ANTIOXIDATIVE AND ANTIDIABETIC EFFECTS OF SOME AFRICAN MEDICINAL PLANTS

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Submitted in fulfillment of the requirements for the award of Doctor of Philosophy degree in Biochemistry

School of Life Sciences

College of Agriculture, Engineering and Science

As the candidate’s supervisor I have approved this thesis/dissertation for submission.

Signed: ………………………… Name: Dr. M.S. Islam Date: ………………………
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DECLARATION II

I, Aminu Mohammed hereby declare that the dissertation entitled “Antioxidative and antidiabetic effects of some African medicinal plants” is the result of my own investigation and research and that it has not been submitted in part or in full for any other degree or to any other university. Where use of the work of others was made, it is duly acknowledged in the text.

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DETAILS OF CONTRIBUTION TO PUBLICATIONS that form part of and/or include research presented in this thesis (include publication 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 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 the correctness of my statistical analysis of data and interpretation of the findings.

Published and/or accepted papers

Publication 1

Publication 2

Publication 3

Publication 4
Publication 5

Papers are intended to submit:

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Publication 7

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Publication 11
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Publication 14

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Presentation 2

Presentation 3
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**Student: Mr. Aminu Mohammed**

**Signature** ………………………
ABSTRACT

Three (3) medicinal plants [Aframomum melegueta K. Schum., Xylopia aethiopica (Dunal.) A. Rich. and Capsicum annuum L.] were selected based on their traditional uses in the treatment of diabetes in Africa. Various crude extracts and fractions from different parts of the plants were screened using several anti-oxidative and anti-diabetic tests \textit{in vitro}. Most active fractions from each plant were used to examine \textit{in vivo} anti-diabetic activity in type 2 diabetes (T2D) rat model. Additionally, possible bioactive compounds from most active extracts and fractions were analyzed by using GC-MS, TLC and NMR spectroscopy. The results showed that ethanolic extracts derived from the fruits of the plants demonstrated excellent anti-oxidative and anti-diabetic activities \textit{in vitro} compared to other extracts from the same or different parts of these plants. After fractionation, ethyl acetate fraction from A. melegueta and acetone fractions from X. aethiopica and C. annuum exhibited strong radical scavenging (IC\textsubscript{50}: 1-120 µg/mL) activity, inhibition of hemoglobin glycation (IC\textsubscript{50}: 100-150 µg/mL), α-amylase (IC\textsubscript{50}: 50-170 µg/mL) and α-glucosidase (IC\textsubscript{50}: 40-87 µg/mL) activities hence were used for the \textit{in vivo} study. The GC-MS analysis of the three (3) most active fractions revealed the presence of mostly phenolic compounds of 4-hydroxy-3-methoxyphenyl derivatives. Furthermore, the data of the \textit{in vivo} study showed that oral intervention of the fractions (150 and 300 mg/kg bw) for 4 weeks demonstrated potent anti-diabetic actions via improving body weight gain, reducing feed and fluid intake and hyperglycemia, improving glucose tolerance ability, insulin sensitivity, amelioration of pancreatic β-cell histology and β-cell functions, improving dyslipidemia in a T2D rat model. Additionally, the pancreatic histopathological damages and other oxidative damages caused by the induction of diabetes were attenuated to near normal in the liver, kidney, heart and pancreas of the treated animals. The bioassay-guided fractionations lead to the isolation of 3 arylalkanes (6-paradol (1), 6-shagaol (2), and 6- gingerol (3)) and oleanolic acid (4) from A. melegueta fruits, when oleanolic acid (4) was the first to be isolated from A. melegueta. Moreover, 6-gingerol (3) and oleanolic acid (4) were similarly isolated for the first time from X. aethiopica fruits as well. These compounds have exhibited significant inhibitions against the α-amylase and α-glucosidase actions and thus are possible anti-diabetic agents and the anti-diabetic action of A. melegueta and X. aethiopica fruits is attributed to the presence of these compounds. This study also confirmed the use of these plants in African anti-diabetic traditional medicines by traditional healers. However, further clinical study is required to confirm these effects in human subjects.
DEDICATION

