014). There was a decrease in body weight of all diabetic animals after STZ injection which again confirmed the induction of T2D. Interestingly, the high dose of CRAQ was able to increase and sustain the body weight of the diabetic animals all through the intervention period. This may be related to the high nutrient level of \textit{C. rubens} (Adjatin et al., 2013b).

The non-fasting and fasting (at the last week) blood glucose levels were measured during the 5-week intervention period, this is because NFBG is a key parameter in understanding the severity of T2D (Group, 1998). From our result (Figure 5.3.3), CRAQ showed potent blood glucose lowering activity. Right from the second week and all through the intervention period, the DCRH group displayed low blood glucose levels. Some processes might be responsible for the observed blood glucose lowering activity of CRAQ, such as improvement of insulin action (Islam and Choi, 2008), pancreatic \(\beta\)-cell stimulation (Remedi and Emfinger, 2016) or improved uptake of glucose by the peripheral tissues (Oyebode et al., 2018). There are reports that plants have \(\beta\)-cell protecting and regenerating abilities (Oh, 2015). Since the DCRH group showed reduced blood glucose level from as early as the second week of intervention, it might be due to CRAQ stimulated the survival of pancreatic \(\beta\)-cells as well as induced insulin production after the oxidative damage caused by STZ. This could also be explained by Figure 5.3.6 where marked regeneration of the damaged pancreatic islets in the DCRH group was observed compared to the DBC group.
Maintaining glucose homeostasis is very important in T2D so as to reduce any risk of micro or macrovascular complications (Chawla et al., 2016). Treatment with CRAQ showed promising improvement in glucose tolerance abilities of the plant (Figure 5.3.4) especially at the high dose of 300 mg/kg bw. This activity might be due to lower insulin resistance and increased insulin secretion that would induce glucose uptake by the peripheral tissues. In addition, the high dose of the extract reduced HOMA-IR index (for insulin resistance), and also improved the HOMA-β (for β-cell function) score (Table 5.3.3 and Figure 5.3.6). Homeostatic model assessment (HOMA) is a technique used for estimating insulin resistance and β-cell function from fasting blood glucose levels and insulin concentration (Wallace et al., 2004). Plants that reduce HOMA-IR scores have been reported to contain potent antidiabetic activities (Vianna et al., 2011). In addition to altering insulin resistance and β-cell function, CRAQ also showed fructosamine reducing ability (Table 5.3.3). Fructosamine is a marker of glucose control showing average serum glycaemic level, in some cases it is regarded as more efficient in detecting early response to treatment (Nansseu et al., 2015).

The CRAQ-treated groups also showed improved levels of hepatic glycogen (Table 5.3.1). Increased phosphorylation of glycogen or reduced activity of glycogen synthase are pathological features observed in STZ-induced T2D, which could be responsible for reduced glycogen level seen in the DBC group (Islam, 2011). The liver glycogen storing activity might also be responsible for the lower blood glucose level in the CRAQ treated group compared to the DBC group. Interestingly, plants have been reported to exhibit hepatic glycogen synthesizing activities, which may contribute to their observed anti-diabetic activity (Bhat et al., 2011, Ezeani et al., 2017).

Hyperglycaemia and insulin resistance lead to various metabolic disorders such as hyperlipidaemia. Hyperlipidaemia occurs due to reduced action of insulin which then increases lipase activity thereby releasing free fatty acids from the adipose tissue (Mbaka et al., 2014). This study showed the efficacy of CRAQ in the modulation of hyperlipidaemia (Figure 5.3.5). Treatment with CRAQ led to significant reduction in triglycerides, total- and LDL-cholesterol, while a promising increase was shown in HDL-cholesterol. From these results, it is possible to say that the extract inhibited lipase enzyme activity as well as mobilized free fatty acids from peripheral fat deposit to the blood vessels. Studies have demonstrated the potency of plant extracts in alleviating hyperlipidaemia (Eze et al., 2012). The reduced CK-MB levels of the CRAQ-treated animals compared to the DBC group in Table 5.3.2 also supports the cardiac-protective ability of this plant. Creatinine kinase is used as a marker for coronary heart disease and increased levels of this enzyme signals increased risk of heart diseases (Patel et al., 2014). The ability of CRAQ to reduce CK-MB levels supports the
hyperlipidaemia-alleviating properties of this plant (Table 5.3.3) which together renders cardio-
protective ability in T2D patients.

Core markers of liver injury are alanine aminotransferase (ALT) and aspartate aminotransferase
(AST) while alkaline phosphatase (ALP) is a marker for cholestasis (liver disease which causes
itching) (Malakouti et al., 2017). Although T2D-induction did not show any impact on serum ALT
levels (Table 5.3.2), increased ALP and AST levels were observed in the DBC group compared to
the NC group; however treatment with CRAQ showed reduced levels of these enzymes which shows
the hepatoprotective ability of the extract.

Serum urea and uric acid are markers of kidney failure. Studies have shown that in T2D, serum urea
and uric acid is usually elevated (Kushiyama et al., 2014). In Table 5.3.2 the DBC groups showed
elevated urea and uric acid levels compared to the normal groups. Treatment with CRAQ alleviated
the effect of these enzymes, which shows the ability of this extract to limit the risk of kidney problems.

5.3.6 Conclusions

In conclusion, the results of this study suggest that the aqueous extract of Crassocephalum rubens
aerial parts dose-dependently portrays anti-diabetic activity, mechanisms may include improving
pancreatic β-cell function, stimulating insulin secretion, improving glucose tolerance, reducing
hyperlipidemia and increasing hepatic glycogen. The extract also ameliorated some T2D-induced
complications. Hence, it can be used for the traditional management of T2D.
5.4  **Aqueous extract of *Crassocephalum rubens* aerial parts ameliorates oxidative stress in a type 2 diabetes model of rats**

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**Preface:** This article reports the detailed *in vivo* antioxidant status of the serum and organs collected from the rats in the above experiment and it is currently awaiting revision before submission to a journal for publication.

**5.4.1 Abstract**

The effects of oral administration of the *Crassocephalum rubens* aqueous extract (CRAQ) on antioxidant status in the serum, pancreas, liver, kidney and heart of type 2 diabetic rats was examined. Alterations in important functional groups in the liver and pancreas were also monitored using Fourier Transform Infrared (FT-IR) spectroscopy. The male SD rats were induced with type 2 diabetes (T2D) and treated with 150 or 300 mg/kg bw of CRAQ. Levels of TBARS (thiobarbituric acid reactive substances), catalase, super oxide dismutase (SOD), reduced glutathione, glutathione reductase and glutathione peroxidase were measured in selected organs and serum were investigated at the end of the intervention period. Induction of type 2 diabetes (T2D) caused a significant (*p* < 0.05) decrease of endogenous antioxidant enzymes, as well as an increment in MDA levels (a marker of lipid peroxidation) in the diabetic rats. However, treatment with CRAQ caused a dose-dependent and significant (*p* < 0.05) increase in reduced glutathione levels and other endogenous antioxidant enzymes in the serum and other organs compared to diabetic control group. The FT-IR analysis showed a reduction of fasting blood glucose levels after the treatment with the high dose of CRAQ which is consistent with reduced FT-IR peaks at 1200-900 cm⁻¹. Results of this study indicate that high dose (300 mg/kg bw) of CRAQ possess strong antioxidant effect in a type 2 diabetic model of rats.

**Keywords:** *Crassocephalum rubens*, oxidative stress, antioxidants, FT-IR, rats
5.4.2 Introduction

Under normal physiological conditions, a complex system of endogenous enzymatic and non-enzymatic antioxidant defences work to combat the deleterious effects of free radicals generated from normal metabolic processes (Kurutas, 2015). These free radicals are implicated in the generation of complications linked to diseases such as diabetes (Asmat et al., 2016). Diabetes mellitus is a complex disease that has quickly become a problem to global public health (Roglic, 2016). Out of the three main types of diabetes, type 2 diabetes (T2D) remains the most prevalent form with 95% of all diabetic cases being type 2 diabetic (IDF, 2017). Persistent hyperglycemia, insulin resistance and partial pancreatic β-cell dysfunction are the main pathogenesis of T2D. Autooxidation of excess glucose in the blood stream as a result of insulin resistance has been linked to the generation of free radicals in T2D (Lyons et al., 1992). These free radicals overwhelm the concentration of antioxidant enzymes in the body thereby leading to the progression of oxidative stress. Oxidative stress causes a host of complications affecting various organs such as heart, liver, kidney, eyes, arteries of patients suffering from T2D. Fortunately, plants have been used successfully as a source of potent antioxidants in curbing the effects of free radicals in oxidative stress conditions. Medicinal plants contain potent hydrogen donating, oxygen quenching, free radical scavenging activities that are beneficial in managing complications of T2D (Garhwal, 2010). The effect of this plants can be due to the presence of potent bioactive components such as tannins, polyphenols, saponins, and others. An example of a medicinal plant with antidiabetic activity is *Crassocephalum rubens*.

*Crassocephalum rubens* belongs to the family Asteraceae; and is a perennial shrub that is mostly eaten and enjoyed as a vegetable in salads and sauces in various parts of Africa. It is successfully used in the treatment and management of diseases such as diabetes. Preliminary *in vitro* α-glucosidase and lipase inhibitory potential of different solvent extracts of aerial parts of *C. rubens* has been reported in Section 5.2. In a previous study, the crude ethanol, ethyl acetate and aqueous extracts of the plant was subjected to different *in vitro*, *ex vivo* and *in silico* antioxidant and anti-diabetic assays from which the aqueous extract was chosen as the most active compared to all other solvent extracts (see section 5.2 above). This also led to investigate the anti-diabetic effect of CRAQ in a type 2 diabetes rat model (Section 5.3). Our results showed that CRAQ has glucose lowering, insulin resistance alleviating and β-cell protective abilities as well as cardiac, hepatic and renal protective abilities in diabetic conditions.
However, another interest of this study was to investigate comprehensively the antioxidant effect of CRAQ since oxidative stress plays a major role in the pathogenesis of T2D and its associated multiple complications, and also to determine whether functional groups in the tissues (liver and pancreas) were altered after the treatment with CRAQ extract.

5.4.3 Materials and methods

Please refer to Chapter 2 for the detailed materials and methods used for C. rubens.

5.4.4 Results

The DBC group showed a significantly ($p < 0.05$) higher level of MDA compared to the normal and treated groups (Figure 5.4.1). This was also replicated in the serum, liver, heart and kidneys of the animals. The MDA levels was seen to reduce upon treatment with the CRAQ which shows the lipid peroxidation alleviating property of the extract.

Figure 5.4.1: Thiobarbituric acid reactive substances (MDA equivalent) in the serum, liver, pancreas, heart and kidneys of all animal groups at the end of the intervention period. Data are reported as mean ± SD of 7 animals. *Values with different letter above the bars for a given sample are significantly different from other group of animals (Tukey's-HSD multiple range post hoc test, $p < 0.05$). NC, Normal control, DBC, Diabetic control, DCRL, Diabetic C. rubens low dose, DCRH, Diabetic C. rubens high dose, DBM, Diabetic metformin, DBG, Diabetic glibenclamide, NCR, Normal C. rubens high dose (toxicological control), MDA, Malondialdehyde.

The reduced glutathione (GSH) concentrations of the serum and organs of all animal groups are reported in Figure 5.4.2. The GSH concentration in the serum, liver, pancreas and heart was significantly ($p < 0.05$) lower in the DBC group upon induction of diabetes compared to the normal groups. However, the reduction in the kidney was not statistically ($p > 0.05$) different among the animal groups. Oral administration of the extract significantly ($p < 0.05$) increased the GSH levels in
the liver of the DCRH group. There was no significant difference between the induction of GSH levels by the DCRL and DCRH groups in the serum and pancreas, while in the heart, the DCRL group showed significantly higher GSH level than the high dose of the extract.

**Figure 5.4.2:** Reduced glutathione (GSH) levels in the serum, liver, pancreas, heart and kidneys of all animal groups at the end of the intervention period. Data are reported as mean ± SD of 7 animals. **a** Values with different letter above the bars for a given sample are significantly different from each other group of animals (Tukey’s-HSD multiple range post hoc test, *p* < 0.05). NC, Normal control; DBC, Diabetic control; DCRL, Diabetic *C. rubens* low dose; DCRH, Diabetic *C. rubens* high dose; DBM, Diabetic metformin; DBG, Diabetic glibenclamide; NCR, Normal *C. rubens* high dose (toxicological control).

**Figure 5.4.3** shows the data for the catalase activity in serum and organs of all animal groups. The catalase activity in the liver, pancreas, heart and kidneys of DBC group was significantly (*p* < 0.05) lower compared to the normal groups. The liver showed the highest catalase activity amongst the serum and organs. However, CRAQ treatment significantly (*p* < 0.05) increased the catalase activity in the organs of diabetic animals with DCRH group showing the better activity than the low dose in the serum and organs.

**Figure 5.4.3:** Catalase activity in the serum, liver, pancreas, heart and kidneys of all animal groups at the end of the intervention period. Data are reported as mean ± SD of 7 animals. **a** Values with different letter above the bars for a given sample are significantly different from each other group of animals (Tukey’s-HSD multiple range post hoc test, *p* < 0.05). NC, Normal control; DBC, Diabetic control; DCRL, Diabetic *C. rubens* low dose; DCRH, Diabetic *C. rubens* high dose; DBM, Diabetic metformin; DBG, Diabetic glibenclamide; NCR, Normal *C. rubens* high dose (toxicological control).
The activities of superoxide dismutase (SOD) in the serum and organs of all animal groups are shown in **Figure 5.4.4**. The SOD activity in the serum and organs of DBC group was significantly ($p < 0.05$) lower compared to the NC group. However, oral administration of CRAQ for 5 weeks significantly ($p < 0.05$) increased SOD activity in the DCRH group compared to the DBC group of the serum, heart and kidneys while in the liver, the low dose group (DCRL) showed the better activity than the high dose. In the pancreas, only metformin group (DBM) showed a lower SOD activity when higher SOD activity was observed in the remaining groups although they were not statistically significant.

![Figure 5.4.4: SOD activity in the serum, liver, pancreas, heart and kidney of all animal groups at the end of the intervention period.](image)

The results of the glutathione reductase (GR) activity in the serum and organs of all animal groups are shown in **Figure 5.4.5**. In the kidneys, the glutathione reductase activity was not altered by diabetes induction. However, the activity of this enzyme was significantly ($p < 0.05$) decreased in the serum and organs of the DBC group as compared to the NC and NCR groups. Oral administration of the CRAQ improved the enzyme activity in the serum and organs. There was no significant difference between the low dose and high dose of the extract in increasing GR activity in the serum, liver and pancreas. While in the heart and kidneys, the high dose (DCRH) group showed a statistically ($p < 0.05$) higher activity than the group given the low dose of the extract.
Figure 5.4.5: Glutathione reductase (GR) activity in the serum, liver, pancreas, heart and kidney of all animal groups at the end of the intervention period. Data are reported as mean ± SD of 7 animals. *Values with different letter above the bars for a given sample are significantly different from other group of animals (Tukey’s-HSD multiple range post hoc test, p < 0.05). NC, Normal control; DBC, Diabetic control; DCRL, Diabetic C. rubens low dose; DCRH, Diabetic C. rubens high dose; DBM, Diabetic metformin; DBG, Diabetic glibenclamide; NCR, Normal C. rubens high dose (toxicological control).

Figure 5.4.6 shows the glutathione peroxidase (GPx) activity in the serum and organs. The GPx activity was significantly (p < 0.05) decreased in the serum and organs of DBC group compared to the NC and NCR groups. However, treatment with CRAQ significantly (p < 0.05) increased the GPx activity in the diabetic treated groups which was comparable to the DBM and DBG groups.

Figure 5.4.6: Glutathione peroxidase (GPx) activity in the serum, liver, pancreas, heart and kidney of all animal groups at the end of the intervention period. Data are reported as mean ± SD of 7 animals. *Values with different letter above the bars for a given sample are significantly different from other group of animals (Tukey’s-HSD multiple range post hoc test, p < 0.05). NC, Normal control; DBC, Diabetic control; DCRL, Diabetic C. rubens low dose; DCRH, Diabetic C. rubens high dose; DBM, Diabetic metformin; DBG, Diabetic glibenclamide; NCR, Normal C. rubens high dose (toxicological control).

The liver and pancreas were chosen for FT-IR analysis since the liver is the major site for metabolism in the body and the pancreas is the major site for production of insulin, when both organs are highly affected in T2D. Alterations in tissue glucose in response to the treatment of CRAQ was investigated
in the FT-IR spectra (Figure 5.4.7). In the liver, all animal groups showed strong bands at 1200-900 cm\(^{-1}\), 1500-1200 cm\(^{-1}\), 1800-1500 cm\(^{-1}\), and 300-2800 cm\(^{-1}\) regions which gave the indication of the presence of sugars, proteins, peptides and lipids, respectively (Table 5.4.1). In T2D, the most observable difference amongst the experimental groups was seen in the range of 1200-900 cm\(^{-1}\) which indicates the presence of sugar (glucose). Table 5.4.1 shows that the glucose peak at 1080 cm\(^{-1}\) was increased in the DBC group, which showed animals with high blood glucose; however, the intensity of the glucose peaks was decreased among the treated groups (DCRL and DCRH) which is consistent with the reduction of blood glucose concentrations by the extract.

![FT-IR absorption spectra of the liver](image)

**Figure 5.4.7:** FT-IR absorption spectra of the liver in the regions of 4000-400 cm\(^{-1}\) from different experimental groups. NC, Normal control; DBC, Diabetic control; DCRL, Diabetic *C. rubens* low dose; DCRH, Diabetic *C. rubens* high dose; DBM, Diabetic metformin; DBG, Diabetic glibenclamide; NCR, Normal *C. rubens* high dose (toxicological control).

**Table 5.4.1: Qualitative analysis of the FT-IR spectra of the liver.**

<table>
<thead>
<tr>
<th>Regions (cm(^{-1}))</th>
<th>NC</th>
<th>DBC</th>
<th>DCRL</th>
<th>DCRH</th>
<th>DBM</th>
<th>DBG</th>
<th>NCR</th>
<th>Assignment</th>
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</thead>
<tbody>
<tr>
<td>1200-900</td>
<td>1080.68</td>
<td>1081.82</td>
<td>1080.29</td>
<td>1080.02</td>
<td>1081.32</td>
<td>1081.79</td>
<td>1081.03</td>
<td>Glucose</td>
</tr>
<tr>
<td>1500-1200</td>
<td>1452.18</td>
<td>1453.12</td>
<td>1452.39</td>
<td>1453.98</td>
<td>1400.23</td>
<td>1452.40</td>
<td>1453.69</td>
<td>Proteins</td>
</tr>
<tr>
<td>1800-1500</td>
<td>1635.39</td>
<td>1638.84</td>
<td>1635.98</td>
<td>1635.98</td>
<td>1634.44</td>
<td>1635.16</td>
<td>1636.13</td>
<td>C=O stretch</td>
</tr>
<tr>
<td>3000-2800</td>
<td>3275.44</td>
<td>3276.02</td>
<td>3275.62</td>
<td>3274.87</td>
<td>3276.9</td>
<td>3274.47</td>
<td>3268.23</td>
<td>OH stretch,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Lipids</td>
</tr>
</tbody>
</table>

NC, Normal control; DBC, Diabetic control; DCRL, Diabetic *C. rubens* low dose; DCRH, Diabetic *C. rubens* high dose; DBM, Diabetic metformin; DBG, Diabetic glibenclamide; NCR, Normal *C. rubens* high dose (toxicological control).
The FT-IR spectra of the pancreatic tissues from different groups are shown in Figure 5.4.8. The IR spectra was divided into 3 regions: 3000-2800 cm\(^{-1}\), 180-1400 cm\(^{-1}\), and 1200-900 cm\(^{-1}\). Same changes in lipid, protein and glucose was seen in the pancreas (Table 5.4.2).

![FT-IR absorption spectra of the pancreas in the regions of 4000-400 cm\(^{-1}\) from different experimental groups. NC, Normal control, DBC, Diabetic control, DCRL, Diabetic C. rubens low dose, DCRH, Diabetic C. rubens high dose, DBM, Diabetic metformin, DBG, Diabetic glibenclamide, NCR, Normal C. rubens high dose (toxicological control).]

**Table 5.4.2**: Qualitative analysis of the FT-IR spectra of the pancreas.

<table>
<thead>
<tr>
<th>Regions (cm(^{-1}))</th>
<th>NC</th>
<th>DBC</th>
<th>DCRL</th>
<th>DCRH</th>
<th>DBM</th>
<th>DBG</th>
<th>NCR</th>
<th>Assignment</th>
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</thead>
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<tr>
<td>1200-900</td>
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<td>1084.04</td>
<td>1081.09</td>
<td>1080.02</td>
<td>1079.74</td>
<td>1081.40</td>
<td>Glucose</td>
</tr>
<tr>
<td>1450-1380</td>
<td>1452.72</td>
<td>1458.41</td>
<td>1463.28</td>
<td>1465.22</td>
<td>1477.09</td>
<td>1453.2</td>
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<td>Proteins</td>
</tr>
<tr>
<td>1800-1500</td>
<td>1634.77</td>
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<td>1628.93</td>
<td>1629.31</td>
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<td>1630.18</td>
<td>C=O stretch</td>
</tr>
<tr>
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<td>3285.13</td>
<td>3283.49</td>
<td>3279.47</td>
<td>OH stretch, Lipids</td>
</tr>
</tbody>
</table>

NC, Normal control; DBC, Diabetic control; DCRL, Diabetic C. rubens low dose; DCRH, Diabetic C. rubens high dose; DBM, Diabetic metformin; DBG, Diabetic glibenclamide; NCR, Normal C. rubens high dose (toxicological control).
5.4.5 Discussion

Reducing the prevalence of obesity, which is closely linked to increased risk of T2D and cardiovascular diseases, needs a dietary focus (Asif, 2014). Higher consumption of whole grains, fruits and vegetables reduces the risk of T2D and its related complications. Plant based phytochemicals provide much of the nutrients, taste and colour in fresh vegetables, therefore it is highly favoured by locals in parts of western Nigeria for its rich taste in making various delicacies. *C. rubens* contains potent free radical and lipid peroxidation inhibiting phytochemicals (Adjatin *et al.*, 2012).

The lipid bilayer of the cell membrane is mostly affected by peroxidation of free radicals, and lipid peroxidation plays a huge role in the initiation of damages to the cell in T2D (Farzaei *et al.*, 2017). Induction of T2D with elevated MDA levels in the diabetic untreated group (DBC) compared to the normal groups. Previous research has demonstrated that pancreatic β-cell damage, increased renal, cardiac and liver function parameters might consequently lead to increase in MDA levels in diabetic rats (Ali *et al.*, 2017). Interestingly, data from this study (*Figure 5.4.1*) showed lipid peroxidation-ameliorating potential of CRAQ. Inhibition of lipid peroxidation (through reduction of MDA levels) in the serum and organs of the diabetic rats further highlights the oxygen quenching effect of CRAQ.

Reduced glutathione is an effective extracellular compound which scavenges hydrogen peroxide and hydroperoxide and donates protons to membrane lipids and protects them from oxidant damages (Nurdiana *et al.*, 2017). The ability of CRAQ to increase the levels of GSH (*Figure 5.4.2*) and activity of glutathione reductase (*Figure 5.4.5*) supports the antioxidant benefit of the plant.

Three first line defence systems were investigated in this study including superoxide dismutase (SOD), catalase and glutathione peroxidase (Ighodaro and Akinloye, 2017). These enzymes, in the same order, dismutates superoxide free radical, transform hydrogen peroxides and hydroperoxides to relatively harmless molecules such as H$_2$O/alcohol and oxygen. The superoxide dismutase (SOD) catalyses the conversion of superoxide radical generated in tissue to hydrogen peroxide (H$_2$O$_2$) and oxygen (Fukai and Ushio-Fukai, 2011). Accumulation of H$_2$O$_2$ causes toxicity to the cells and tissues. In the presence of iron (Fe$^{2+}$), H$_2$O$_2$ is converted to hydroxyl radical through the Fenton reaction (Birben *et al.*, 2012). Catalase breaks down H$_2$O$_2$ into water and molecular oxygen consequently reducing free radical damage. However, the absence of catalase in the mitochondria makes glutathione peroxidase responsible for breaking-down the H$_2$O$_2$ to water (Ighodaro and Akinloye, 2017). Our study showed the ability of CRAQ to increase the activity of these first line defence
enzymes (Figure 5.4.4, 5.4.3 and 5.4.6) in the serum and organs of type 2 diabetic animals which again confirm its antioxidative potential.

An important finding of the present work is the changes in liver and pancreas which are supported by the FT-IR peaks. Previous studies have shown that the alterations in the IR spectra is related to the subsequent changes in structure and function of the liver (Baker et al., 2014, Nurdiana et al., 2017). Most importantly, reduction of FBG is suspected by reduced FT-IR peaks at 1200-900 cm\(^{-1}\), while induction of T2D with STZ increased the intensity of glucose peak in the pancreas (Figure 5.4.8 and Table 5.4.2) of diabetic animals is indicative of insulin deprivation in the cells.

5.4.6 Conclusions

In conclusion, the high dose of CRAQ was found to provide better antioxidant effect than the low dose; this result is comparable to a recent study where the protective effect of 300 mg/kg bw of aqueous extract of \textit{C. rubens} was tested in rats with rifampicin-induced oxidative damage (Omoregie et al., 2015). From overall findings of that study, it was suggested that the presence of flavonoids and proanthocyanins might be responsible for the observed free radical scavenging activity of the plant which protected hepatic cells from oxidative damage.
CHAPTER 6
THE ANTIOXIDANT AND ANTIDIABETIC ACTIVITIES OF BRIDELIA FERRUGINEA IN VITRO AND IN VIVO

6.1 Background

*Bridelia ferruginea* Benth (Euphorbiaceae) ([Figure 6.1](#)) is a woody shrub up to 8 meters tall, though it can grow up to a tree of 15 meters in height in suitable environments. It thrives in the Savannah and rain forests of Africa, especially in moist regions extending from Guinea to Zaire. The bark is scaly, dark grey and rough with crooked bole branches hanging low.

![Bridelia ferruginea](image)

**Figure 6.1:** *Bridelia ferruginea* Benth (Euphorbiaceae); Common names: Iralodan (Yoruba, Nigeria), Ola (Igbo, Nigeria), Kirni (Hausa, Nigeria). Adapted without permission from Brunken U. (2008).
6.1.1 Ethnobotanical uses

Ethnobotanically, the aqueous extract of the stem bark is effective in the treatment of ulcer, diarrhoea, boils (Akuodor et al., 2012), reduction of high blood sugar levels (Bakoma et al., 2013, Onunkwo et al., 1996), antimicrobial activity and treatment of chest infections (Magistretti et al., 1988), anti-inflammatory (Olajide et al., 1999) and water treatment to drinkable conditions (Kolawole and Olayemi, 2003). The roots of B. ferruginea are used in Togo as chewing sticks and in treatment of bladder, intestinal and skin problems (Olaide et al., 2014).

6.1.2 Biological activities

The aqueous extract of the stem bark of B. ferruginea has been reported to possess quinones, alkaloids, flavonoids, tannins, saponins and reducing compounds (Akuodor et al., 2011). In two separate studies, Bakoma et al. investigated the effect of the crude extracts and fractions from the root bark on insulin resistance in fructose-fed mice (Bakoma et al., 2011, Bakoma et al., 2014). The results from the study showed marked improvement of insulin sensitivity and glucose tolerance. Another study was also done to evaluate the effect of B. ferruginea aqueous extract on gestational diabetes; improved glucose tolerance was observed in the adult pregnant rats used in this study (Taiwo et al., 2012).

B. ferruginea stem bark ethanolic extract has been reported to act as an antioxidant by protecting brain and liver homogenates from iron (II) sulphate-induced oxidative stress in normal rat tissue homogenates (Oloyede and Babalola, 2012). Although, a very high dose (4000 mg/kg bw) of the aqueous extract of stem bark has also been reported to cause lipid peroxidation and sperm damage, therefore low doses (less than 1000 mg/kg bw) should be used with caution (Awodele et al., 2015). The aqueous stem bark extract of this plant has also been reported to possess anti-inflammatory activities through p38 MAPK and NF-kB signalling. Akuodor et al. (2011) reported that the aqueous extract from the stem bark of B. ferruginea exhibited promising pain relieving and antipyretic activities in experimental mice and rats.

Different parts of B. ferruginea have also been successfully used in the management of blood glucose level in experimental diabetes-induced animal models. A 100 mg/kg bw of the aqueous extract of the bark of this plant has been reportedly used in improving blood glucose levels in alloxan-induced diabetic rats and also render hypoglycaemic activity in normal rats (Adewale and Oloyede, 2012).
According to the literature, limited work has been done on the anti-diabetic activity of the different parts of *B. ferruginea*, which prompted the need to investigate the detailed anti-diabetic potentials together with antioxidant properties of the leaves, stem and root bark extracts of this plant and then proceed to an *in vivo* study using the most active fraction.
6.2 Extracts of *Bridelia ferruginea* scavenges free radicals and modulates key anti-diabetic enzymes

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Preface: This article reports the detailed *in vitro* antioxidant and anti-diabetic effects of the crude extracts from *B. ferruginea*. It is currently awaiting revision before submission to a journal for publication.

6.2.1 Abstract

This study investigates the *in vitro* antioxidant activities of extracts from three parts of *Bridelia ferruginea*. Sequential extraction was performed on the leaves, stem and root bark samples with solvents of increasing polarity and these extracts were assayed for total phenolic content, *in vitro* antioxidant and anti-diabetic activities using different experimental models. The electron donating, 1,1-diphenyl-2-picryl-hydrazyl (DPPH), nitric oxide and hydroxyl radical scavenging activities of all the extracts were investigated in this study. The ability of these extracts to inhibit α-glucosidase, α-amylase and lipase enzymes were also examined *in vitro*. The ethanol extract of the leaves showed the highest total phenolic content compared to other extracts. All extracts showed free radical scavenging abilities in the different models. The ethanol and aqueous extracts significantly (*p* < 0.05) inhibited the key enzymes linked to diabetes. GC-MS analysis of the different plant parts revealed the presence of sterols, aromatics, aliphatic acids and esters. In particular, β-amyrin, 4-Phenylbenzophenone and lupenone showed strong binding affinities for α-glucosidase, α-amylase and lipase enzymes respectively when docked with the molecular structures of these enzymes. Data from this study suggest that the different parts of *B. ferruginea*, especially the ethanol and aqueous extracts of the leaves, possesses potent anti-oxidant activities that could be further explored in the area of anti-diabetic drug research.

Key words: Medicinal plant, type 2 diabetes, oxidative stress, hyperglycaemia, *Bridelia ferruginea*, molecular docking.
**6.2.2 Introduction**

Medicinal plants have long been regarded as better alternatives to pharmaceutical drugs and have gained much interest in the field of drug discovery. This can be attributed to the fact that they contain necessary and substantial concentration of antioxidants and bioactive agents that have been reported in the management and treatment of diseases such as diabetes (Dewanjee *et al.*, 2011).

A host of plant sources are found to be rich in polyphenols such as herbs, fruits, flowers and these plants are found in abundance everywhere in the world especially in Africa where there is a high reliance on medicinal plants for the treatment of diverse ailments (Hamzah *et al.*, 2013).

Plants are rich in polyphenolic agents which are highly beneficial to nature as they can react with reactive oxygen species/reactive nitrogen species (ROS/RNS) to bring about reduction in deleterious effects to cell biomolecules (Gach *et al.*, 2015). Plants antioxidants are capable of attenuating damages to biomolecules (lipids, proteins and DNA); these damages range from lipid peroxidation to cell membrane damage through oxidation (Hossain and Shah, 2015).

Oxidative stress occurs as a result of imbalance between free radical-generating enzymes (reactive oxygen species [ROS]) and free radical-scavenging enzymes (antioxidant enzymes) and plays a key role in the progression of diseases (Asmat *et al.*, 2016). This triggers an increased level of free radicals which initiates cellular damage and has been recognized as the main contributing factor to late complications of ailments such as diabetes and cancer (Son, 2012).

Diabetes has become a global health problem with over 425 million people currently suffering from this disease (IDF, 2017). Type 1 diabetes (T1D), type 2 diabetes (T2D) and gestational diabetes remains the 3 main types of diabetes of which the most prevalent is T2D (ADA, 2016). Insulin resistance, hyperglycaemia and pancreatic β-cell dysfunction are involved in the pathogenesis of T2D. Oxidative stress has been implicated as a major cause of the progression of diabetic complications (Asmat *et al.*, 2016). These complications range from macro and microvascular disorders such as heart, kidney, eyes and nerve problems. There are many synthetic anti-diabetic medications available, these drugs although effective sometime come with various side effects such as nausea, constipation, lactic acidosis, weight gain and others. Fortunately, medicinal plants with oxidative stress and hyperglycaemia inhibiting properties are available in nature. These plants are widely available, inexpensive and can be used to boost socio-economic importance. An example of such medicinal plant is *Bridelia ferruginea*. *B. ferruginea* Benth, (*Euphorbiaceae*) is a shrub or short tree which grows to about 15 m in height, it is found majorly in the Savannah and rainforest area of...
Africa. However, very little is known about the detail antioxidative and antidiabetic effects of the different parts of this plant either under in vitro, ex vivo or in vivo conditions.

Hence, this study was aimed to investigate the antidiabetic and antioxidant effects of the various extracts of leaves, stem bark and root bark extracts of B. ferruginea, as well as the identification and molecular docking of potent bioactive compounds present in the plant.

6.2.3 Materials and methods

Please refer to Chapter 2 for the detailed materials and methods used for B. ferruginea.

6.2.4 Results

The ethyl acetate extract of the leaves showed significantly ($p < 0.05$) higher total phenol content compared to other extracts followed by the aqueous extract of the stem bark (Figure 6.2.1).

![Figure 6.2.1](image)

**Figure 6.2.1:** Total phenolic contents of B. ferruginea extracts. Data are reported as mean ± SD of three replicas. *abc* Values with different letters above the bars for a given extract are significantly different from each other ($p < 0.05$, Tukey's HSD-multiple range post-hoc test, IBM SPSS for Windows).

The ethanol extracts of all the plant parts showed a significantly higher ($p < 0.05$) total reducing power than extracts obtained from other solvents (ethyl acetate and water), which was even higher than the standards (Trolox and ascorbic acid) used in this experiment. The ethanol extract of the root bark showed the highest total reducing power compared to the other extracts from the different parts of the plant (Figure 6.2.2 a-c).

The DPPH radical scavenging activities of the different solvent extracts of B. ferruginea parts are shown in Figure 6.2.2 (d-f). All the extracts from all parts of the plant showed a dose-dependent inhibition of DPPH, while the ethanol extracts from all parts showed a significantly higher ($p < 0.05$)
free radical scavenging activity than other solvent extracts. Interestingly, the ethyl acetate extract of the leaves and the ethanol extract of the root bark gave the lowest IC\textsubscript{50} values compared to other extracts from the different parts of the plant (Table 6.2.1).

Figure 6.2.2: (a-c) Ferric reducing antioxidant power (FRAP) and (d-f) DPPH scavenging activity of extracts from leaves, stem bark and root barks of *B. ferruginea*. Data are reported as mean ± SD of three replicas. *\textsuperscript{a-e} Different letters above the bars for a given extract are significantly different from each other (*p* < 0.05, Tukey's-HSD-multiple range post-hoc test, IBM SPSS for Windows) EtAc; Ethyl acetate, EtOH; Ethanol, GA; Gallic acid, AA; Ascorbic acid.
Figure 6.2.3 (a-c) shows the nitric oxide (NO) inhibitory activities of *B. ferruginea* parts solvents extracts. The ethyl acetate extract of the plant parts showed a dose-independent inhibition of NO while the ethanol extracts showed a dose-dependent inhibition of NO which was significantly (*p* < 0.05) higher than other solvents. Furthermore, the ethanol extract of the leaves and the ethyl acetate extract of the root bark showed lowest IC$_{50}$ values than the other extracts (Table 6.2.1).

**Figure 6.2.3:** (a-c) Nitric oxide and (d-f) Hydroxyl radical scavenging activity of extracts from leaves, stem bark and root barks of *B. ferruginea*. Data are reported as mean ± SD of three replicas. Different letters above the bars for a given extract are significantly different from each other (*p* < 0.05, Tukey's-HSD-multiple range post-hoc test, IBM SPSS for Windows). EtAc; Ethyl acetate, EtOH; Ethanol, GA; Gallic acid, AA; Ascorbic acid.
The hydroxyl (OH) radical scavenging activity of the extracts is shown in Figure 6.2.3 (d-f). The ethanol extract of the leaves and root bark showed OH radical (obtained through the Fenton reaction) scavenging activity. The ethyl acetate extracts from all the parts showed a dose-independent OH radical scavenging activity. However, the aqueous extract of the stem bark showed a dose dependent inhibition of OH radical. The ethyl acetate extract of the root bark and leaves showed the lowest IC
\(_{50}\) values compared to other extracts (Table 6.2.1).

Figure 6.2.4 shows the \(\alpha\)-glucosidase and \(\alpha\)-amylase inhibitory activities of the extracts. The ethanol and aqueous extracts of all the plant parts showed a dose-dependent inhibition of \(\alpha\)-glucosidase enzyme activity (Figure 6.2.4 a-c). The ethanol extract of the root bark and leaves showed the lowest IC
\(_{50}\) values which was significantly \((p < 0.05)\) lower than even the tested standard drug (acarbose) (Table 6.2.1). \(\alpha\)-amylase was also inhibited by some solvents of \(B.\ ferruginea\) plant parts (Figure 6.2.4 d-f). On the table showing the IC
\(_{50}\) values, the aqueous extract showed the lowest IC
\(_{50}\) value compared to the other extracts.
Figure 6.2.4: (a-c) α-glucosidase and (d-f) α-amylase inhibiting activity of extracts from leaves, stem bark and root barks of *B. ferruginea*. Data are reported as mean ± SD of three replicas. a–d Different letters above the bars for a given extract are significantly different from each other (*p* < 0.05, Tukey’s-HSD-multiple range post-hoc test, IBM SPSS for Windows). EtAc; Ethyl acetate, EtOH; Ethanol.

The pancreatic lipase inhibitory activities of the extracts are reported on Figure 6.2.5. All extracts from the different parts of *B. ferruginea* showed a dose-dependent inhibition of lipase enzyme. The ethanol extract of the leaves showed a significantly lower (*p* < 0.05) IC₅₀ value compared to the other extracts.
Figure 6.2.5 (a-c) Lipase inhibiting activity of extracts from leaves, stem bark and root barks of *B. ferruginea*. Data are reported as mean ± SD of three replicas. *a-d* Different letters above the bars for a given extract are significantly different from each other (*p* < 0.05, Tukey’s-HSD-multiple range *post-hoc* test, IBM SPSS for Windows). EtAc; Ethyl acetate, EtOH; Ethanol.

Based on the antioxidant and antidiabetic activities, the ethanol extracts from the leaves, stem bark and root barks were selected for GC-MS analysis. The main compounds identified from these extracts are steroids, terpenoids, aliphatic and aromatic compounds. **Figure 6.2.5 and 6.2.6** shows the structures of the identified compounds. **Table 6.2.2** shows the molecular formula, retention time, molecular mass and relative abundance of the different compounds identified from the plant.
Figure 6.2.6: Sterols and other terpenoids from *Bridelia ferruginea*
Figure 6.2.7: Aromatic and aliphatic compounds from *Bridelia ferruginea*
Table 6.2.1: IC₅₀ values of various extracts of *B. ferruginea* parts in different anti-oxidative, anti-diabetic and anti-obesogenic models

<table>
<thead>
<tr>
<th></th>
<th>DPPH (µg/mL)</th>
<th>NO (µg/mL)</th>
<th>OH (µg/mL)</th>
<th>α-glucosidase (µg/mL)</th>
<th>α-amylase (µg/mL)</th>
<th>Lipase (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Leaves</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EtAc</td>
<td>2.54±0.55a</td>
<td>76.24±16.54c</td>
<td>52.34±13.27b</td>
<td>8.17±1.61c</td>
<td>326.34±7.31g</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>EtOH</td>
<td>14.61±0.09d</td>
<td>16.95±1.73a</td>
<td>46.94±12.99b</td>
<td>3.63±1.03a</td>
<td>224.75±9.32f</td>
<td>1.34±1.02b</td>
</tr>
<tr>
<td>Aqueous</td>
<td>23.03±0.71e</td>
<td>151.86±23.61c</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>5.01±2.41a</td>
<td>&gt;10000</td>
</tr>
<tr>
<td><strong>Stem bark</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EtAc</td>
<td>6.24±0.11c</td>
<td>27.53±4.82b</td>
<td>49.29±11.82b</td>
<td>4.89±2.51b</td>
<td>75.96±2.7d</td>
<td>&gt;10000</td>
</tr>
<tr>
<td>EtOH</td>
<td>&gt;1000</td>
<td>148.57±25.71c</td>
<td>301.5±28.4c</td>
<td>8.39±1.26c</td>
<td>100.69±16.73d</td>
<td>&gt;10000</td>
</tr>
<tr>
<td>Aqueous</td>
<td>&gt;1000</td>
<td>105.51±16.35d</td>
<td>303.16±41.69c</td>
<td>&gt;1000</td>
<td>13.32±75b</td>
<td>&gt;1000</td>
</tr>
<tr>
<td><strong>Root bark</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EtAc</td>
<td>7.46±0.46c</td>
<td>15.76±9.53a</td>
<td>44.48±3.51b</td>
<td>&gt;1000</td>
<td>188.59±24.92d</td>
<td>&gt;10000</td>
</tr>
<tr>
<td>EtOH</td>
<td>5.29±0.08b</td>
<td>35.77±7.62b</td>
<td>47.31±13.8b</td>
<td>2.42±0.92a</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>Aqueous</td>
<td>2.34±0.10e</td>
<td>108.66±11.48d</td>
<td>30.95±17.35b</td>
<td>&gt;1000</td>
<td>519.1±15.92b</td>
<td>&gt;10000</td>
</tr>
<tr>
<td><strong>Standard</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GA</td>
<td>ND</td>
<td>191.12±23.05f</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>AA</td>
<td>2.19±0.05a</td>
<td>196.71±27.45f</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Trolox</td>
<td>5.79±0.43b</td>
<td>19.40±1.03a</td>
<td>3.54±0.91a</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Acarbose</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>36.27±1.84d</td>
<td>53.77±3.95c</td>
<td>ND</td>
</tr>
<tr>
<td>Orlistat</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.03±0.12a</td>
<td>ND</td>
</tr>
</tbody>
</table>

DPPH, 1,1-diphenyl-2-picrylhydrazyl radical, NO, nitric oxide radical, OH, hydroxyl radical; EtAc, Ethyl acetate, EtOH, Ethanol, GA, Gallic acid, AA, Ascorbic acid, ND, not determined. Data are reported as mean ± SD values of three replicas. *Values with different letters within a column are for a given test are significantly different from each other (Tukey’s-HSD multiple range post hoc test, p < 0.05).
Table 6.2.2: Compounds identified in the ethanol extract of the leaves of *B. ferruginea* by GC-MS

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Molecular formula</th>
<th>RT (min)</th>
<th>Molecular mass</th>
<th>Relative abundance (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cymene</td>
<td>C_{10}H_{14}</td>
<td>6.91</td>
<td>134.22</td>
<td>0.47</td>
</tr>
<tr>
<td>Phytol acetate</td>
<td>C_{22}H_{42}O_{2}</td>
<td>16.43</td>
<td>338.32</td>
<td>2.64</td>
</tr>
<tr>
<td>Phytol</td>
<td>C_{20}H_{36}O</td>
<td>16.87</td>
<td>296.31</td>
<td>0.99</td>
</tr>
<tr>
<td>Stigmasta-3,5-dien-7-one</td>
<td>C_{29}H_{44}O</td>
<td>18.64</td>
<td>410.67</td>
<td>3.32</td>
</tr>
<tr>
<td>Z-6-octadecenoic acid</td>
<td>C_{18}H_{36}O_{2}</td>
<td>19.35</td>
<td>282.46</td>
<td>1.29</td>
</tr>
<tr>
<td>4-phenylbenzophenone</td>
<td>C_{19}H_{16}O</td>
<td>20.07</td>
<td>258.31</td>
<td>0.74</td>
</tr>
<tr>
<td>3β-acetoxystigmastadien-7-one-14,16,22-tetraenolide</td>
<td>C_{26}H_{32}O_{4}</td>
<td>20.20</td>
<td>408.53</td>
<td>0.35</td>
</tr>
<tr>
<td>Lupeol</td>
<td>C_{30}H_{50}O</td>
<td>21.97</td>
<td>426.72</td>
<td>1.39</td>
</tr>
<tr>
<td>9-((4-methoxyphenyl) xanthene</td>
<td>C_{20}H_{16}O_{2}</td>
<td>22.40</td>
<td>288.34</td>
<td>3.26</td>
</tr>
<tr>
<td>β-ameyrin</td>
<td>C_{30}H_{50}O</td>
<td>23.25</td>
<td>426.72</td>
<td>2.68</td>
</tr>
<tr>
<td>α-ameyrin acetate</td>
<td>C_{32}H_{52}O_{2}</td>
<td>24.47</td>
<td>468.40</td>
<td>26.13</td>
</tr>
<tr>
<td>Tocopherol acetate</td>
<td>C_{31}H_{30}O_{3}</td>
<td>27.39</td>
<td>472.74</td>
<td>0.57</td>
</tr>
<tr>
<td>Stigmasterol</td>
<td>C_{29}H_{40}O</td>
<td>28.97</td>
<td>412.69</td>
<td>0.43</td>
</tr>
<tr>
<td>Sitosterol</td>
<td>C_{29}H_{40}O</td>
<td>29.76</td>
<td>414.71</td>
<td>0.54</td>
</tr>
</tbody>
</table>

The compounds reported in the table are those which matched similar compounds in the NIST library.

Table 6.2.3: Compounds identified in the ethanol extract of the stem bark of *B. ferruginea* by GC-MS

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Molecular formula</th>
<th>RT (min)</th>
<th>Molecular mass</th>
<th>Relative abundance (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,3,6-trimethylhept-3-en-1-ol</td>
<td>C_{10}H_{20}O</td>
<td>7.20</td>
<td>156.23</td>
<td>5.04</td>
</tr>
<tr>
<td>Citronellyl butyrate</td>
<td>C_{14}H_{26}O_{2}</td>
<td>7.54</td>
<td>226.36</td>
<td>4.25</td>
</tr>
<tr>
<td>3,7-dimethyl-2-octen-1-ol</td>
<td>C_{10}H_{20}O</td>
<td>7.73</td>
<td>156.15</td>
<td>4.27</td>
</tr>
<tr>
<td>p-menth-4-en-3-one</td>
<td>C_{10}H_{16}O</td>
<td>9.64</td>
<td>152.23</td>
<td>0.34</td>
</tr>
<tr>
<td>3β-hydroxydamascone</td>
<td>C_{13}H_{20}O_{2}</td>
<td>14.00</td>
<td>208.30</td>
<td>0.88</td>
</tr>
<tr>
<td>4-hydroxy-3-methoxyphenyl-2-propenal</td>
<td>C_{10}H_{16}O_{3}</td>
<td>15.44</td>
<td>178.18</td>
<td>1.35</td>
</tr>
<tr>
<td>3,5-dimethoxy-4-hydroxycinnamaldehyde</td>
<td>C_{11}H_{12}O_{4}</td>
<td>17.97</td>
<td>208.21</td>
<td>0.90</td>
</tr>
<tr>
<td>4-phenylbenzophenone</td>
<td>C_{18}H_{14}O</td>
<td>20.08</td>
<td>258.31</td>
<td>0.58</td>
</tr>
<tr>
<td>9-((4-methoxyphenyl) xanthene</td>
<td>C_{20}H_{16}O_{2}</td>
<td>22.40</td>
<td>288.34</td>
<td>2.12</td>
</tr>
</tbody>
</table>

The compounds reported in the table are those which matched similar compounds in the NIST library.
Table 6.2.4: Compounds identified in the ethanol extract of the root bark of *B. ferruginea* by GC-MS

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Molecular formula</th>
<th>RT (min)</th>
<th>Molecular mass</th>
<th>Relative abundance (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-amyrin</td>
<td>C_{30}H_{50}O</td>
<td>19.10</td>
<td>426.72</td>
<td>0.55</td>
</tr>
<tr>
<td>α-amyrin</td>
<td>C_{30}H_{50}O</td>
<td>19.31</td>
<td>426.72</td>
<td>2.85</td>
</tr>
<tr>
<td>Olean-12-en-3-one</td>
<td>C_{30}H_{48}O</td>
<td>19.43</td>
<td>424.70</td>
<td>1.51</td>
</tr>
<tr>
<td>α-amyrin acetate</td>
<td>C_{32}H_{52}O_{2}</td>
<td>19.51</td>
<td>468.40</td>
<td>1.37</td>
</tr>
<tr>
<td>Lupenone</td>
<td>C_{30}H_{48}O</td>
<td>20.64</td>
<td>424.70</td>
<td>0.54</td>
</tr>
<tr>
<td>Lupeol</td>
<td>C_{30}H_{48}O</td>
<td>21.94</td>
<td>426.72</td>
<td>2.41</td>
</tr>
<tr>
<td>9-(4-methoxyphenyl)xanthen</td>
<td>C_{20}H_{16}O_{2}</td>
<td>22.40</td>
<td>288.34</td>
<td>0.90</td>
</tr>
<tr>
<td>24-methyleneycloartenol</td>
<td>C_{31}H_{32}O</td>
<td>23.48</td>
<td>440.74</td>
<td>0.68</td>
</tr>
<tr>
<td>Lupeol acetate</td>
<td>C_{32}H_{52}O_{2}</td>
<td>24.56</td>
<td>468.75</td>
<td>36.99</td>
</tr>
</tbody>
</table>

The compounds reported in the table are those which matched similar compounds in the NIST library software.

The binding affinities of some (compounds with high relative abundance) constituents with α-glucosidase, α-amylase and lipase is shown in Table 6.2.5. β-amyrin, Lupenone and 4-Phenylbenzophenone showed the highest binding affinities for the enzymes: α-glucosidase (-8.5 kcal/mol), α-amylase (-6.8 kcal/mol) and lipase (-10.2 kcal/mol).

Table 6.2.5: Binding affinities (kcal/mol) of compounds enzymes

<table>
<thead>
<tr>
<th>Compounds</th>
<th>α-glucosidase</th>
<th>α-amylase</th>
<th>Lipase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cymene</td>
<td>-4.7</td>
<td>-4.4</td>
<td>-6.5</td>
</tr>
<tr>
<td>β-amyrin</td>
<td><strong>-8.5</strong></td>
<td>-6.2</td>
<td>-9.5</td>
</tr>
<tr>
<td>α-amyrin acetate</td>
<td>-7.2</td>
<td>-6.4</td>
<td>-9.1</td>
</tr>
<tr>
<td>4-Phenylbenzophenone</td>
<td>-6.5</td>
<td>6.3</td>
<td><strong>-10.2</strong></td>
</tr>
<tr>
<td>Lupenone</td>
<td>-7.7</td>
<td><strong>-6.8</strong></td>
<td>-9.9</td>
</tr>
<tr>
<td>Lupeol acetate</td>
<td>-6.2</td>
<td>-8.7</td>
<td></td>
</tr>
<tr>
<td>2,3,6-trimethylhept-3-en-1-ol</td>
<td><strong>-4.2</strong></td>
<td>-4.3</td>
<td><strong>-5.9</strong></td>
</tr>
</tbody>
</table>
The 3D illustrations of the interactions of β-amyrin, Lupenone and 4-Phenylbenzophenone with α-glucosidase, α-amylase and lipase respectively is shown in Figure 6.2.8, 6.2.9 and 6.2.10.

Figure 6.2.8: Illustration of 3-D (a) and 2-D (b) interactions with amino acid residues of docked β-amyrin-α-glucosidase complex with the highest binding affinity.

Figure 6.2.9: Illustration of 3-D (a) and 2-D (b) interactions with amino acid residues of docked 4-Phenylbenzophenone-Lipase complex with the highest binding affinity.
6.2.5 Discussion

Plants with antioxidant abilities have been exploited in recent times for management of disease such as diabetes whose complications arise as a consequence of oxidative stress. The thriving of oxidative stress in the body leads to the progression of diverse complications in diabetes. *B. ferruginea* has been reported to contain a high antioxidant potential in combating the damages of oxidative stress (Oloyede and Babalola, 2012). This study investigated the antioxidant and anti-diabetic activities of the leaves, stem bark and root bark extracts (different solvents) in order to further explain the importance of *B. ferruginea* in treatment of diseases associated with oxidative stress.

Plants rich in polyphenols have been reported to perform antioxidant abilities through their reactive oxygen species (ROS) neutralising capacities (Aiyegoro and Okoh, 2010). The solvents used in this study extracted a good quantity of phenols. The ethyl acetate extract of the leaves showed the highest total phenolic content which is a similar finding to an earlier reported study (Bhandari et al., 2008).

The ability of bioactive compounds to donate electrons as a show of their reducing power is an indication of their antioxidant potentials (Benzie and Strain, 1996). Antioxidants function as redox reactants where they are able to donate electrons to oxidants. Thus, the reduction of Fe$^{2+}$ to Fe$^{3+}$ could be due to the presence of reductants in the plant which was investigated in the FRAP assay. The strong reducing capacity of the leaves, stem and root bark ethanol extracts (*Figure 6.2.2 a-c*) showed the phytochemical components of *B. ferruginea* exhibiting high redox potential are extractable by ethanol.
The DPPH radical is a stable free radical that is commonly used to investigate the free radical scavenging activity of plant extracts, fractions or pure compounds in vitro (Kedare and Singh, 2011). Antioxidants inhibit DPPH by donating a hydrogen atom. The potent DPPH radical scavenging activities of the different solvent extracts especially from the leaves and root even comparable to standard antioxidants could suggest that bioactive compounds with hydrogen donating ability could serve as inhibitors of free radicals. However, the aqueous extract of the root bark showed the lowest IC$_{50}$ (Table 6.2.1) value similar to ascorbic acid and even lower than Trolox (a standard antioxidant). Interestingly, a recent research examined the DPPH scavenging potential of free and bound phenolics extracted from the leaves of B. ferruginea (Afolabi et al., 2018). The IC$_{50}$ values obtained from the assay was not as low as the IC$_{50}$ obtained from our study which shows that root bark just like the leaves might contain phenolics with better DPPH scavenging capacity.

Under aerobic conditions, nitric oxide is unstable and generates stable nitrates and nitrites by reacting with O$_2$. Griess reagent is used to estimate nitric oxide, where the amount of nitrous acid is reduced in the presence of an antioxidant (Boora et al., 2014). The ethanol and ethyl acetate extracts of the leaves and root bark respectively, showed the lowest IC$_{50}$ values compared to other extracts (Table 6.2.1). This further suggests that the extracts from B. ferruginea contain potent antioxidant activity capable of inhibiting free radical generation and oxidative stress.

Hydroxyl radicals are short-acting highly reactive species that are capable of initiating damages to important macromolecules such as proteins and lipids (Nita and Grzybowski, 2016). These radicals carry out destructive activities through polymerization, auto-oxidation and breakage of biomolecules. Therefore, medicinal plants with hydrogen radical-scavenging activities are potential targets in the treatment of diseases where oxidative stress causes diverse complications. From the present study (Figure 6.2.3 d-f), the ethanol extracts of the leaves and root barks showed a dose-dependent inhibition of OH radical with low IC$_{50}$ values.

An effective therapeutic method for managing T2D is through the reduction of post-prandial high blood glucose levels. This is achieved by reducing the rise in glucose levels by inhibiting carbohydrate-hydrolysing enzymes: α-glucosidase and α-amylase both active in the digestive tract (Tundis et al., 2010). Inhibiting these enzymes causes a stretch in the digestion of carbohydrates in food thereby giving more time to insulin to effectively offset the glucose load in the blood stream. A number of medicinal plants have been reported to exhibit reduction of glucose production from carbohydrates in the intestine (Choudhury et al., 2017). The ethanol extracts of the leaves and root bark showed dose-dependent inhibition (Figure 6.2.4), low IC$_{50}$ values (Table 6.2.1) for α-
glucosidase and α-amylase (aqueous extract of the leaves) than other counterparts, which suggests the presence of better groups that could inhibit the breakdown of complex carbohydrates to glucose thereby reducing post-prandial hyperglycaemia. Obesity, one of the major risk factors of T2D is caused by high calorie intake, which can be reduced by inhibiting pancreatic lipase activity thereby delaying the absorption of lipids (Padwal and Majumdar, 2007). The ethanol extract of the leaves showed dose-dependent lipase inhibition (Figure 6.2.5) and low IC50 values for the anti-lipase activity, which in combination with the α-glucosidase result shows the anti-diabetic and anti-obesogenic potency of B. ferruginea.

A recent study by Afolayan et al. (2018) reported that 14 compounds were identified through bioassay guided fractionation and purification from the methanol leaves of B. ferruginea. Our study went further to identify the potent bioactive constituents present in the ethanol extracts of the leaves, stem and root bark. Fourteen compounds were identified in the ethanol extracts of the leaves, while nine compounds were identified each from the stem bark and root bark extracts. The compounds identified were mainly phenols like sterols, triterpenes and other aliphatic compounds (Figure 6.2.6). Some aromatic compounds were also identified in the GC-MS analysis (Figure 6.2.7). β-Amyrin and α-amyrin acetate found in this plant are known pentacyclic triterpenes found distributed in plants and have been reported to have diverse biological properties like anti-inflammatory, anti-microbial, cytotoxic, and trypanocidal activity amongst others (Vázquez et al., 2012). The anti-diabetic properties of these triterpenes have also been established (Nair et al., 2014, Santos et al., 2012). 4-phenylbenzophenone and Lupenone identified in our study have been reported to exhibit promising anti-diabetic activity (Lin et al., 2009, Xu et al., 2014). Conversely, the observed anti-diabetic and anti-obesogenic activities of B. ferruginea might be due to the synergistic effect rendered by the identified bioactive constituents. To further verify this claim, some of the identified compounds were docked with key anti-diabetic (α-glucosidase and α-amylase) and anti-obesogenic (lipase) enzymes using in silico molecular docking analysis (Table 6.2.5). Interestingly, β-amyrin, 4-phenylbenzophenone and Lupenone had the highest binding energies for α-glucosidase, α-amylase and lipase enzymes respectively, which justified the biological properties of the compounds. The 3D interactions of these compounds with the enzymes is shown in Figure 6.2.8-10. Previous studies have also reported high binding energies of plant constituents with anti-diabetic enzymes (Bharatham et al., 2008, Oyebode et al., 2018).
In the light of the results obtained for all the antioxidant and anti-diabetic models used in this study, it is possible to conclude that the ethanol extract of the leaves in particular shows better activities in all models and should be exploited in future studies to identify and extract the exact bioactive constituent. Further studies will entail bioassay guided fractionation of the ethanol extract of the leaves.

Postscript: From the above experiment, the ethanol crude extract from the leaves was found to have relatively higher antioxidant and anti-diabetic activity observed across various types of assays. It was therefore selected for further investigations.
6.3 Fractions from Bridelia ferruginea leaves attenuate oxidative stress, inhibit key carbohydrate digesting enzyme and intestinal glucose absorption but enhance muscle glucose uptake

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Preface: This chapter reports the detailed in vitro and ex vivo anti-diabetic effects of the fractions obtained from the ethanol extract of the leaves. It is currently awaiting revision before submission to a journal for publication.

6.3.1 Abstract

The antioxidant, anti-hyperglycaemic and anti-lipidaemic potentials of leaves of Bridelia ferruginea have been documented, but the modes of action remain elusive. The present study investigated not only the antioxidant potentials, but also the anti-hyperglycaemic mode of action of fractions obtained from the crude ethanol extract of leaves of B. ferruginea using several in vitro and ex vivo models. The crude ethanol extract of the leaves of B. ferruginea was subjected to solvent fractionation to yield four fractions: butanol, dichloromethane (DCM), ethyl acetate and aqueous fractions. The total phenolic content of these fractions was first determined. The abilities of these fractions to activate antioxidant enzymes in Fe²⁺-induced pancreatic oxidative injuries were also investigated ex vivo. Then, α-glucosidase inhibitory activity was estimated in vitro. The ex vivo effects of the fractions on glucose absorption in isolated rat jejunum as well as glucose uptake in isolated rat psoas muscle was also investigated. All fractions exhibited a moderate total phenolic content and DPPH inhibitory activities. Catalase, SOD and reduced glutathione levels were significantly (p < 0.05) increased by the fractions as compared to the untreated group. The butanol fraction showed a more potent antioxidant activity which might be due to the presence of high phenolic content. The butanol fraction also showed a dose-dependent inhibition of α-glucosidase enzyme and a high glucose uptake activity in isolated rat jejunum. The fractions of B. ferruginea were also able to enhance glucose uptake in isolated rat psoas muscle. GC-MS analysis revealed the presence of some potent bioactive constituents which could render synergistic effects to the observed antioxidant and anti-diabetic
activities of *B. ferruginea*. Data from this study suggest that *B. ferruginea* may not only ameliorate oxidative stress but may also exert glycaemic control via enhancing muscle glucose uptake and impairing carbohydrate and glucose absorption. Thus, it may be further investigated as a useful dietary supplement for managing hyperglycaemia and oxidative stress.

**Key words:** *Bridelia ferruginea*, oxidative stress, anti-oxidant, glucose absorption, glucose uptake, enzyme inhibition

### 6.3.2 Introduction

The increased oxidative stress in diseases related to metabolic syndrome has been long established (Asmat *et al.*, 2016). Prolonged oxidative stress leads to β-cell dysfunction, insulin resistance which in the long run leads to diseases such as type 2 diabetes (Tiwari *et al.*, 2013). Oxidative stress occurs when there is an imbalance between reactive oxygen species (ROS) and antioxidants in favour of ROS. This imbalance causes ROS to become harmful to macro molecules like lipids, proteins and nucleic acids due to the inactivity of the natural defence system of the cell (antioxidants) (Styskal *et al.*, 2012). This imbalance leads to reduction in endogenous antioxidants enzymes such as catalase and superoxide dismutase (SOD) as well as *in vivo* antioxidants and reduced glutathione. The destruction caused by oxidative stress leads to macro- and micro-vascular complications of type 2 diabetes which encompasses eyes, kidney and heart problems and ultimately, death (Folli *et al.*, 2011). The ability of natural antioxidants to reduce ROS formation by increasing the activities of endogenous antioxidants can be exploited for treatment of diseases caused by oxidative stress such as diabetes.

Persistent hyperglycaemia has also been implicated in leading to multiple diabetic complications (Marcovechio *et al.*, 2011). The level of post prandial hyperglycaemia keeps rising due to continuous breakdown and digestion of dietary carbohydrates. The inhibition of carbohydrate hydrolysing enzymes such as α-glucosidase and α-amylase can delay the digestion, as well as the absorption of sugar from carbohydrates, which is a key focus in anti-diabetic drug research (Asif, 2014). The ability of natural products from plant sources to inhibit the activity of these carbohydrate-hydrolysing enzymes have been paid more attention in recent times (Kalita *et al.*, 2018).

*Bridelia ferruginea* Benth. (*Euphorbiaceae*) is a ligneous shrub or tree (as climate and soil favours) that grows in various parts of Africa and is used by traditional healers for the treatment of ailments like boils, arthritis, diabetes, bruises and burns (Awodele *et al.*, 2015; Olajide *et al.*, 2012). From the
work done on this plant so far, there is no report on the effects of the fractions obtained from the leaves of the plant on intestinal glucose absorption and muscle glucose uptake.

Hence, the present study was conducted to examine the effects *Bridelia ferruginea* fraction on antioxidative status, inhibition of carbohydrate digesting enzymes, intestinal glucose absorption and muscle glucose uptake using a number of experimental models.

### 6.3.3 Materials and methods

Please refer to Chapter 2 for the detailed materials and methods used for *B. ferruginea*.

### 6.3.4 Results

The total phenol contents of the fractions (dichloromethane, ethyl acetate, butanol and aqueous) are shown on Table 6.3.1. The DCM fraction showed the significantly (*p* < 0.05) higher concentration of total phenol than other fractions.

The ability of the fractions to scavenge free radicals was investigated using the DPPH method and is shown in Figure 6.3.1. Only butanol fraction showed a consistent dose-dependent inhibition of DPPH. The butanol fraction also showed a significantly (*p* < 0.05) higher inhibition of DPPH compared to other fractions which was also similar to the activity of the standards (Trolox and ascorbic acid) used in this experiment. The lowest DPPH scavenging activity was displayed by the DCM fraction. The butanol and aqueous fractions both showed low IC$_{50}$ values for DPPH inhibition as reported in Table 6.3.1.

![Figure 6.3.1: DPPH inhibiting activity of *B. ferruginea* fractions. Data are reported as mean ± SD. *a*–*d* Values with different letter above the bars for a given concentration are significantly different from each other (*p* < 0.05, Tukey’s HSD-multiple range post-hoc test, IBM SPSS for Windows). EtAc, DCM and AA represent Ethyl acetate, Dichloromethane fractions and Ascorbic acid respectively, DPPH, 2,2-Diphenyl-1-picrylhydrazyl.](image-url)
Figure 6.3.2 shows the free radical antioxidant power of the fractions, which was measured by the ability of the fractions to convert the ferricyanide complex (Fe$^{3+}$) to ferrous form (Fe$^{2+}$). The butanol fraction gave the significantly ($p < 0.05$) higher activity compared to other fractions. The butanol fraction was closely followed by the DCM and ethyl acetate fractions and the lowest activity was exhibited by the most polar or aqueous fraction.

Oxidative stress was induced in the pancreatic homogenates by incubating with FeSO$_4$. A reduction in reduced glutathione level, catalase and SOD enzymes activity in the untreated group showed a successful induction of pancreatic oxidative injury (Figure 6.3.3a-c). The reduced levels of these antioxidant enzymes were further justified by an increase in malondialdehyde (MDA) levels as an indicator of lipid peroxidation. Interestingly, treatment with different concentrations of the fractions significantly ($p < 0.05$) increased the endogenous antioxidant enzymes activity and GSH level and concomitantly reduced MDA levels to almost normal levels. The butanol fraction showed the highest ability to induce GSH levels (Figure 6.3.3a) in the treated sample which was also replicated by the low IC$_{50}$ value in Table 6.3.1 and was comparable to the standard ascorbic acid. For catalase activity, only the DCM and butanol fractions showed a dose-dependent induction of catalase (Figure 6.3.3b). In Figure 6.3.3c, all fractions showed a dose-dependent induction of SOD activity, interestingly, the high dose (250 µg/mL) of all the fractions showed statistically significant and comparable results to the standard (ascorbic acid). The fractions also showed a dose-dependent reduction of lipid peroxidation, the butanol and aqueous fractions showed significantly ($p < 0.05$) lower MDA levels.
compared to other fractions which demonstrate the lipid peroxidation reducing properties of these fractions (Figure 6.3.3d).

**Figure 6.3.3:** Effect of fractions of *B. ferruginea* leaves on a) Reduced glutathione, b) catalase, c) Superoxide dismutase (SOD) and d) Lipid peroxidation in oxidative pancreatic injury. Data are reported as mean ± SD of three replicas Data are reported as mean ± SD of three replicas. ‘Significantly different from untreated sample and ‘Significantly different from normal sample (*p* < 0.05, Tukey’s-HSD-multiple range post-hoc test, IBM SPSS for Windows). EtAc, DCM and AA represent Ethyl acetate, Dichloromethane fractions and ascorbic acid respectively.

The α-glucosidase enzyme-inhibiting activity of the fractions is shown on **Figure 6.3.4.** All the fractions inhibited the enzyme in a dose-dependent manner, the butanol fraction showed significantly (*p* < 0.05) higher inhibition of α-glucosidase compared to other fractions. The enzyme inhibitory activity of the butanol and aqueous fractions at the highest dose (250 µg/mL) was even significantly
(p < 0.05) higher than the standard drug (acarbose) used. The butanol fraction gave the lowest IC₅₀, as low as the standard drug, acarbose (Table 6.3.1).

![Graph showing inhibition of α-glucosidase activity](image)

**Figure 6.3.4:** α-glucosidase inhibitory activity of *B. ferruginea* leaves fractions. Data are reported as mean ± SD of three replicas. a-d Different letters over the bars for a given concentration for each extract indicate a significant difference from each other (p < 0.05, Tukey’s-HSD-multiple range post-hoc test, IBM SPSS for Windows). EtAc and DCM represents Ethyl acetate and Dichloromethane fractions respectively.

The effect of the butanol and aqueous fractions (fractions with the best results in all the models tested) on glucose absorption and glucose uptake in harvested organs was investigated (Figure 6.3.5 and 6.3.6).

![Graph showing glucose absorption](image)

**Figure 6.3.5:** Effect of the butanol and aqueous fractions of the leaves of *B. ferruginea* on glucose absorption in isolated rat jejunum. Data are reported as mean ± SD of three replicas. a-c Different letters over the bars for a given concentration are significantly different from each other (p < 0.05, Tukey’s-HSD-multiple range post-hoc test, IBM SPSS for Windows).
**Table 6.3.1:** IC$_{50}$ values of the different solvent fractions of ethanol extract of *B. ferruginea* leaves in different antioxidant and antidiabetic models.

<table>
<thead>
<tr>
<th>Solvent Fraction</th>
<th>TPC (mg/g GAE)</th>
<th>DPPH</th>
<th>α-glucosidase</th>
<th>Catalase</th>
<th>SOD</th>
<th>GSH</th>
<th>LPO</th>
</tr>
</thead>
<tbody>
<tr>
<td>DCM</td>
<td>65±1.2$^d$</td>
<td>108.7±1.3$^d$</td>
<td>110.1±24.3$^c$</td>
<td>15.6±2.3$^b$</td>
<td>3.5±1.7$^b$</td>
<td>28.1±2.3$^c$</td>
<td>21.1±3.4$^c$</td>
</tr>
<tr>
<td>EtAc</td>
<td>39.9±0.8$^c$</td>
<td>184.1±1.1$^c$</td>
<td>122.9±16.8$^c$</td>
<td>31.2±0.6$^d$</td>
<td>2.4±1.6$^b$</td>
<td>18.6±2.7$^b$</td>
<td>25.6±4.2$^c$</td>
</tr>
<tr>
<td>Butanol</td>
<td>23.0±0.2$^b$</td>
<td>4.7±0.8$^b$</td>
<td>36.0±3.7$^a$</td>
<td>25.4±0.3$^c$</td>
<td>1.7±0.5$^b$</td>
<td>6.3±1.1$^a$</td>
<td>10.1±1.2$^b$</td>
</tr>
<tr>
<td>Aqueous</td>
<td>17.4±0.1$^a$</td>
<td>6.7±1.5$^c$</td>
<td>52.6±5.0$^b$</td>
<td>12.6±1.3$^b$</td>
<td>1.4±0.2$^b$</td>
<td>15.4±1.4$^b$</td>
<td>12.4±2.6$^b$</td>
</tr>
</tbody>
</table>

**Standards**

<table>
<thead>
<tr>
<th></th>
<th>TPC (mg/g GAE)</th>
<th>DPPH</th>
<th>α-glucosidase</th>
<th>Catalase</th>
<th>SOD</th>
<th>GSH</th>
<th>LPO</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>ND</td>
<td>2.2±0.4$^a$</td>
<td>ND</td>
<td>1.3±0.4$^a$</td>
<td>1.1±0.04$^a$</td>
<td>7.4±1.1$^a$</td>
<td>9.5±1.7$^a$</td>
</tr>
<tr>
<td>Trolox</td>
<td>ND</td>
<td>5.8±1.3$^c$</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Acarbose</td>
<td>ND</td>
<td>36.3±4.3$^a$</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

DPPH-1,1-diphenyl-2-picylhydrazyl radical, ND-not determined, EtAc, Ethyl acetate fraction, AA, Ascorbic acid, TPC, Total phenol content, SOD, Superoxide dismutase, GSH, Reduced glutathione, LPO, Lipid peroxidation. Data are reported as mean ± SD values of three replicas. $^{a-c}$Values with different superscript letters within a column are significantly different from each other (Tukey’s-HSD multiple range post hoc test, $p < 0.05$): the same letters stand for non-significant difference.

**Figure 6.3.6:** Effect of the a) butanol and b) aqueous fraction of the leaves of *B. ferruginea* on glucose uptake in isolated rat psoas muscle. Data are reported as mean ± SD of three replicas. $^{a-c,w-z}$Different letters over the bars for a given concentration are significantly different from each other ($p < 0.05$, Tukey’s-HSD-multiple range post-hoc test, IBM SPSS for Windows). Metformin: 2mg/dL.

The phytochemical compounds present in the DCM, ethyl acetate, butanol and aqueous fractions of *B. ferruginea* was identified using GC-MS analysis. The main compounds identified from these extracts are phenols, steroids, terpenoids, aliphatic and aromatic compounds. **Figure 6.3.7** shows the structures of the identified compounds. **Table 6.3.2** shows the molecular formula, retention time, molecular mass and relative abundance of the different compounds identified from the plant.
Figure 6.3.7: Identified bioactive components in the DCM, butanol, aqueous and ethyl acetate fractions of the *B. ferruginea*.
Table 6.3.2: Compounds identified in the fractions of *B. Ferruginea* leaves ethanol extract by GC-MS

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Molecular formula</th>
<th>RT (min)</th>
<th>Molecular mass</th>
<th>Relative abundance (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Aqueous</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-methoxy-3-(2-propenyl) phenol</td>
<td>C₁₀H₁₂O₂</td>
<td>11.58</td>
<td>164.20</td>
<td>0.33</td>
</tr>
<tr>
<td><strong>Butanol</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pyrogallol</td>
<td>C₆H₆O₃</td>
<td>11.81</td>
<td>126.11</td>
<td>0.37</td>
</tr>
<tr>
<td>Sitosterol</td>
<td>C₂₉H₄₅O</td>
<td>29.68</td>
<td>414.71</td>
<td>0.54</td>
</tr>
<tr>
<td><strong>Ethyl acetate</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pyrogallol</td>
<td>C₆H₆O₃</td>
<td>11.81</td>
<td>126.11</td>
<td>0.28</td>
</tr>
<tr>
<td>1-hydroxy-3-(4-hydroxy-3-methoxyphenyl)‐2‐propanone</td>
<td>C₁₀H₁₂O₄</td>
<td>16.10</td>
<td>196.20</td>
<td>0.16</td>
</tr>
<tr>
<td>phytol acetate</td>
<td>C₂₃H₄₂O₂</td>
<td>17.30</td>
<td>338.56</td>
<td>0.21</td>
</tr>
<tr>
<td>5,8,8a,9-tetrahydro-5-(3,4,5-trimethoxyphenyl)-furo[3′,4′:6,7] naphtho[2,3d]-1,3-dioxol-6-(5aH)-one</td>
<td>C₂₃H₂₂O₇</td>
<td>28.89</td>
<td>398.41</td>
<td>0.25</td>
</tr>
<tr>
<td>Stigmasterol</td>
<td>C₂₉H₄₈O</td>
<td>29.10</td>
<td>412.69</td>
<td>0.22</td>
</tr>
<tr>
<td>Sitosterol</td>
<td>C₂₉H₅₂O</td>
<td>29.68</td>
<td>414.71</td>
<td>0.66</td>
</tr>
<tr>
<td><strong>Dichloromethane</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-coumaranone</td>
<td>C₈H₆O₂</td>
<td>9.94</td>
<td>134.13</td>
<td>0.19</td>
</tr>
<tr>
<td>Pyrogallol</td>
<td>C₆H₆O₃</td>
<td>11.84</td>
<td>126.11</td>
<td>0.27</td>
</tr>
<tr>
<td>5-hydroxy-5-(1-hydroxy-1-isopropyl)-2-methyl-2-cyclohexen-1-one</td>
<td>C₁₀H₁₆O₃</td>
<td>14.85</td>
<td>184.23</td>
<td>2.22</td>
</tr>
<tr>
<td>5,5,8a-trimethyl-3,5,6,7,8,8a-hexahydro-2H-chromene</td>
<td>C₁₂H₂₆O</td>
<td>16.86</td>
<td>180</td>
<td>1.00</td>
</tr>
<tr>
<td>phytol acetate</td>
<td>C₂₃H₄₂O₂</td>
<td>17.30</td>
<td>338.56</td>
<td>0.76</td>
</tr>
<tr>
<td>Stigmasterol</td>
<td>C₂₉H₄₈O</td>
<td>29.10</td>
<td>412.69</td>
<td>0.32</td>
</tr>
<tr>
<td>Sitosterol</td>
<td>C₂₉H₅₂O</td>
<td>29.68</td>
<td>414.71</td>
<td>0.92</td>
</tr>
</tbody>
</table>

The compounds reported in the table are those which matched similar compounds in the NIST library software.

6.3.5 Discussion
The damage caused by free radicals when left unmanaged leads to the progression of diverse complications in T2D. Although research has shown that plants contain high amounts of polyphenols, hence detailed screening is necessary in order to validate this claim. The moderately high total phenol content was found in the fractions of *B. ferruginea* in our study (*Table 6.3.1*), showed the potential as an antioxidative agent. The stem bark aqueous fraction of *B. ferruginea* has been reported to contain antioxidant activity which supports results from our data (Olaide *et al.*, 2014).

The ability of the fractions to scavenge free radicals was investigated using the DPPH method. This method due to its ease of execution is usually the first line of action in detecting the antioxidant activities of plants (Kedare and Singh, 2011). The DPPH radical has an odd nitrogen electron which can be reduced by accepting a hydrogen atom from a potential antioxidant to form a hydrazine complex. *In vitro* DPPH analysis of the fraction (*Figure 6.3.1*) showed a dose dependent inhibition of DPPH exhibited only by the butanol fraction. This could be due to the presence of compounds that can donate a hydrogen atom to form a stable complex in the polar butanol fraction. Plants like *B. ferruginea* have been shown to possess promising antioxidant activity using the DPPH method (Olaide *et al.*, 2014).

The FRAP assay is a sensitive, precise and simple method for understanding the interaction between antioxidant status and disease risk (Benzie and Strain, 1999). The principle of the FRAP assay is based on the ability of an antioxidant in a sample to form a coloured complex with ferric chloride, potassium ferricyanide and trichloroacetic acid measured at 700 nm using a UV-spectrophotometer (Vijayalakshmi and Ruckmani, 2016). The highest reduction of the reactive Fe$^{3+}$ to an inactive Fe$^{2+}$-polyphenol complex was exhibited by the butanol fraction (*Figure 6.3.2*).

Transition metals like iron have major roles to play in the generation of free radicals thereby leading to oxidative stress (Yoshino and Murakami, 1998). This justifies the use of FeSO$_4$ to induce experimental oxidative pancreatic injury in normal pancreatic homogenate. Iron over load causes an increase in free radicals because the reactive superoxide anion is produced through oxygen reduction by ferrous ion, then dismutated to hydrogen peroxide through enzymatic and non-enzymatic systems (Halliwell and Gutteridge, 1990). When left untransformed, hydrogen peroxide is converted to extremely reactive hydroxyl radical (OH) through the Haber-Weiss reactions using iron or copper (Aust *et al.*, 1985). Iron also binds to molecular oxygen to form perferryl ion which is highly reactive in combination with hydroxyl radical and act as an initiator of lipid peroxidation (Yoshino and Murakami, 1998). Induction of oxidative injury by iron was done in the pancreas because the β-cells (due to high insulin action) are parts of the most metabolically active tissues and benefit highly from
oxidative phosphorylation for ATP generation (Wang and Wang, 2017). The secretion of insulin relies on oxygen utilisation due to increased blood glucose levels, thereby making the β-cells prone to the deleterious effects of ROS. Again, reduction of endogenous antioxidant enzymes also makes the β-cells vulnerable to oxidative stress (Lenzen et al., 1996, Li et al., 2008, Robertson et al., 2003).

From our result (Figure 6.3.3 a-c), incubation of the pancreatic homogenate with FeSO₄ (as earlier explained) caused a marked reduction of GSH levels, and catalase and SOD enzyme activities in the untreated cells of the antioxidan-deficient pancreatic β-cells. Reduced glutathione (GSH) has been reported as being low during the progression of complications of T2D (Tiwari et al., 2013). The ability of treatment with the different fractions of B. ferruginea in our study (Figure 6.3.3 a) shows the antioxidant potential of the fractions. SOD catalyses the superoxides to hydrogen to generate hydrogen peroxide, which is in turn dismutated by catalase to molecular oxygen and water (Robertson et al., 2003). If not cleared by catalase, hydrogen peroxide will react hitherto with Fe³⁺ (as earlier explained) to produce reactive hydroxyl radicals which causes lipid peroxidation by attacking the lipid membrane (Aslan et al., 2000). There was marked increase in the activity of catalase and SOD enzymes and concomitant reduction of lipid peroxidation after the treatment with varying doses of the fractions (Figure 6.3.3 b & c). Indeed, the antioxidant activity noticed from the fractions of B. ferruginea may have been brought about by different mechanisms such as inhibition of chain initiation, binding of transition metal enzymes, elimination of peroxides, reducing effect, radical scavenging and others (Diplock, 1997).

Effective anti-diabetic agents must have good postprandial glucose lowering activities by reduction of the digestion of carbohydrates in food to glucose. This is done by inhibiting the action of α-glucosidase enzyme (Ghadyale et al., 2012). From our results (Figure 6.3.4), the butanol fraction showed the highest α-glucosidase inhibitory activity. Research have shown that α-glucosidase inhibitors have been successfully isolated from medicinal plants (Yin et al., 2014). This leads us to suggest that B. ferruginea can be exploited as potent candidate for isolation of α-glucosidase inhibitors which will be effective in therapeutic use for T2D.

The anti-diabetic potential of fractions of B. ferruginea was also shown by the inhibition of glucose absorption in intestinal tissues (Figure 6.3.5) and concomitant induction of glucose uptake in the muscle (Figure 6.3.6). The butanol and aqueous fractions alone were used for these assays since they showed better potential as antioxidant and anti-diabetic agents. Motility and ease of exit of luminal content in the small intestine are markers of glucose absorption and in turn potential pharmacological
targets for the treatment of T2D (Thazhath et al., 2016). Postprandial glucose rise occurs when glucose is absorbed quickly in the small intestine (i.e. within the jejunal and duodenal areas) (Oyebode et al., 2018) and leaving this unmanaged may have adverse consequences in T2D (Hassan et al., 2010). Our result showed the ability of the fractions especially the aqueous fraction to reduce the absorption of glucose in the intestine (Figure 6.3.5); this together with the inhibition of α-glucosidase activity by the fractions show its potential to delay the digestion and absorption of carbohydrates might be due to the presence of phenolic compounds (Table 6.3.1). Such activity has been reported in plants containing phenolic compounds in some previous studies (Inthongkaew et al., 2017; Toma et al., 2014).

The skeletal muscle is important in glucose homeostasis as it is the main region for exercise-stimulated glucose disposal and it also interacts with the pancreas to induce secretion of insulin for effective utilization of glucose (Mizgier et al., 2014). The muscle can secrete myokines and GLUT4 which aid in endocrine functions such as glucose uptake (Mizgier et al., 2014). Induction of muscle glucose uptake notwithstanding insulin levels has been reported as a key therapy for control of hyperglycaemia (Pereira et al., 2017). Interestingly, the butanol and aqueous fractions showed induction of glucose abilities both in the presence of insulin and without, which was even as comparable as the activity of the standard drug (metformin) used. This shows that the fractions on its own (Figure 6.3.6) can induce glucose uptake in peripheral tissues. Taken together with the results from all the anti-diabetic models shows the potential of fractions of B. ferruginea in modulating hyperglycaemia.

GC-MS analysis of the fractions was conducted in order to identify the presence of bioactive components that might be responsible for the observed antioxidant and anti-diabetic activities. 2-methoxy-3-(2-propenyl) phenol found solely present in the aqueous fraction which has been reported to act as a cytotoxic agent (Espineli et al., 2014), while, pyrogallol has been reported to act as an anti-inflammatory agent (Nicolis et al., 2008). Phytol acetate has been reported to have insulin resistance alleviating properties (Nicolis et al., 2008). Of utmost importance however is the sitosterol found present in the fractions. Sitosterol is a dietary phytosterol mainly found in plant and studies have elucidated its anti-diabetic activity (Balamurugan et al., 2011; Gupta et al., 2011; Karan et al., 2012; Zeb et al., 2017). Conversely, the observed antioxidant and anti-diabetic activity of the fractions could be due to the synergistic effects of the identified bioactive compounds.

Summarily, this study suggests that the fractions (especially the butanol and aqueous) of B. ferruginea leaves contains potent antioxidant agents coupled with α-glucosidase and intestinal glucose
absorption inhibitors as well as peripheral tissue glucose uptake inducers, which could be further exploited for the development of a therapeutic strategy for the management and control of postprandial hyperglycemia in type 2 diabetes and its macrovascular complications. Future work will entail a detailed study of the active fraction in a type 2 diabetes model of rats.

Postscript: The butanol fraction was picked for the in vivo experiment or animals study since it had high antioxidant and antidiabetic activity in the tested models and also it had a high yield.
6.4 Butanol fraction of *Bridelia ferruginea* leaves regulates pancreatic β-cell functions, stimulates insulin secretion and ameliorates diabetic complications in a type 2 diabetes model of rats

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Preface: This article reports the detailed *in vivo* anti-diabetic studies of the butanol fraction and it is currently awaiting revision before submission to a journal for publication.

6.4.1 Abstract

This study reports the anti-diabetic effects of the butanol fraction of *B. ferruginea* (BFBF) leaves on streptozotocin induced diabetic rats. Seven weeks old male Sprague dawley (SD) rats were given a single intraperitoneal injection of STZ (40 mg/kg bw), after initially being fed 10% fructose solution for two weeks to induce type 2 diabetes. The diabetic animals were thereafter given an oral dose (150 or 300 mg/kg bw) of BFBF. Treatments with the plant extract significantly (*p* < 0.05) reduced the concentration of blood glucose and improved glucose tolerance compared to the untreated groups. There was also a marked improvement in β-cell function, serum insulin levels and insulin sensitivity, liver glycogen, serum lipids alterations and increased activity of liver function enzymes. Histopathological analysis of the pancreas by H and E staining also revealed the ability of BFBF to protect and regenerate the pancreatic β-cells after partial damage induced by STZ injection. The findings of this study suggest that BFBF possesses promising anti-T2D activity, which is probably mediated through the modulation of β-cell function and stimulation of insulin secretion.

**Key words:** Type 2 diabetes, histopathology, serum analysis, *Bridelia ferruginea*, SD rats

6.4.2 Introduction

Type 2 diabetes (T2D) is a complex disease, which occurs due to an increased level of postprandial glucose. This spike is caused by defective insulin transport of the elevated glucose for effective utilization by peripheral organs (such as muscle and adipose tissues) (Geijselaers *et al.*, 2017; Nolan *et al.*, 2015). Insulin resistance leads to chronic hyperglycaemia a precursor to anomalies in carbohydrate, protein and fat metabolism (Hardy *et al.*, 2012).
Presently, over 422 million cases of diabetes have been recorded, which means 1 in 11 people in the world have diabetes and there will be an estimated increase by 2030 to 640 million cases (i.e. 1 in 10 people) (Conway et al., 2018; WHO, 2016). This increase can be linked to factors such as change in diet, urbanization, sedentary lifestyles, aging and increasing population. There has been a global rise of diabetic cases as compared to the year 1980 where only 108 million cases were recorded (IDF, 2015). In South Africa, the prevalence of diabetes increased from 5.5% to 9% during 2000 to 2009, making it the second highest mean of health care expenditure in the African region as a whole (Manyema et al., 2015). T2D remains the most prevalent type of diabetes and therefore the focus of this study is T2D.

Many long-term and short-term complications are associated with the progression of T2D (Fonseca, 2009) which include eye, kidney, nerve and heart problems. Diabetes can be controlled with the use of different types of treatment modalities, while the most common one is the use of synthetic oral hypoglycemic drugs. These drugs, though sometimes effective when used singly or in combination with other drugs, are associated with a number short- or long-term side effects such as nausea, weigh gain, lactic acidosis, gastrointestinal disturbances (Stein et al., 2013). Interestingly traditional medicine is able to play a role in the treatment of diverse diseases. Because, plants contain phytochemicals which are able to render synergistic effects in the treatment of diseases such as diabetes (Kooti et al., 2016). Several studies have highlighted the efficacy of medicinal plants in the treatment of diabetes (Choudhury et al., 2017; Mohammed et al., 2014). The vast array of land filled with medicinal plants gives more opportunities for researchers to carry out more studies with a view to harness the spread of diseases through the use of medicinal plants.

*Bridelia feruginea* Benth (Euphorbiaceae) is a traditional medicinal plant commonly used in parts of Africa for the treatment of diabetes mellitus (Bakoma et al., 2014). The stem bark is boiled, and the water infusion is drunk to cure ailments such as diabetes, diarrhoea (Akuodor et al., 2012), arthritis (Olajide et al., 2012), boils and others (Olajide et al., 2012). The methanol leaves extract of *B. ferruginea,* was found in a recent study to possess hypoglycaemic activity in glucose-intolerant rats (Njamen et al., 2012). The hypoglycaemic activity of the aqueous extract of the stem bark of this plant has also been investigated in both normal and alloxan-induced diabetic rats (Adewale and Oloyede, 2012). However, the anti-diabetic effect of the butanol fraction of the leaves of *B. ferruginea* has not been tested using a model that accurately mimics T2D in rats. Therefore, this study was conducted to comprehensively investigate the in vivo anti-diabetic mechanism of action of butanol fraction of *B. ferruginea* using a better T2D animal model.
6.4.3 Materials and methods

Please refer to Chapter 2 for detailed materials and methods that affect *B. ferruginea*.

6.4.4 Results

The mean feed and fluid intake with weekly body weight changes of all animals were monitored all through the experimental period. The diabetic rats showed significantly \((p < 0.05)\) higher feed and fluid consumption as well as lower body weight gains compared to the normal rats. Oral dosing of BFBF to the diabetic rats significantly \((p < 0.05)\) decreased the fluid intake compared to the untreated diabetic rats (Figure 6.4.1). Feed intake was also reduced upon intervention with BFBF and metformin as seen in Figure 6.4.1.

![Figure 6.4.1: Mean feed and fluid intakes of different groups during the entire experimental period. Data are reported as the mean ± SD of seven animals. **Different superscript alphabets over the bars for a given parameter indicate significant difference (Tukey's-HSD multiple range post hoc test, \(p < 0.05\)). NC, Normal control, DBC, Diabetic control, DBFL, Diabetic *B. ferruginea* low dose, DBFH, Diabetic *B. ferruginea* high dose, DBM, Diabetic metformin, DBG, Diabetic glibenclamide, NBF, Normal *B. ferruginea* high dose (toxicological control). Interestingly, animals in the DBFH and DBM group showed a better body weight gain than the low dose, standard and untreated groups towards the end of the experiment (Figure 6.4.2).](image-url)
Figure 6.4.2: Mean body weight change for all groups of experimental animals over the seven weeks experimental period. Data are reported as the mean ± SD of seven animals. \(^{a-c}\)Values with different letters for a given week are significantly different from each other (Tukey’s-HSD multiple range post hoc test, \(p < 0.05\)). NC, Normal control, DBC, Diabetic control, DBFL, Diabetic \(B.\ ferruginea\) low dose, DBFH, Diabetic \(B.\ ferruginea\) high dose, DBM, Diabetic metformin, DBG, Diabetic glibenclamide, NBF, Normal \(B.\ ferruginea\) high dose (toxicological control), STZ, Streptozotocin.

After STZ injection, the diabetic groups showed significantly higher blood glucose concentrations compared to the normal groups. However, as soon as treatment of the animals started the DBFL and the DBFH groups portrayed a dose-dependent and significantly lower (\(p < 0.05\)) blood glucose levels than the untreated group (DBC) throughout the entire intervention period (Figure 6.4.3).

Figure 6.4.3: Weekly blood glucose concentrations in different animal groups during the intervention period. Data are reported as the mean ± SD of seven animals. \(^{a-e}\)Values with different letters for a given week are significantly different from each other (Tukey’s-HSD multiple range post hoc test, \(p < 0.05\)). NC, Normal control, DBC, Diabetic control, DBFL, Diabetic \(B.\ ferruginea\) low dose, DBFH, Diabetic \(B.\ ferruginea\) high dose, DBM, Diabetic metformin, DBG, Diabetic glibenclamide, NBF, Normal \(B.\ ferruginea\) high dose (toxicological control), STZ, Streptozotocin, NFBG, Non-fasting blood glucose, FBG, Fasting blood glucose.
The data for OGTT done on the last week (week 5) of the intervention period and the corresponding area under the curve (AUC) are shown in Figure 6.4.4. The glucose tolerance of DBFL and DBFH groups were significantly \((p < 0.05)\) lower than the DBC and DBG group and even comparable to the metformin group (DBM). At the 120 min mark, the DBFH group exhibited a better glucose tolerance ability which was even lower than the 0-min blood glucose level. The total area under the curve (AUC) of DBFL and DBM groups were significantly lower than the other diabetic groups (DBC, DBFH, and DBG) (Figure 6.4.4) which signifies better tolerance of the glucose overload in the blood.

Figure 6.4.4: Oral glucose tolerance test (OGTT) and corresponding area under the curve (AUC) of different animal groups in the last week of the intervention period. Data are reported as the mean ± SD of seven animals. *Values with different letters for a given time are significantly different from each other (Tukey’s-HSD multiple range post hoc test, \(p < 0.05\)). NC, Normal control; DBC, Diabetic control; DBFL, Diabetic B. ferruginea low dose; DBFH, Diabetic B. ferruginea high dose; DBM, Diabetic metformin; DBG, Diabetic glibenclamide; NBF, Normal B. ferruginea high dose (toxicological control); AUC, Area under the curve.

The liver weight, relative liver weight and liver glycogen level of different animal groups are shown in Table 6.4.1. There was no significant difference in the liver weight of all animal groups, however, the relative liver weight of the normal groups (NC and NBF) were significantly \((p < 0.05)\) lower than that of the diabetic groups. The DBFL group showed a significantly \((p < 0.05)\) lower relative liver weight compared to the DBC group. The treatment with BFBF significantly increased the liver glycogen level compared to the untreated group (Table 6.4.1).
Table 6.4.1: The effects of different doses of BFBF on liver weight, relative liver weight and liver glycogen levels in the different animal groups at the end of the intervention period.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>NC</th>
<th>DBC</th>
<th>DBFL</th>
<th>DBFH</th>
<th>DBM</th>
<th>DBG</th>
<th>NBF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver weights (g)</td>
<td>10.7±0.8</td>
<td>8.7±0.9</td>
<td>9.0±0.7</td>
<td>10.6±0.9</td>
<td>9.6±0.7</td>
<td>9.9±0.9</td>
<td>9.9±0.8</td>
</tr>
<tr>
<td>Rel. liver weights (%)</td>
<td>2.9±0.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.8±0.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.2±0.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.4±0.5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.7±0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.4±0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.8±0.3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Liver glycogen (mg/g tissue)</td>
<td>36.0±0.9&lt;sup&gt;e&lt;/sup&gt;</td>
<td>11.8±1.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>23.6±0.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>31.8±0.1&lt;sup&gt;d&lt;/sup&gt;</td>
<td>31.4±1.1&lt;sup&gt;d&lt;/sup&gt;</td>
<td>21.8±0.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>36.4±0.7&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SD (n=7). <sup>a-e</sup>Different superscript letters along a row for a given parameter indicate the significance of difference (Tukey’s-HSD multiple range post hoc test, p < 0.05).

At the end of the experimental period after sacrificing the animals, the blood samples were taken and the serum lipid profile of all the animals were taken and it is reported in Figure 6.4.5. The DBC group showed a significantly (p < 0.05) higher total and LDL-cholesterol compared to the normal groups. However, treatment with BFBF reduced the levels of these cholesterols as seen in the DBFL, DBFH and DBM groups, the DBG group showed a statistically similar result to DBC for total and HDL-cholesterol. Interestingly, the DBFL group showed even lower LDL-cholesterol than the normal group. On the other hand, the NC group showed a significantly (p < 0.05) higher amount of HDL-cholesterol just like the other groups more than the DBC group. T2D-induction caused a significant (p < 0.05) elevation of serum triglycerides level which was reduced in the DBFH and DBM groups.

Figure 6.4.5: Serum lipid profile of different animal groups at the end of the intervention period. Data are reported as the mean ± SD of seven animals. <sup>a-e</sup>Values with different letters for a given parameter significantly different from each other (Tukey’s-HSD multiple range post hoc test, p < 0.05). NC, Normal control, DBC, Diabetic control, DBFL, Diabetic <i>B. ferruginea</i> low dose, DBFH, Diabetic <i>B. ferruginea</i> high dose, DBM, Diabetic metformin, DBG, Diabetic glibenclamide, NBF, Normal <i>B. ferruginea</i> high dose (toxicological control), HDL, High density lipoprotein, LDL, Low density lipoprotein.
Data of other serum indices of diabetic complications investigated are shown on Table 6.4.2. The induction of T2D using STZ intraperitoneal injection had no effect of serum ALT levels in all animals in all groups. Serum levels of ALP, AST and urea were increased in the DBC group compared to the NC and NBF groups. Interestingly, BFBF treatment significantly ameliorated the ALP, AST, urea and creatinine levels in the respective groups.

**Table 6.4.2:** Serum biochemical parameters in different animal groups at the end of the experimental period.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>NC</th>
<th>DBC</th>
<th>DBFL</th>
<th>DBFH</th>
<th>DBM</th>
<th>DBG</th>
<th>NBF</th>
</tr>
</thead>
<tbody>
<tr>
<td>AST (U/L)</td>
<td>49.5±5.6a</td>
<td>147±16.3e</td>
<td>73.5±5.7bc</td>
<td>59.5±9.7ab</td>
<td>102.5±12.5b</td>
<td>85±8.8cd</td>
<td>50±14.3a</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>87.8±1.1</td>
<td>87.3±2.0</td>
<td>86.3±1.8</td>
<td>87±0.7</td>
<td>85.5±2.1</td>
<td>86.3±1.3</td>
<td>86±5.8</td>
</tr>
<tr>
<td>ALP (U/L)</td>
<td>72.3±6.2a</td>
<td>308.3±44.8de</td>
<td>259.3±23cd</td>
<td>142±24ab</td>
<td>206.8±59.5bc</td>
<td>395.9±86.2e</td>
<td>68.3±13.9a</td>
</tr>
<tr>
<td>Urea (mg/dL)</td>
<td>52.6±4.8b</td>
<td>73.8±3.4c</td>
<td>56.5±8.8b</td>
<td>41.3±2.8a</td>
<td>51±5.8ab</td>
<td>71.5±3.9c</td>
<td>46.3±5.8ab</td>
</tr>
<tr>
<td>UA (mg/dL)</td>
<td>0.78±0.43a</td>
<td>3.5±0.9c</td>
<td>2.2±0.3b</td>
<td>1.1±0.5ab</td>
<td>1.3±0.5ab</td>
<td>1.6±0.7ab</td>
<td>1.2±0.4ab</td>
</tr>
<tr>
<td>CK-MB (u/L)</td>
<td>118.9±14.7a</td>
<td>305.5±27.8d</td>
<td>250.9±35c</td>
<td>171.9±22.9b</td>
<td>113.9±17.7a</td>
<td>185.9±7.6b</td>
<td>153±22.3ab</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SD (n=7). *Different superscript letters for a given parameter along a row indicate the significance of difference (Tukey’s-HSD multiple range post hoc test, p < 0.05). NC, Normal control; DBC, Diabetic control; DBFL, Diabetic *B. ferruginea* low dose; DBFH, Diabetic *B. ferruginea* high dose; DBM, Diabetic metformin; DBG, Diabetic glibenclamide; NBF, Normal *B. ferruginea* high dose (toxicological control); AST, Aspartate aminotransferase; ALT, Alanine aminotransferase; ALP, Alanine phosphatase; UA, Uric acid; CK-MB, Creatinine kinase-myocardial band.

Serum insulin levels were significantly (*p < 0.05*) higher in DBFH and normal groups compared to the remaining groups (Table 6.4.3). The HOMA-IR score in the NC group was significantly lower than that of the DBC and treatment with BFBF was able to reduce to HOMA-IR scores. In the other case, the HOMA-β scores of the DBC group was seen to be significantly lower than that of the NC groups and the DBFH group showed a moderate increase in HOMA-β scores. Serum fructosamine data also showed a difference in the DBC group compared to the NC. Interestingly, treatment with DBFH significantly (*p < 0.05*) improved these parameters at the end of the experiment.
Table 6.4.3: Serum insulin, and fructosamine concentrations as well as calculated HOMA-IR and HOMA-β scores of different animal groups at the end of the experimental period.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>NC</th>
<th>DBC</th>
<th>DBFL</th>
<th>DBFH</th>
<th>DBM</th>
<th>DBG</th>
<th>NBF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum Insulin (pmol/L)</td>
<td>65.2±1.1c</td>
<td>23.5±1.8a</td>
<td>46±3.9b</td>
<td>58.7±1.5c</td>
<td>59.8±6.7c</td>
<td>41.8±2.3b</td>
<td>62.8±3.5a</td>
</tr>
<tr>
<td>Fructosamine</td>
<td>495.8±17.9a</td>
<td>990±10.4c</td>
<td>896.3±117.5c</td>
<td>575.8±11.6a</td>
<td>572.5±9.2a</td>
<td>743.6±37.4b</td>
<td>492±43.6a</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>2.1±0.3c</td>
<td>14.0±0.3c</td>
<td>6.2±0.5d</td>
<td>4.9±0.1b</td>
<td>6.5±0.7d</td>
<td>5.2±0.3c</td>
<td>2.1±0.1a</td>
</tr>
<tr>
<td>HOMA-β</td>
<td>100.3±1.7f</td>
<td>2.7±0.2a</td>
<td>7.0±0.6b</td>
<td>16.3±0.4d</td>
<td>11.9±1.3c</td>
<td>7.0±0.4b</td>
<td>95±4.6c</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SD (n=7). **Different superscript letters for a given parameter along a row indicate the significance of difference (Tukey’s-HSD multiple range post hoc test, p < 0.05). NC, Normal control, DBC, Diabetic control, DBFL, Diabetic B. ferruginea low dose, DBFH, Diabetic B. ferruginea high dose, DBM, Diabetic metformin, DBG, Diabetic glibenclamide, NBF, Normal B. ferruginea high dose (toxicological control), HOMA-IR and HOMA-β; Homeostatic model assessment for IR (insulin resistance) and β (β-cell function).

The pancreas was subjected to histopathological analysis Figure 6.4.6. Examination of the DBC pancreas showed a reduction or shrinkage of the pancreatic islets (red arrow). Microscopic examination of the normal (NC) pancreas sections showed the normal appearance of the pancreatic islets which looked lightly stained than the surrounding acinar cells (green arrow). The acinar cells are generated from pyramidal cells with basal nuclei and apical acidophilic cytoplasm. Induction of T2D caused a severe destruction to both the exocrine and endocrine components of the pancreatic islets in the DBC group (red arrow). The acinar cells appear swollen and vacuoles were seen in almost all acinar cells compared to the pancreas of NC group. The β-cells of pancreatic islets are almost entirely lost in the untreated STZ-injected rats (red arrow). Similar findings were seen in the DBG group (purple arrow). However, high dose (yellow arrow) of BFMB treatment rendered protection and regeneration abilities to the T2D-associated destruction of β-cells because atrophic alterations of the acinar cells were less severe and larger islets when low dose (grey arrow) could not show any such protection of pancreatic β-cells (Figure 6.4.6).
Figure 6.4.6: Histopathological images of the pancreas of different animal groups at the end of the experiment. NC, Normal control, DBC, Diabetic control, DBFL, Diabetic B. ferruginea low dose, DBFH, Diabetic B. ferruginea high dose, DBM, Diabetic metformin, DBG, Diabetic glibenclamide, NBF, Normal B. ferruginea high dose (toxicological control).

6.4.5 Discussion

Africa is home to many indigenous plants which have anti-diabetic activity. The cost, unavailability and unwanted side effects of common synthetic drugs makes medicinal plants of high importance in T2D-drug research. Medicinal plants showing anti-diabetic activities must possess glucose lowering effect and must be able to keep glucose levels close to normal at all times.
The T2D-induction model used in this study was carried out using two distinct pathological features of T2D. The first is induction of insulin resistance by high fructose diet (giving the animals 10% fructose for two weeks) and the second is induction of partial pancreatic β-cell destruction (a single intraperitoneal injection of 40 mg/kg bw STZ) (Wilson and Islam, 2012).

Common physical manifestation of T2D includes polydipsia (frequent thirst), polyphagia (insatiable appetite), weight loss due to diarrhoea, lethargy, blurred vision, frequent urination and others (Kohei, 2010). Successful induction of T2D in our experiment was confirmed by the induction of feed and fluid intake of the diabetic animals compared to the normal animals (Figure 6.4.1) and loss of body weight (Figure 6.4.2). The ability of these symptoms to improve significantly upon treatment with extracts suggests the anti-diabetic potential of this plant fraction.

A main pathological feature of T2D is persistent high blood glucose level which was exhibited in the untreated diabetic (DBC) group all through the intervention period (Figure 6.4.3). Maintaining normal blood glucose level is the main goal of T2D treatment modalities so as to stop the diseases from progressing into major macrovascular complications (Asif, 2014). The ability of the high dose of the fraction (DBFH) to reduce blood glucose concentrations to almost normal level might be due to its enhancing glycogenolysis and cellular glucose uptake.

A high dose (300 mg/kg bw) of the BFBF showed promising blood glucose lowering abilities (Figure 6.4.3), improved glucose clearance (Figure 6.4.4), displayed insulin resistance alleviating properties (Table 6.4.3) and even demonstrated pancreatic β-cell protective abilities (HOMA-β and histopathological analysis). This can be explained better by suggesting that the fraction favoured insulin secretion, which then induced effective glucose uptake by the cells. The ability of the fraction to protect the β-cells of the pancreas after STZ-injection might be due to the antioxidant potential of the fraction which was reported in the last section (Section 6.3) since oxidative stress has been implicated to induce damage to the β-cell of the pancreas (Li et al., 2008).

The reduction of liver glycogen level is another pathological feature of experimental induction of diabetes (Chaudhury et al., 2017). This observed reduction is caused by decrease in the action of glycogen synthase and glycogen phosphorylase (Adeva-Andany et al., 2016). Studies have reported on the ability of medicinal plants to stimulate glycogen production by increasing glycogenesis in the liver thereby potentiating anti-diabetic activities. Interestingly high glycogen levels seen in the DBFH group (Table 6.4.1) compared to the untreated group showed the anti-diabetic activity is not only

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limited to insulin production, β-cell protection and increasing glucose uptake but also by improving the synthesis of liver glycogen level.

Hyperlipidaemia is another feature of experimentally-induced T2D where elevation of serum insulin levels suggests increased risk of coronary heart disease and stroke in T2D (Gomes, 2013); a major complication of T2D that increases the mortality rate of patients (Einarson et al., 2018). Significantly, increased levels of serum total and LDL-cholesterol was seen in the DBC group compared to the normal group (Figure 6.4.5). The serum HDL-cholesterol on the other hand was increased in the normal groups compared to the diabetic groups. Interestingly, the BFBF groups all had same HDL-cholesterol levels as the normal and standard drug-treated groups. Hypertriglyceridemia (high serum triglyceride levels) is a suggested marker for coronary heart disease. Induction of T2D elevated the level of serum triglycerides, which was reduced in the BFBF treated groups shows the ability of the plant fraction to protect the heart against cardiac problems and atherosclerosis associated with T2D. A number of plant-based phytoconstituents have been reported to elicit hypolipidemic abilities in experimentally induced diabetes (Dhulasavant et al., 2010, Gaikwad et al., 2014).

Data from other parameters of complications in T2D also showed a significant increase in AST, ALP, urea, uric acid and CK-MB. No significant change was seen amongst the serum ALT of all the animals in different groups. Insulin deficiency in T2D leads to increased serum levels of transaminase enzymes due to initiation of gluconeogenesis and ketogenesis (Ghosh and Suryawanshi, 2001). Increase levels of AST, ALP and ALT are markers of liver malfunction as a result of insulin deficiency. Interestingly, treatment with BFBF in our study significantly reversed the increased levels of these enzymes thereby signifying hepatoprotective ability of the fraction.

Diabetic kidney disease is a major complication of T2D. Hyperglycaemia destroys the microvascular blood vessels thereby causing impaired renal activity and diabetic nephropathy. Studies have shown that high serum uric acid levels show the prognosis of chronic kidney disease in T2D (Kushiyama et al., 2014), which predicts an imbalance in uric acid production and excretion. Induction of T2D in our study caused an increased level of serum uric acid (Table 6.4.2), however, daily treatment with the fraction significantly reduced the uric acid levels, thereby showing the nephroprotective ability of the fraction in T2D complications.

The presence of sitosterol a known phenolics in B. ferruginea might be responsible for the observed cardio, renal and hepatoprotective effect of the high dose of the butanol fraction as demonstrated in a study by Olaiya et al. (2015).
In conclusion, the findings of this study indicate that oral administration of BFBF in male SD rats can reduce hyperglycaemia, diabetic complications and protect against STZ-induced damage to the pancreatic β-cell. The phyto-chemical constituents of *B. ferruginea* might be responsible for these pharmacological activities. We can therefore conclude that *B. ferruginea* is a non-toxic (even at a high dose) source of natural antioxidants that can be effectively used in the treatment of diabetes and its complications. However, a detailed molecular study should be done to better understand the mechanism of action of its biochemical constituents.
CHAPTER 7
THE ANTIOXIDANT AND ANTIDIABETIC ACTIVITIES OF BOERHAVIA DIFFUSA IN VITRO, EX VIVO AND IN SILICO

7.1 Background

Boerhaavia diffusa is a perennial, ground-spreading shrub (as long as 2000 m) found growing richly in unfertilized soil and is native to both India and Brazil and parts of Africa (Figure 7.1). The leaves are simple, thick, fleshy, hairy and oblong in shape. The small pinkish flowers with short stalk has both staminate and carpellate parts. The roots of B. diffusa are well developed, stout, fusiform and cylindrical in shape with yellowish brown colour. The stem is prostrate (creeping plant) in nature, succulent, slender and cylindrical in shape.

Figure 7.1: Boerhavia diffusa Linn (Nyctaginaceae): Common names: Arowojeja (Yoruba, Nigeria), red spiderling, spreading hogweed (English). Adapted without permission from Indiamart (2009).

7.1.1 Ethnobotanical uses

B. diffusa is a super food used successfully in folk medicine, and the leaves are eaten in salad or sauces. In parts of Nigeria, the whole plant is eaten in curries and soups while the seeds and roots are used to make pancakes. Traditionally, B. diffusa is planted in gardens as snake and scorpion
repellents. The plant is used locally tied around a pregnant woman’s waist to induce labour, and when ground and mixed with honey it is used to treat conjunctivitis. In Ayurveda, *B. diffusa* is commonly used for treatment of jaundice, liver diseases, diabetes, edema, oliguria, anemia, inflammatory edema, diseases of the eye, and aging (Murti et al., 2010).

### 7.1.2 Biological activities

Some bioactive constituents have been isolated from the aerial parts of *B. diffusa* and they include ursolic acid, β-sitosterol, boeravinone, punarnavoside, alkaloids, sterols, sugars and flavones and a host of others (Chakraborti and Handa, 1989, Nayak and Thirunavoukkarasu, 2016; Pereira et al., 2009.).

A study reported that oral administration of leaf extract of *B. diffusa* at 200 mg/kg body weight (bw) elicited a significant reduction in lipid peroxidation with a concomitant induction of endogenous antioxidant enzymes like catalase, superoxide dismutase, reduced glutathione, glutathione peroxidase and S-transferase in the kidney and liver of alloxan-induced diabetic rats (Satheesh and Pari, 2004).

*B. diffusa* root extract showed hepatoprotective ability in experimentally-induced hepatotoxicity in mice (Rajpoot and Mishra, 2011). Oral administration of the aqueous leaves extract of *B. diffusa* in normal and alloxan-induced diabetic rats showed a decrease in blood glucose levels and a significant rise in plasma insulin levels, which was even more significant than the effect of glibenclamide (a standard anti-diabetic drug) (Chude et al., 2001; Pari and Amarnath Satheesh, 2004; Satheesh and Pari, 2004). At a dose of 200 mg/kg bw for four weeks, the aqueous leaf extract of *B. diffusa* showed significant (*p* < 0.05) change in blood glucose concentration and activity of hepatic gluconeogenic enzymes (increase in hexokinase activity and decrease in glucose-6-phosphatase and fructose-1, 6-phosphatase activity) in both normal and alloxan induced diabetic rats (Pari and Satheesh, 2004). The chloroform extract of the leaves of this plant was tested on streptozotocin-induced type 2 diabetic rats and it also showed a dose-dependent reduction of blood sugar levels even comparable to the action of glibenclamide (Nalamolu et al., 2004) thus supporting its anti-diabetic role in Ayurvedic medicine.
7.2 *Boerhaavia diffusa* inhibits key enzymes linked to type 2 diabetes *in vitro* and *in silico*; and modulates abdominal glucose absorption and muscle glucose uptake *ex vivo*

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**Preface:** This article reports the detailed *in vitro* antioxidant and anti-diabetic effects of the crude extracts from *B. diffusa*, which has been published in Journal of Biomedicine and Pharmacotherapy.

7.2.1 **Abstract**

The present study investigated the *in vitro* and *ex vivo* antioxidant, anti-diabetic and anti-obesogenic potentials of different solvent (ethyl acetate, ethanol and water) extracts from the aerial parts of *Boerhaavia diffusa*. The ferric reducing antioxidant power (FRAP), DPPH scavenging activity and the ameliorative effects of the extracts on Fe$^{2+}$-induced oxidative injury was investigated both *in vitro* and *ex vivo*. The glucosidase and pancreatic lipase inhibitory potentials of the extracts were examined in vitro, while the effects of the ethanol extract on abdominal glucose intake and muscle glucose uptake were determined in freshly harvested tissues *ex vivo*. The extracts were subjected to Gas Chromatography-Mass Spectrometry (GC–MS) analysis to identify their possible bioactive components. The ethanol extract showed the most potent FRAP and DPPH radical scavenging activities compare to other extracts. All extracts increased catalase and SOD activities, and GSH levels in oxidative pancreatic injury. Both ethanol and aqueous extracts exhibited remarkable enzyme inhibitory activities, which was significantly higher than ethyl acetate extract and acarbose but was not comparable to orlistat. The ethanol extract portrayed a dose-dependent inhibitory effect on jejunal glucose uptake and enhancement of muscle glucose
uptake. 9-(4 methoxyphenyl) xanthene, xanthone and stigmasterol showed strong binding affinities for α-glucosidase and lipase enzymes tested. Data from this study suggest that aerial parts of B. diffusa (particularly the ethanol extract) may not only exhibit antioxidant potentials but may also mediate anti-lipidemic and anti-hyperglycemic effects via inhibiting fat and carbohydrate digestion as well as abdominal glucose intake and enhancing muscle glucose uptake.

7.2.2 Introduction

Diabetes mellitus (DM) is regarded as the most common endocrine disorder in humans. According to the World Health Organization (WHO, 2016), at least 1.5 million deaths in 2015 alone were directly caused by diabetes globally. Among the different occurrences of diabetes, type 2 diabetes (T2D) remains the most common, and major public health challenge of the 21st century (Canivell and Gomis, 2014). It is described as a versatile and degenerative illness that is affiliated with several metabolic flaws and organ malfunction or damage (Pratley, 2013). The eminent pathogenic features of T2D include pancreatic beta cell impairment and insulinemia which ultimately results in defective insulin secretion and persistent hyperglycemia (Gilbert and Pratley, 2015). Insulin resistance is also associated with poor lipid metabolism, which aggravates metabolic syndrome and lipidemia in the progression of T2D (Kahn et al., 2014).

In peripheral tissues like adipose and muscle tissues, insulin resistance is an indicator for inadequate cellular glucose uptake, which leads to hyperglycemia in T2D (Paneni et al., 2014, Pratley and Weyer, 2001). Hyperglycemia-induced oxidative stress has been implicated in the symptoms, progression and complications of T2D (Röder et al., 2014, Tangvarasittichai, 2015). Persistent hyperglycemia is responsible for elevated levels of mitochondrial energy production, glucose oxidation, protein glycosylation and lipid peroxidation, which leads to an increased output of free radicals and reactive oxygen species (ROS) and eventually, oxidative stress (Gargari et al., 2011). The pancreas is one of the most vulnerable organs to oxidative stress, because of its low levels of free radical scavenging ability compared to other organs (Eleazu et al., 2013). Free radicals such as nitric oxide and hydroxyl radical are the potent culprit in the oxidative damage of pancreatic beta cells, which further worsens diabetic conditions (Eleazu et al., 2013).

The use of traditional medicinal plants has been an effective approach in ameliorating oxidative stress, hyperglycemia and metabolic syndrome (Nistor BL et al., 2010). This is because of their rich therapeutic phytochemical constituents including phenolics, flavonoids, tannins, saponins, glycosides etc. (Eid and Haddad, 2014), which can potentiate several bioactivities including anti-oxidant, anti-
hyperglycemic and anti-lipidemic activities. Additionally, medicinal plants are relatively accessible and affordable compared to commercial synthetic drugs and are void of most side effects associated with these commercial synthetic drugs (El-Houri et al., 2015). Amongst such plants is *Boerhaavia diffusa* L.

*B. diffusa* (family: Nyctaginaceae) is a perennial creeping weed with spreading branches whose roots are tough and tapered (Nayak and Thirunavoukkarasu, 2016). The stem is woody, purplish, hairy and inspissates at the nodes. Its leaves are fleshy, hairy and green arranged in unequal pairs with minute pinkish-red flowers. Its leaves, roots and the aerial parts are used in India in the treatment of kidney disorders, rheumatism, liver complaints and other diseases (Mishra et al., 2014). The leaf extracts have been reported to contain anti-hyperglycemic activities in various animal models (Prakash et al., 2015, Sharma et al., 2014). The root extract has also been reported to show no α-amylase inhibitory activity (Prashanth D et al., 2001) but when it was used in combination with seeds of *Silybum marianum* it showed hepatoprotective abilities on animals with induced non-alcoholic fatty liver disease (Sachin et al., 2013). The extracts from the leaves and roots of this plant have also been tested on animal models to investigate their anti-hyperglycemic abilities and *B. diffusa* has also shown some inhibitory effects on carbohydrate absorption in experimental animals (Kanagavalli et al., 2015, Malhotra et al., 2014). Previous phytochemical studies on *B. diffusa* have indicated the presence of a wide range of phytochemicals such as essential oils, rotenoids, alkaloids, flavonoids, alkamide, and N-trans-feruloyltyramine, xanthones and phenolic compounds (Wajid et al., 2017, Olaoluwa et al., 2018, Agrawal et al., 2011, Do et al., 2013).

However, no study in the literature has reported on compounds found in the ethanol and aqueous extracts of the aerial parts or shown the ability of these compounds to effectively bind key anti-diabetic (α-glucosidase) and anti-obesogenic enzyme (lipase). This shows that some studies on the anti-hyperglycemic and anti-lipidemic potentials of the aerial part of this plant and the possible mode of actions are still lacking. Hence, this study examined the antioxidant potentials of *B. diffusa* aerial parts and its effect on carbohydrate and lipid digestion, intestinal glucose absorption and muscle glucose uptake using several *in vitro* and *ex vivo* experimental and computational models.

### 7.2.3 Results

The data showing the total phenolic content of the extracts as presented in Figure 7.2.1. A significantly higher ($p < 0.05$) phenolic content was observed in the ethanol extract compared to the
ethyl acetate and aqueous extract, when ethyl acetate extract showed significantly higher total phenolic content compared to the aqueous extract.

**Figure 7.2.1:** Total phenolic contents of *B. diffusa* extracts. Data are reported as mean ± SD of three replicas. *a-c* Values with different letters above the bars for a given extract are significantly different from each other (*p* < 0.05, Tukey's HSD-multiple range post-hoc test, IBM SPSS for Windows). EtAc: Ethyl acetate extract.

The FRAP results of the different solvent extracts are presented in **Figure 7.2.2**. All solvent extracts exhibited a dose-dependent reduction of Fe$^{3+}$ radical, when ethanol extract showed a significantly higher (*p* < 0.05) activity than ethyl acetate and aqueous extracts and positive control, Trolox.

**Figure 7.2.2:** Ferric reducing antioxidant power (FRAP) of *B. diffusa* aerial part extracts. Data are reported as mean ± SD of three replicas. *a-c* Values with different letter above the bars for a given concentration are significantly different from each other (*p* < 0.05, Tukey's HSD-multiple range post-hoc test, IBM SPSS for Windows). EtAC, Ethyl acetate; EtOH, Ethanol.

The results of the DPPH radical scavenging activity of the different extracts *B. diffusa* aerial parts are presented in **Figure 7.2.3** while their calculated EC$_{50}$ (minimum extract concentration for 50% inhibition) values are presented in **Table 7.2.1**. All extracts exhibited concentration-dependent
scavenging activities of DPPH, where the ethanol extract exhibited the most potent \( p < 0.05 \) activity \( (EC_{50} = 9.92 \pm 0.5 \mu g/mL) \).

![Figure 7.2.3: DPPH scavenging activity of B. diffusa aerial part extracts. Data are reported as mean ± SD three replicas. Values with different letters above the bars for a given concentration are significantly different from each other \( p < 0.05 \), Tukey’s HSD-multiple range post-hoc test, IBM SPSS for Windows). EtAc, Ethyl acetate; EtOH, Ethanol; AA, Ascorbic acid.](image)

Oxidative injury was successfully induced by incubation of the samples with FeSO\(_4\) as depicted by significantly \( p < 0.05 \) lower catalase activity in the untreated group compared to the normal group (Figure 7.2.4). After treatment with varying doses of the extracts, there was noticeable increase in catalase levels (Figure 7.2.4 A) which shows the antioxidative potentials of B. diffusa. The ethanol and aqueous extracts of B. diffusa showed significantly higher \( p < 0.05 \) catalase increasing activity.

On induction of oxidative injury in the pancreas, the SOD activities were significantly \( p < 0.05 \) reduced in the untreated group, treatment with varying doses of the extracts, showed marked increase in SOD activities (Figure 7.2.4 B). The ethanol and aqueous extracts exhibited higher activities compared with the ethyl acetate extract (Figure 7.2.4 B and Table 7.2.1).

There was a reduction in the levels of reduced GSH upon induction of oxidative stress in the pancreas (Figure 7.2.4 C) but treatment with the different doses of the extracts led to significant \( p < 0.05 \) increase in GSH level. This could further ascertain the antioxidative potentials of the extract in correlation with the \textit{in vitro} results which have already been discussed above. The induction of oxidative injury in the pancreas caused significant \( p < 0.05 \) increase in malondialdehyde (MDA) levels (Figure 7.2.4 D). All the extracts were able to reduce the MDA level, with the aqueous extract showing the highest reducing capacity as suggested by its lowest EC\(_{50}\) value (Table 7.2.1).
Figure 7.2.4: Effect of extracts of *B. diffusa* aerial parts on (A) catalase activity, (B) SOD activity, (C) GSH level and (D) lipid peroxidation in oxidative pancreatic injury. Data are reported as mean ± SD of three replicas. *Significantly different from untreated sample and #Significantly different from normal sample (*p* < 0.05, Tukey's-HSD-multiple range post-hoc test, IBM SPSS for Windows). Normal = normal tissues, Untreated = tissues oxidatively injured but not treated. AA; Ascorbic acid

The results of α-glucosidase and lipase enzymes inhibitory effects of *B. diffusa* extracts are presented in Figure 7.2.5 A and B respectively and their calculated EC$_{50}$ values are shown in Table 7.2.1. All extracts exhibited concentration-dependent inhibition of α-glucosidase (Figure 7.2.5 A) and pancreatic lipase (Figure 7.2.5 B) inhibitory activities. Both ethanol and aqueous extract showed significantly (*p* > 0.05) more potent inhibitory activity compared to ethyl acetate extract, while the lipase inhibitory activity of the aqueous extract was significantly (*p* < 0.05) greater than that of the ethanol extract (Figure 7.2.5 B). There was no virtual difference on α-glucosidase inhibitory activities between the ethanol and aqueous extracts when compared with the positive control, acarbose (Figure 7.2.5 A and Table 7.2.1).
Figure 7.2.5: (A) $\alpha$-glucosidase, (B) Lipase inhibitory activity of aerial part extracts of *B. diffusa*. Data are reported as mean ± SD of three replicas. $^{a-d}$Different letters over the bars for a given concentration for each extract are significantly different from each other ($p < 0.05$, Tukey's-HSD-multiple range post-hoc test, IBM SPSS for Windows). EtAC, Ethyl acetate; EtOH, Ethanol.

The data of the effects of the different concentrations of the ethanol on glucose absorption in harvested rat jejunum and glucose uptake in harvested rat psoas muscle are presented in Figure 7.2.6 A and B, respectively. Incubation of isolated rat jejunum with ethanol extract resulted in a significant ($p < 0.05$) and dose-dependent reduction in glucose absorption compared to the control (Figure 7.2.6 A). On the other hand, the ethanol extract significantly and dose dependently increased glucose uptake in rat psoas muscle (Figure 7.2.6 B). Glucose uptake at 200 and 250 mg/dL concentrations of ethanol
extracts was significantly \((p < 0.05)\) higher than that of the control, 50, 100 and 150 mg/dL concentrations of ethanol extracts and metformin (Figure 7.2.6 B).

![Figure 7.2.6: Effect of the ethanol extract of the aerial part of B. diffusa on (A) glucose absorption in isolated rat jejunum and (B) glucose uptake in rat psoas muscle. Data are reported as mean ± SD of three replicas. Different letters over the bars for a given concentration are significantly different from each other \((p < 0.05,\) Tukey’s-HSD-multiple range post-hoc test, IBM SPSS for Windows).](image)

The GC–MS result (Figure 7.2.7) showed the presence of 9 and 3 compounds respectively from the ethanol and aqueous extracts of B. diffusa aerial parts. Their retention time (min), molecular formula, and mass spectra data (amu) are shown in Table 7.2.2. Figure 7.2.8 A–C shows the binding affinity of xanthone, 9-(4-methoxyphenyl) xanthene and stigmasterol to complexed \(\alpha\)-glucosidase, while Figure 7.2.8 D–F shows the binding affinities of these compounds with lipase enzyme.
Table 7.2.1: EC50 summary table for inhibitory effects of different extracts of *B. diffusa*

<table>
<thead>
<tr>
<th>Sample</th>
<th>DPPH (µg/mL)</th>
<th>Catalase (µg/mL)</th>
<th>SOD (µg/mL)</th>
<th>GSH (µg/mL)</th>
<th>Lipid peroxidation (µg/mL)</th>
<th>α-glucosidase (µg/mL)</th>
<th>Anti-lipase (µg/mL)</th>
<th>Glucose absorption (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Aerial parts</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>36.7±5.6c</td>
<td>5.21±1.0b</td>
<td>13.21±2.1d</td>
<td>7.34±1.1d</td>
<td>16.42±2.5c</td>
<td>39.01±5.3c</td>
<td>134.39±9.2d</td>
<td>ND</td>
</tr>
<tr>
<td>Ethanol</td>
<td>9.92±0.5c</td>
<td>1.13±0.5a</td>
<td>2.05±0.1a</td>
<td>2.14±0.4b</td>
<td>11.83±1.0b</td>
<td>2.82±0.3b</td>
<td>17.43±3.1c</td>
<td>1.60±1.03</td>
</tr>
<tr>
<td>Aqueous</td>
<td>25.70±1.1d</td>
<td>1.26±0.8a</td>
<td>5.72±1.0c</td>
<td>1.09±0.1a</td>
<td>5.71±0.4a</td>
<td>1.08±0.5a</td>
<td>5.79±2.9b</td>
<td>ND</td>
</tr>
<tr>
<td><strong>Standard</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>2.19±0.05a</td>
<td>1.01±0.04a</td>
<td>3.91±1.2b</td>
<td>3.65±0.7c</td>
<td>15.39±6.2c</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Trolox</td>
<td>5.79±0.4b</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Acarbose</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>36.27±1.84c</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Orlistat</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.03±0.12a</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

DPPH-1,1-diphenyl-2-picylhydrazyl radical, NO-nitric oxide radical, ND-not determined. Data are reported as mean ± SD values of three replicas. Different letters stand for significantly different values from each other within a column (Tukey’s-HSD multiple range post hoc test, p < 0.05): the same letters stand for non-significant difference.

Table 7.2.2: Phytochemical compounds identified from the ethanol (F) and aqueous (BD) extracts of *B. diffusa* aerial parts by GC-MS

<table>
<thead>
<tr>
<th>No.</th>
<th>Compounds</th>
<th>Retention time (min)</th>
<th>Molecular formula</th>
<th>Mass spectral data (amu)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>Ethanol extract</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>D-Limonene</td>
<td>6.09</td>
<td>C_{10}H_{16}</td>
<td>136 [M+]</td>
</tr>
<tr>
<td>2</td>
<td>Tetradecanoic acid</td>
<td>15.58</td>
<td>C_{14}H_{26}O_{2}</td>
<td>228 [M+]</td>
</tr>
<tr>
<td>3</td>
<td>Phytol acetate</td>
<td>16.88</td>
<td>C_{22}H_{44}O_{2}</td>
<td>278 [M+ - acyl - H_{2}O]</td>
</tr>
<tr>
<td>4</td>
<td>Xanthone</td>
<td>17.02</td>
<td>C_{13}H_{30}O</td>
<td>196 [M+]</td>
</tr>
<tr>
<td>5</td>
<td>Phytol</td>
<td>19.11</td>
<td>C_{20}H_{40}O</td>
<td>123, 71 (base peak)</td>
</tr>
<tr>
<td>6</td>
<td>Octadecanoic acid</td>
<td>19.56</td>
<td>C_{18}H_{36}O_{2}</td>
<td>284 [M+]</td>
</tr>
<tr>
<td>7</td>
<td>9-(4-Methoxyphenyl) xanthene</td>
<td>22.40</td>
<td>C_{20}H_{16}O_{2}</td>
<td>288 [M+], 181 (base peak)</td>
</tr>
<tr>
<td>8</td>
<td>Stigmasterol</td>
<td>28.99</td>
<td>C_{29}H_{44}O</td>
<td>412 [M+]</td>
</tr>
<tr>
<td>9</td>
<td>Sitosterol</td>
<td>29.78</td>
<td>C_{29}H_{50}O</td>
<td>414 [M+]</td>
</tr>
<tr>
<td></td>
<td><strong>Aqueous extract</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Dodecanoic acid methyl ester</td>
<td>12.82</td>
<td>C_{13}H_{26}O_{2}</td>
<td>87, 74 (base peak)</td>
</tr>
<tr>
<td>11</td>
<td>Tetradecanoic acid methyl ester</td>
<td>15.17</td>
<td>C_{15}H_{30}O_{2}</td>
<td>143, 87, 74 (base peak)</td>
</tr>
<tr>
<td>12</td>
<td>Hexadecanoic acid methyl ester</td>
<td>17.29</td>
<td>C_{17}H_{34}O_{2}</td>
<td>87, 74 (base peak)</td>
</tr>
</tbody>
</table>
Figure 7.2.7: Structures of the compounds isolated from the ethanol (F) and aqueous (BD) extracts of *B. diffusa* aerial parts.

Table 7.2.3: Binding affinities and hydrogen bonding (HB) obtained from molecular docking for α-glucosidase

<table>
<thead>
<tr>
<th>No.</th>
<th>Compounds</th>
<th>Binding affinities (kcal/mol)</th>
<th>HB Distance (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>D-Limonene</td>
<td>-7.2</td>
<td>6.51</td>
</tr>
<tr>
<td>2</td>
<td>Tetradecanoic acid</td>
<td>-3.5</td>
<td>7.30</td>
</tr>
<tr>
<td>3</td>
<td>Phytol acetate</td>
<td>-3.4</td>
<td>7.13</td>
</tr>
<tr>
<td>4</td>
<td>Xanthone</td>
<td>-9.8</td>
<td>2.35</td>
</tr>
<tr>
<td>5</td>
<td>Phytol</td>
<td>-8.5</td>
<td>3.12</td>
</tr>
<tr>
<td>6</td>
<td>Octadecanoic acid</td>
<td>-4.7</td>
<td>6.25</td>
</tr>
<tr>
<td>7</td>
<td>9-(4-Methoxyphenyl) xanthene</td>
<td>-10.3</td>
<td>4.52</td>
</tr>
<tr>
<td>8</td>
<td>Stigmasterol</td>
<td>-10.9</td>
<td>2.05</td>
</tr>
<tr>
<td>9</td>
<td>Sitosterol</td>
<td>-8.8</td>
<td>5.26</td>
</tr>
<tr>
<td>10</td>
<td>Dodecanoic acid methyl ester</td>
<td>-6.7</td>
<td>3.7</td>
</tr>
<tr>
<td>11</td>
<td>Tetradecanoic acid methyl ester</td>
<td>-8.2</td>
<td>2.1</td>
</tr>
<tr>
<td>12</td>
<td>Hexadecanoic acid methyl ester</td>
<td>-7.1</td>
<td>3.2</td>
</tr>
</tbody>
</table>
Table 7.2.4: Binding affinities and hydrogen bonding (HB) obtained from molecular docking for anti-lipase activity

<table>
<thead>
<tr>
<th>No.</th>
<th>Compounds</th>
<th>Binding affinities (kcal/mol)</th>
<th>HB Distance (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>Ethanol extract</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>D-Limonene</td>
<td>-4.5</td>
<td>4.20</td>
</tr>
<tr>
<td>2</td>
<td>Tetradecanoic acid</td>
<td>-5.4</td>
<td>4.11</td>
</tr>
<tr>
<td>3</td>
<td>Phytol acetate</td>
<td>-6.2</td>
<td>4.52</td>
</tr>
<tr>
<td>4</td>
<td>Xanthone</td>
<td>-10.3</td>
<td>3.30</td>
</tr>
<tr>
<td>5</td>
<td>Phytol</td>
<td>-8.4</td>
<td>3.52</td>
</tr>
<tr>
<td>6</td>
<td>Octadecanoic acid</td>
<td>-5.7</td>
<td>6.11</td>
</tr>
<tr>
<td>7</td>
<td>9-(4-Methoxyphenyl)xanthene</td>
<td>-12.7</td>
<td>2.33</td>
</tr>
<tr>
<td>8</td>
<td>Stigmasterol</td>
<td>-11.8</td>
<td>3.14</td>
</tr>
<tr>
<td>9</td>
<td>Sitosterol</td>
<td>-7.9</td>
<td>4.44</td>
</tr>
<tr>
<td></td>
<td><strong>Aqueous extract</strong></td>
<td></td>
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<tr>
<td>10</td>
<td>Dodecanoic acid methyl ester</td>
<td>-6.7</td>
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<td>12</td>
<td>Hexadecanoic acid methyl ester</td>
<td>-7.1</td>
<td>4.73</td>
</tr>
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Figure 7.2.8: 3D structure showing the interacting residues (hydrogen bonding and hydrophobic) of extracts with highest binding affinities around binding pocket of (A) and (D) Xanthone (B) and (E) 9-(4-Methoxyphenyl) xanthene and (C) and (F) Stigmasterol to complexed α-glucosidase and Lipase respectively

7.2.4 Discussion

The derangement in lipid metabolism in diabetic conditions is one of the major influencing factors in the complications associated with diabetes especially T2D (Odegaard and Chawla, 2012) and oxidative stress is a known culprit (Fowler, 2008). Different parts of B. diffusa plants have been shown to exhibit antioxidant and anti-hyperglycemic potentials both in vivo and in vitro (Prakash et
al., 2015, Sharma et al., 2014, Prashanth D et al., 2001) but the possible anti-lipidemic potentials and mode of action behind their antioxidative and anti-hyperglycemic potentials remains elusive. Our present study revealed that different solvent extract (particularly, ethanol extract and aqueous extract) of *B. diffusa* aerial part exhibited not only antioxidant activities but anti-lipidemic and anti-hyperglycemic potentials via inhibiting lipid and carbohydrate digestion and abdominal glucose intake as well as enhancing muscle glucose uptake.

It is well documented that plants rich in polyphenols can boost antioxidant activity of the plasma, thus limiting the effects of oxidative stress associated diseases such as T2D (Zhang and Tsao, 2016, Kanner et al., 2017). The presence of polyphenols in all studied extracts of the aerial parts of *B. diffusa* (*Figure 7.2.1*) gives credence to its role in the traditional treatment of various ailments, particularly the ethanol extract with the highest phenolic content.

Scavenging free radicals has been demonstrated as an ameliorative effect against oxidative stress (Shon et al., 2007). The DPPH scavenging and total reducing power of the extracts, portray an anti-oxidative effect. These activities depict a therapeutic potential against oxidative complications particularly in T2D, where oxidative stress has been linked to the pathogenesis and progression of T2D (Erunkainure et al., 2017a).

Fe²⁺-induced oxidative stress in the rat pancreatic homogenates led to an increased generation of superoxide, hydrogen peroxide, and hydroxy radicals. Diverse methods have been used to reduce the damage caused by oxidative stress and the most potent of all is the use of natural endogenous antioxidants (Gil et al., 2017). The protective ability of catalase and SOD has been reported, SOD catalyzes the dismutation of superoxide to hydrogen peroxide and catalase in turn catalyzes the conversion of the resulting hydrogen peroxide to water and molecular oxygen (Liu and Kokare, 2017). The reduced catalase and SOD levels seen in the untreated groups (*Figure 7.2.4 A and B*) occurred as a result of oxidative injury caused by incubation with FeSO₄. The ability of the extracts, especially the ethanol and aqueous extracts, to increase the activities of these enzymes in the treated groups portrays their antioxidant abilities.

A reduction in the GSH levels has been reported in cases of pancreatic β-cell dysfunction which could ultimately lead to the complications of T2D (Tiwari et al., 2013). At the cellular level, GSH is a direct marker of oxidative stress and has been described as a first line of defence against oxidative damage (Erunkainure et al., 2017a, Gil et al., 2017). The reduced levels of GSH seen in the untreated groups
indicates an occurrence of oxidative damage and this was increased upon incubation with the extracts (Figure 7.2.4 C).

Lipid peroxidation has been well reported to occur due to iron overload and if the residual hydrogen peroxide is not removed by catalase leads to a build-up of hydroxy radicals through the Fenton reaction (Andrews, 1999, Rajpathak et al., 2009). These radicals cause lipid peroxidation on the cell membrane thereby leading to the production of reactive moieties such as acrolein, 4-hydroxynonenal, 4-oxononenal and MDA (Saddala et al., 2013). High MDA levels have been reported in cases of T2D (Saddala et al., 2013). After incubation with FeSO$_4$, there was an increase in levels of MDA which portrays an occurrence of lipid peroxidation (Figure 7.2.4 D). Treatment with the extracts was able to reduce the levels of MDA, an indication of the ability of the extract to reduce the level of lipid peroxidation in the tissues. This also relates with the ability of the extracts to increase the activity of catalase and SOD, and GSH levels thereby portraying the anti-peroxidative ability of the plant.

Lipase and α-glucosidase are major enzymes in the breakdown of dietary lipids and carbohydrates. The effective inhibition of these enzymes can control the postprandial rise in blood glucose as well as minimize the accumulation of fat as seen in obese-T2D patients (Dalar et al., 2015, Wu et al., 2013). The inhibitory effect of the extracts on these enzymes (Figure 7.2.5 A and B) demonstrates a delayed breakdown of carbohydrates and lipids in the intestine, thereby reducing the amount of glucose and fatty acids in the blood stream (Erukainure et al., 2017a). Thus, demonstrating an anti-hyperglycemic and antilipolytic potential of B. diffusa aerial parts. This is further portrayed by the inhibition of glucose absorption in isolated intestinal tissues incubated with B. diffusa ethanol extract (Figure 7.2.6 A), also reducing the amount of glucose in the blood stream. Dietary glucose resulting from carbohydrate breakdown is rapidly absorbed in the small intestine, especially in the first quarter jejunal and duodenal regions (Rider et al., 1967, Riesenfeld et al., 1980). Its rapid absorption results in elevated blood glucose which could be detrimental, especially in diabetic individuals if not normalized (Hassan et al., 2010). Thus, the delayed carbohydrate digestion and concomitant inhibition of intestinal glucose absorption by B. diffusa extracts may validate its folkloric use in the treatment of diabetes and its complications. Similar effects have been reported for phenolics compounds (Toma et al., 2014, Inthongkaew et al., 2017), thus these activities may also be attributed to the total phenolic content of the extracts (Figure 7.2.1).

Skeletal muscle plays an important role in the regulation of glucose homeostasis and carbohydrate metabolism (Sinacore and Gulve, 1993). They are particularly rich in GLUT-4 which aids glucose uptake (Satoh, 2014). Defects in muscle glucose uptake have been attributed to the pathogenesis of
insulin resistance (Sinacore and Gulve, 1993, Satoh, 2014), and is thus a major contributor to hyperglycemia. Stimulation of muscle glucose uptake regardless of insulin level has been recognized as a major therapy for glycemic control in T2D (Pereira et al., 2017). Thus, the increased glucose uptake in muscles incubated with B. diffusa ethanol extract (Figure 7.2.6 B) further indicates an anti-hyperglycemic activity of the plant and its potential to modulate hyperglycemia.

The presence of various bioactive compounds in the aerial part of the plant justifies the use of this plant in the treatment and management of various ailments. Thus, the phytoconstituents analysed by GC–MS gives a comprehensive presentation of the pharmaceutical and therapeutic potential of the plant. Previous studies on the leaves and root of the plant showed the presence of diverse phytochemicals which have been shown to possess anti-hyperglycemic abilities (Agrawal et al., 2011, Shanmugapriya and Maneemegalai, 2017). The presence of xanthones and their derivatives in the ethanol extract of the aerial part of B. diffusa has been reported in this study (Figure 7.2.7, Table 7.2.2). Xanthones have been shown to possess hypoglycemic abilities (Taher et al., 2016).

Limonene also seen in the ethanol extract (Table 7.2.3) has been documented to act as an antioxidant by lowering oxidative stress in streptozotocin-induced diabetic rats (Bacanli et al., 2017). The ethanol extract showed the presence of stigmasterol in the GC–MS analysis; interestingly, the role of stigmasterol in the fight against diseases such as cancer and diabetes has also been reported (Ramu et al., 2016). Plant sterols and esters have also been reported to possess potent anti-hyperglycemic and anti-obesogenic abilities (Hallikainen et al., 2011, Gylling et al., 2014). The observed anti-hyperglycemic and anti-obesogenic abilities of B. diffusa could be as a result of the synergistic effect rendered by these constituents identified by GC–MS.

Molecular docking studies were performed to gain further insight to the inhibition potentials of ethanol and aqueous extract. Docking result revealed ethanol and aqueous extracts showed inhibition potentials with 9-(4 methoxyphenyl) xanthene, xanthone and stigmasterol having highest binding affinities compared to other compounds. This is also evident in the hydrogen bond interactions (Tables 7.2.3 and 7.2.4) with amino acid residues. Similar protocol was performed by Bharatham, Bharatham, Park and Lee (Bharatham et al., 2008). For stigmasterol with the highest binding affinity, the amino acid residues are: ASN54, ASP57, GLU 58, ARG 84 and GLY 83. Comparing the inhibitory potential of compounds between α-glucosidase and lipase, Figure 7.2.8 A–C and D–F revealed that 9-(4 methoxyphenyl) xanthene and stigmasterol had the strongest binding affinities of -12.7 and 11.8 kcal/mol respectively for lipase. The high binding energy of stigmasterol and 9-(4
methoxyphenyl) xanthene is very likely due to the hydroxyl and methoxy groups as well as hydrophobic interactions (Bharatham et al., 2008). The binding mode analysis of the compounds with some amino acid residues provides important information of catalytic site which is essential for proposing the catalytic mechanism.

7.2.5 Conclusions

These results demonstrate the antioxidant and antidiabetic activities of the aerial parts of *B. diffusa* as shown by its free radical scavenging, and carbohydrate and lipid digesting enzymes inhibitory activities. These activities can be attributed to the total phenolic content and identified bioactive compounds, particularly in the ethanol extract. The ability of the ethanol extract to inhibit small intestinal glucose absorption as well as stimulate muscle glucose uptake, further portrays the therapeutic potential of the plant against T2D and its complications.
CHAPTER 8

GENERAL DISCUSSION AND CONCLUSIONS

8.1 General discussion

In recent times, increase in mortality, morbidity and amputations in Africa has occurred due to the rise in diabetes mellitus. Sedentary lifestyle, dietary habits and urbanization have encouraged the spread of this disease (IDF, 2017). Microvascular and macrovascular complications linked to diabetes increase mortality in Africa and other parts of the world. A number of synthetic drugs are currently available for treatment of this disease, but the high cost and unwanted side effects makes them less desirable to people living with diabetes. In most undeveloped parts of Africa, people rely on traditional medicine as a means of avoiding the heavy monetary burden due to using expensive drugs. However, so many locals are more appreciative of traditional medicine since they claim, “it has been in use since the time of our fore-fathers”. The plants used in this study are plants which are used in traditional medicine for treatment of diabetes mellitus and they include: *Alstonia boonei, Acalypha wilkesiana, Crassocephalum rubens, Bridelia ferruginea and Boerhaavia diffusa*. The claims of most locals on the efficacy of these plants in treating diabetes led to the need to ascertain and confirm their hypothesis. Thus, this study was undertaken to give a sound scientific proof for future exploration of these plants in the pharmaceutical industry either in the crude form or as standard formulations for treatment of diabetes mellitus and its associated complications. This study was also done to identify the potent bioactive constituents responsible for anti-diabetic action and most importantly, to ascertain the level of toxicity of these plants at different doses, thereby enlightening the locals who still depend on traditional medicine for the treatment of diseases.

To fully perform a detailed screening on these plants, the leaves, stem bark and root barks of *A. boonei, A. wilkesiana* and *B. ferruginea* were used while the aerial parts of *C. rubens* and *B. diffusa* were exploited. The first screening test done using these plant parts was to investigate the *in vitro* and *ex vivo* antioxidant potential of these plants since diabetic complications and etiology abounds due to actions of oxidative stress (Asmat *et al.*, 2016). The crude extract (within a plant) with the best activity was selected for further analysis.

*Alstonia boonei* was the first plant studied in this research. After various *in vitro* and *ex vivo* antioxidant and antidiabetic study on the crude extracts, the ethanol extract of the leaves was chosen for fractionation (*Section 3.2*). Four fractions were obtained after fractionation and the butanol
fraction in particular showed the best inhibition of key enzymes linked to type 2 diabetes and also showed potent glucose absorption inhibition in the small intestine together with induction of glucose uptake in peripheral tissues (Section 3.3). However, results for the in vivo assay showed the low dose (150 mg/kg bw) as more effective in eliciting glucose lowering and organ protection than the high dose (300 mg/kg bw) (Section 3.4). This could be due to the reported toxicity of a high dose of this fraction (Olanlokun and Olorunsogo, 2018). The low dose of the fraction also showed a better oxidative stress alleviating potential than the higher dose, which further verifies the toxicity of this fraction at a higher dose. The GC-MS analysis revealed the presence of potent phyto-phenols like 2-hydroxy-1-(hydroxymethyl) ethyl hexadecanoate, which was predominantly present in the butanol and aqueous fraction. Interestingly, this compound has been reported to possess antioxidant and hypocholesterolemic activity (Jegadeeswari et al., 2012; Tyagia and Argawak, 2017). Considering the results from this study, it is suggested that further screening be done using a low dose of the fraction to fully verify the efficacy of the plant in alternate therapies for treatment of diabetes.

*Acalypha wilkesiana* was the second plant to be investigated. The in vitro antioxidant activity of the extracts from different plant parts of *A. wilkesiana* was first done coupled with the free radical scavenging activity in Fe$^{2+}$-induced oxidative pancreatic injury (Section 4.2). The ethanol extract of the root bark showed potent antioxidant effect in the ex vivo study. The crude ethanol extract obtained from the stem bark and root bark also showed promising $\alpha$-glucosidase and $\alpha$-amylase inhibitory potential (Section 4.3). GC-MS analysis revealed the presence of biologically active plant phenols such as xanthenes and sterols which have been reported to elicit anti-diabetes activity (Ghosh et al., 2015, Takato et al., 2017).

*Crassocephalum rubens* the next plant investigated is a traditional leafy vegetable eaten as a salad in parts of Africa. Only solvent extracts from the aerial parts were investigated, and no fractionation was done due to the unavailability of the plant in large quantity. In vitro and ex vivo antioxidant and anti diabetic activity revealed the aqueous extract as the most potent extract (Section 5.2). No study in the literature has reported the presence of bioactive constituents in any part of *C. rubens*, so this study is the first of its kind to detect the presence of bioactive compounds in the leaves of this plant. LC-MS analysis of the aqueous extract revealed the presence of phytochemicals like sanguisorbic acid dilactone (an ellagitannin), 8-methoxy-6,7-methylenedioxyxycoumarin, 3,4-methylenedioxybenzoic acid, all of which have potent antioxidant and antidiabetic activity (García-Villalba et al., 2015; Tan et al., 2017, Tk, 2012, Venugopala et al., 2013). To further verify the antioxidant and antidiabetic potential, these bioactive constituents were docked with key antidiabetic ($\alpha$-glucosidase and lipase)
and antioxidants (superoxide dismutase, catalase and glutathione reductase) using in silico molecular docking techniques. Interestingly, sanguisorbic acid dilactone showed highest binding energies for all the enzymes tested, which could provide valuable information towards proposing the catalytic mechanism of these compounds (Section 5.2). Although LC-MS assay identified some phytochemicals present, there is need to isolate and characterize a pure compound before we can be sure of the bioactive constituents. The aqueous extract (since it showed best activity) was subjected to in vivo analysis using a type 2 diabetic animal model (Section 5.3). Interestingly the extract retained its anti-diabetic activity in a dose-dependent manner with no toxicity seen; this confirms an earlier report on the toxicity of C. rubens (Adjatin et al., 2013a). It was also able to ameliorate diabetes-induced oxidative stress thereby rendering cardiac, renal and hepatoprotective effects in complications of diabetes (Section 5.4). In general, C. rubens could play a promising role in area of drug research.

For Bridelia ferruginea, the crude ethanol extract of the leaves and root bark showed the best antioxidant and anti-diabetic activity (Section 6.2). The ethanol extract of the leaves was consequently fractionated, while the butanol fraction was discovered to show best results for the in vitro antioxidant and antidiabetic models (Section 6.3). The fraction also demonstrated potent anti-diabetic effects and seems to do this via different mechanisms. This might be a result of the synergistic effects of the bioactive constituents present in the plant. GC-MS analysis of the extracts showed the presence of 32 compounds most of which were phenols. β-amyrin and α-amyrin acetate found in this plant are known pentacyclic triterpenes found distributed in plants and have been reported to have diverse biological properties like anti-inflammatory, anti-microbial, cytotoxic, and trypanocidal activity amongst others (Vázquez et al., 2012). The anti-diabetic properties of these triterpenes have also been established (Nair et al., 2014; Santos et al., 2012). 4-phenylbenzophenone and lupenone identified in our study have also been reported to exhibit promising anti-diabetic activity (Lin et al., 2009; Xu et al., 2014). Interestingly, β-amyrin, 4-phenylbenzophenone and lupenone had the highest binding energies for α-glucosidase, α-amylase and lipase enzymes respectively, which justified the biological properties of the compounds. With the observed anti-diabetic potential of the leaves of this plant, it can be recommended for further screening for possible usage as an anti-diabetic herbal drug, which could consequently be made available for public consumption.

Boerhaavia diffusa was the last plant investigated. The aerial part of the plant was subjected to in vitro, ex vivo and in silico antioxidant and anti-diabetic assays. Both ethanol and aqueous extracts exhibited remarkable enzyme inhibitory activities, which were significantly higher than ethyl acetate
extract and acarbose but was not comparable to orlistat. The ethanol extract portrayed a dose-dependent inhibitory effect on jejunal glucose uptake and enhancement of muscle glucose uptake in isolated normal rat tissue (Section 7.2). 9-(4 methoxyphenyl) xanthene, xanthone and stigmasterol showed strong binding affinities for α-glucosidase and lipase enzymes using in silico molecular docking analysis. Due to the observed antioxidant and anti-diabetic activity of this plant, *B. diffusa* should be recommended to be eaten in soups and salads of individuals with high blood glucose levels.

**Figure 8.1:** Pictures of the plants used in this study with their mechanism of action. a) *Alstonia boonei*, b) *Acalypha wilkesiana* c) *Crassocephalum rubens*, d) *Bridelia ferruginea*, e) *Boerhaavia diffusa*, TPC, Total phenol content, EtOH ext, Ethanol extract, ABBF, *Alstonia boonei* butanol fraction, GC-MS, Gas chromatography-mass spectroscopy, CRAQ, *Crassocephalum rubens* aqueous extract, LC-MS, Liquid chromatography-mass spectrometry, AP, Aerial parts, BFBF, *Bridelia ferruginea* butanol fraction.
8.2 General conclusions
Three out of the five medicinal plants used in this study have exhibited promising anti-diabetic activity in all the models tested, even similar to the standard drug metformin and sometimes better than the action of glibenclamide. Except for one plant at a high dose, these plants did not show signs of toxicity for most of the parameters measured in this investigation. These findings are important for the relevant government agencies, pharmaceutical industries, scientific community and local people suffering from diabetes because it might open an avenue for the development of viable and cost effective anti-diabetic drugs.

8.3 Recommendations
The next step for confirmation of the anti-diabetic activity of these plants should entail human-based cell culture studies followed by a detailed clinical-trial studies in human subjects with diabetes mellitus. The identified bioactive compounds should be subjected to a thorough in vivo anti-diabetic study with the most effective fractions determined in this study. This may eventually lead to development of novel anti-diabetic drugs. However, toxicological studies in human-based cell lines as well as clinical trials in humans are warranted to understand their safety and efficacy in humans.
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APPENDIX A

ANIMAL ETHICS APPROVAL LETTER

11 May 2017

Ms Cleumoke Arinola Daramola (216068797)
School of Life Sciences
Westville Campus

Dear Ms Daramola,

Protocol reference number: AREC/003/017D
Project title: Studies on the antidiabetic and anti-hyperglycaemic potentials of Acalypha wilkesiana, Boerhavia diffusa and Alostria boonei in a rat model of type 2 diabetes

Full Approval – Research Application

With regards to your revised application received on 28 April 2017. The documents submitted have been accepted by the Animal Research Ethics Committee and FULL APPROVAL for the protocol has been granted.

Any alteration(s) to the approved research protocol, i.e. Title of Project, Location of the Study, Research Approach and Methods must be reviewed and approved through the amendment/modification prior to its implementation. In case you have further queries, please quote the above reference number.

Please note: Research data should be securely stored in the discipline/department for a period of 5 years.

The ethical clearance certificate is only valid for a period of one year from the date of issue. Renewal for the study must be applied for before 11 May 2018.

Attached to the Approval letter is a template of the Progress Report that is required at the end of the study, or when applying for Renewal (whichever comes first). An Adverse Event Reporting form has also been attached in the event of any unanticipated event involving the animals’ health/wellbeing.

I take this opportunity of wishing you everything of the best with your study.

Yours faithfully

Dr Sandi Singh
Deputy Chair, Animal Research Ethics Committee

C C Professor Shahidul Islam
C C Dean & Head of School: Dr A Olaniran
C C Registrar: Mr Simon Mokoena
C C NSPCA: Ms Stephanie Keulder
C C BRU – Dr R Singh

Animal Research Ethics Committee (AREC)
Ms Marielle Snyman (Administrator)
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Website: http://research.ukzn.ac.za/ResearchEthics/AnimalEthics.asp

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APPENDIX B

PUBLICATIONS FROM THIS THESIS

https://doi.org/10.2478/acph-2018-0037

Acalypha wilkesiana 'Java white':
Identification of some bioactive compounds by GC-MS
and their effects on key enzymes linked to type 2 diabetes

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OCHUKO L. EBUKAINURE1
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In this study, we identified bioactive compounds from the ethanolic extracts of the leaves, stem bark and root bark of Acalypha wilkesiana through GC-MS analysis and investigated the effects of these extracts on some of the enzymes linked to type 2 diabetes. Plant parts were extracted sequentially with ethyl acetate, ethanol and water. GC-MS analysis revealed the presence of long-chain alkyl acids, esters, ketones and alcohols including phytol and phytol acetate along with some secondary metabolites such as xanthone, vitamin E and various types of sterols including stigmasterol, campesterol and sitosterol. Ethanolic extracts of all the parts showed a dose-dependent inhibition of α-glucosidase and α-amylase activity. The extracts also demonstrated anti-lipase activity. The ethanolic extract of root bark showed the highest inhibition of enzymes compared to other extracts. The EC₅₀ values (concentrations for 50% inhibition) of α-glucosidase, α-amylase and lipase inhibition were 35.75 ± 1.95, 6.25 ± 1.05 and 101.33 ± 5.21 µg mL⁻¹, resp. The study suggests that A. wilkesiana ethanolic extracts have the ability to inhibit the activity of enzymes linked to type 2 diabetes. Further studies are needed to confirm the responsible bioactive compounds in this regard.

Keywords: Acalypha wilkesiana, α-glucosidase, α-amylase, lipase, type 2 diabetes, ethanolic extract

Accepted June 21, 2018
Published August 29, 2018
Original research article

*Boerhaavia diffusa* inhibits key enzymes linked to type 2 diabetes *in vitro* and *in silico*; and modulates abdominal glucose absorption and muscle glucose uptake *ex vivo*


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**ARTICLE INFO**

**Keywords:**
- *Boerhaavia diffusa*
- Antioxidant
- Glucose absorption
- Enzyme inhibition

**ABSTRACT**

The present study investigated the in vitro and ex vivo antioxidant, anti-diabetic and anti-obesogenic potentials of different solvent (ethyl acetate, ethanol and water) extracts from the aerial parts of *Boerhaavia diffusa*. The ferric reducing antioxidant power (FRAP), DPPH scavenging activity and the ameliorative effects of the extracts on FeCl₂-induced oxidative injury was investigated both in vitro and ex vivo. Alpha glucosidase and pancreatic lipase inhibitory potentials of the extracts were examined in vitro, while the effects of the ethanol extract on abdominal glucose intake and muscle glucose uptake were determined in freshly harvested tissues ex vivo. The extracts were subjected to Gas Chromatography-Mass Spectrometry (GC-MS) analysis to identify their possible bioactive components. The ethanol extract showed the most potent FRAP and DPPH radical scavenging activities compared to other extracts. All extracts increased catalase and SOD activities, and GSH levels in oxidative pancreatic injury. Both ethanol and aqueous extracts exhibited remarkable enzyme inhibitory activities, which was significantly higher than ethyl acetate extract and scabrosac but was not comparable to orlistat. The ethanol extract portrayed a dose-dependent inhibitory effect on jejunal glucose uptake and enhancement of muscle glucose uptake. 9-(4-methoxyphenyl) xanthine, xanthine and xanthostearon showed strong binding affinities for α-glucosidase and lipase enzymes tested. Data from this study suggest that aerial parts of *B. diffusa* (particularly the ethanol extract) may not only exhibit antioxidant potentials but may also mediate anti-lipemic and anti-hyperglycemic effects via inhibiting fat and carbohydrate digestion as well as abdominal glucose intake and enhancing muscle glucose uptake.
APPENDIX C

OTHER PUBLICATIONS

Biomedicine & Pharmacotherapy 06 (2017) 17–17

Original article

Dacyodes edulis enhances antioxidant activities, suppresses DNA fragmentation in oxidative pancreatic and hepatic injuries; and inhibits carbohydrate digestive enzymes linked to type 2 diabetes

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b School of Chemistry and Physics, University of KwaZulu-Natal (Westville Campus), Durban 4000, South Africa
c Nutrition and Toxicology Division, Federal Institute of Industrial Research, Ilogbo, Nigeria

ARTICLE INFO

Keywords
Antioxidants
Dacyodes edulis
Type 2 diabetes
Medicinal plant

ABSTRACT

The leaves of Dacyodes edulis were investigated for their anti-oxidative and anti-diabetic potentials in vitro. Extracts from sequential extraction with solvents of increasing polarity (n-hexane, ethyl acetate, ethanol and aqueous) of the leaves were subjected to in vitro antioxidant assays using the 2,2’-diphenyl-1-picrylhydrazyl (DPPH) scavenging and Ferric reducing antioxidant power (FRAP) protocols respectively. Their inhibitory effects were investigated on α-glucosidase, pancreatic lipase, pancreatic ALTase and glucose-6-phosphate dehydrogenase activities. Their antioxidant and anti-apoptotic effects on Fe 3+ - induced oxidative injuries in pancreatic and hepatic tissue were also investigated ex vivo. The ethanol extract was subjected to Gas chromatography mass spectrometry (GC-MS) and Fourier transform infrared (FTIR) spectroscopic analysis to identify bioactive chemical constituents. The extracts showed potent free radical scavenging activity and significantly (p < 0.05) inhibited all studied enzymes, with the ethanol extract showing greater activities. Superoxide Dismutase (SOD) and Catalase (CAT) activities were significantly (p < 0.05) increased in both pancreatic and hepatic tissues with concomitant elevation of reduced glutathione (GSH) levels as well as reduced levels of malondialdehyde (MDA). The extracts significantly inhibited DNA fragmentation. These activities were dose - dependent. Amongst compounds identified, only Kaurene, Urs-12-ene-3-ol acetate and 2,3,4-trihydroxy-9,12-en-28-ol acetate showed strong binding affinities when docked with α glucosidase (PDB ID: 3T0N). These results indicate the anti-oxidative, anti-diabetic and anti-atherogenic potentials of D. edulis leaves, which give credence to its anti-diabetic folkloric claims.

1. Introduction

The world has witnessed an increase in the prevalence of diabetes insulin resistance and pancreatic β-cell dysfunction, resulting in chronic hyperglycemia. In the progression of T2D, hyperglycemia results in elevated reactive oxygen species (ROS) production which causes ox-
Caffeine – rich infusion from *Cola nitida* (kola nut) inhibits major carbohydrate catabolic enzymes; abates redox imbalance; and modulates oxidative dysregulated metabolic pathways and metabolites in Fe$^{2+}$-induced hepatic toxicity

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**Article Info**

**Keywords:**
Antioxidative
Cola nitida
Kola nut
Metabolomics
Type 2 diabetes

**Abstract**

The antioxidative and antidiabetic effects and toxicity of caffeine-rich infusion of *Cola nitida* were investigated using in vitro and in silico models. *C. nitida* was infused in boiling water and allowed to cool before concentrating at 50°C. HPLC analysis of the infusion revealed a caffeine content of 80.0%. The infusion showed potent in vitro antioxidative activity by significantly ($\rho < 0.05$) scavenging 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS$^+$). It significantly ($\rho < 0.05$) inhibited α-glucosidase and α-amylase activities. Treatment of Fe$^{2+}$-induced oxidative hepatic tissues with the infusion led to increase superoxide dismutase (SOD) and catalase activities, and glutathione (GSH) levels as well as decreased malondialdehyde (MDA) levels. FTIR-spectroscopy of hepatic metabolites revealed restoration of oxidative-induced depleted functional groups by the infusion. LC-MS analysis of the metabolite also revealed restoration of most depleted metabolites with concomitant generation of 4-O-methylgallic acid (4-O-MGA), 4-hydroxyphenylethyl amine, 6-amino nicotinic acid, 4-aminopyridine, 4-amino nicotinic acid, and 6-aminopyridine in the treated tissues. Treatment with the infusion restored 4 metabolic pathways common to the normal tissue and further activated 4 additional pathways. Prediction of oral toxicity of caffeine showed it to belong to class 3, with a LD50 of 157 mg/kg. Its toxicity target was predicted as Adenosine Receptor 2A. It was also predicted to be an inhibitor of CYP1A2. These results suggest the antioxidative and antidiabetic properties of *C. nitida* infusion, with caffeine as the major constituent.

1. Introduction

devotes the production of reactive oxygen species (ROS) which triggers
Concentrated hot water-infusion of *Phragmanthera incana* improves muscle glucose uptake, inhibits carbohydrate digesting enzymes and abates Fe^{2+}-induced oxidative stress in hepatic tissues

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**ARTICLE INFO**

**Keywords**
Antioxidants
Enzyme inhibition
Glucose uptake
*Phragmanthera incana*

**ABSTRACT**

Chronic hyperglycemia has been implicated in the development of oxidative stress and as a major factor in etiology of secondary complication in diabetes. In the present study, the antidiabetic potential of *Phragmanthera incana* (P. incana) hot infusion and its possible inhibitory effects on carbohydrate digesting enzymes, promotion of muscle glucose uptake, and the antioxidative potentials in Fe^{2+}-induced oxidative stress in hepatic tissue were investigated. The infusion significantly (p < 0.05) scavenged free radicals (DPPH) and displayed favourable ferric reducing antioxidant power (FRAP) with increasing concentrations. It also significantly ameliorated Fe^{2+}-induced oxidative stress in hepatic tissues by increasing superoxide dismutase (SOD) and catalase activities and depleting malondialdehyde (MDA) level. The results further showed that the infusion significantly (p < 0.05) inhibited amylase and a-glucosidase activity, and enhanced muscle glucose uptake, with and without insulin. Liquid Chromatography-Mass Spectrometry (LCMS) analysis of the infusion revealed the presence of 5-methoxyxiacene, 1-cysteine, nicotinic acid S-methyl-1-cysteine, 6-quinoline, 1-methyl, and 1H-indole-1,3-dione5-methyl. The results of this study suggest that the observed antidiabetic and antioxidative potentials of *P. incana* could be attributed to its identified phytochemical constituents, however, this supports folkloric medicinal use of this plant.

1. Introduction

Diabetes mellitus (DM) has, over the years, become one of the leading threats to human health [1]. Available data reveal that the prevalence of diabetes has increased from 4.7% in 1980 to 8.5% in 2014 [1,2]. The International Diabetes Federation (IDF) projected a leading to micro- and macro-vascular complications [6]. Most oral synthetic antidiabetic drugs have been targeted towards the control of hyperglycemia, thus limiting its progression to secondary complications [7]. These drugs exert their actions by improving pancreatic β-cell function, reducing glucotoxicity, decreasing glucose absorption in the kidney, increasing insulin sensitivity in the muscle.