To my parent Alhaji Abubakar Mohammed and Hajiya Hauwa’u Aminu for their guidance, support and wisdom.
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<td>ADA</td>
<td>American diabetes association</td>
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<tr>
<td>AI</td>
<td>Atherogenic index</td>
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<td>ALP</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>ALT</td>
<td>Alanine transaminase</td>
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<td>AMEF</td>
<td><em>Aframomum melegueta</em> ethyl acetate fraction</td>
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<tr>
<td>AST</td>
<td>Aspartate transaminase</td>
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<tr>
<td>AUC</td>
<td>Area under the curve</td>
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<tr>
<td>BRU</td>
<td>Biomedical Resource Unit</td>
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<td>CAAF</td>
<td><em>Capsicum annuum</em> acetone fraction</td>
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<tr>
<td>CK-MB</td>
<td>Creatine kinase</td>
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<td>CRI</td>
<td>Coronary artery risk index</td>
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<td>DAMH</td>
<td>Diabetic <em>Aframomum melegueta</em> high dose</td>
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<td>DAML</td>
<td>Diabetic <em>Aframomum melegueta</em> low dose</td>
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<tr>
<td>DBC</td>
<td>Diabetic control</td>
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<td>DCAH</td>
<td>Diabetic <em>Capsicum annuum</em> high dose</td>
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<td>DCAL</td>
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<tr>
<td>DETAPAC</td>
<td>Diethylenetriaminepentaacetic acid</td>
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<td>DM</td>
<td>Diabetes mellitus</td>
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<tr>
<td>DMF</td>
<td>Diabetic metformin</td>
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<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<td>DNS</td>
<td>Dinitrosalicylic acid</td>
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<tr>
<td>DPP-4</td>
<td>Dipeptidyl peptidase-4</td>
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<tr>
<td>DPPH</td>
<td>1, 1-Diphenyl-2-picrylhydrazyl radical</td>
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<td>DXAH</td>
<td>Diabetic <em>Xylopia aethiopica</em> high dose</td>
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<td>DXAL</td>
<td>Diabetic <em>Xylopia aethiopica</em> low dose</td>
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<td>EMA</td>
<td>European medicines agency</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<td>EtOAc</td>
<td>Ethyl acetate</td>
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<td>EtOH</td>
<td>Ethanolic</td>
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<td>FBG</td>
<td>Fasting blood glucose</td>
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<td>FDA</td>
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<td>FRAP</td>
<td>Ferric reducing anti-oxidant power</td>
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GADA  Glutamic acid decarboxylase antibodies
GAE  Gallic acid equivalent
GC-MS  Gas chromatography-mass spectroscopic
GDM  Gestational diabetes mellitus
GLUT 2  Glucose transporter type 2
GLUT 4  Glucose transporter type 4
GLP  Glucagon-like peptide
GPx  Glutathione peroxidase
GR  Glutathione reductase
HOMA-β  Homeostatic model assessment-β cell function
HOMA-IR  Homeostatic model assessment-insulin resistance
IDDM  Insulin dependent diabetes mellitus
IDF  International Diabetes Federation
IFG  Impaired fasting glucose
IGT  Impaired glucose tolerance
LDH  Lactate dehydrogenase
MDA  Malondialdehyde
MODY  Maturity-onset diabetes of the young
NAMH  Normal Aframomum melegueta high dose
NC  Normal control
NCAH  Normal Capsicum annuum high dose
NFBG  Non-fasting blood glucose
NIDDM  Non-insulin dependent diabetes mellitus
NMR  Nuclear magnetic resonance spectroscopy
pNPG  p-Nitropheynyl glucopyranoside
NXAH  Normal Xylopia aethiopica high dose
OGTT  Oral glucose tolerance test
PPAR  Peroxisome proliferator activated receptor
QE  Quercetin equivalent
ROS  Reactive oxygen species
SOD  Superoxide dismutase
STZ  Streptozotocin
TBARS  Thiobarbituric acid reactive substance
T1D  Type 1 diabetes
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<td>TLC</td>
<td>Thin layer chromatography</td>
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<td>WHO</td>
<td>World health organization</td>
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<td>XAAF</td>
<td><em>Xylopia aethiopica</em> acetone fraction</td>
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CHAPTER 1

Introduction and Literature Review

1.0 Introduction and background of the study

Diabetes mellitus (DM) is a group of complex and chronic metabolic disorders with diverse multiple etiologies. It is characterized by high blood glucose (hyperglycemia) resulting from malfunction in insulin secretion and/or insulin action, both leading to impair metabolism of carbohydrates, lipids and proteins (ADA, 2015). The alterations in the utilization of complex biomolecules by the most affected tissues (liver, muscle and adipose tissue) due to hyperglycemia initiate a sequence of oxidative processes that cause dysfunction and failure of other organs in the body. Long-term complications may affect the organs such as kidneys, eyes, nerves, heart and blood vessels, and in absence of effective treatment result into death (ADA, 2015; Surampud et al. 2009; Maritim et al. 2003).

At present, different approaches are used to control diabetes using modern synthetic anti-diabetic drugs, insulin injection and life style modification. The synthetic anti-diabetic drugs include sulphonylureas, glucosidase inhibitors, dipeptidyl peptidase-4 (DPP-4) inhibitors and biguanide. However, these synthetic drugs have characteristic profiles of serious side effects, which include hypoglycemia, weight gain, gastrointestinal discomfort and nausea, liver and heart failure, and diarrhea (Hung et al. 2012; Michael et al. 2005). This is in addition to being rather costly and not affordable by the majority of people in developing countries especially for African populations. These limitations coupled with an exponential increase in the prevalence of diabetes motivate researchers to scientifically validate the folkloric use of a number of medicinal plants and/or their isolated bioactive compounds as possible alternative therapies for diabetes. The prime target for such research is to pave the way for the development of newer plant-derived anti-diabetic compounds that could be used to ameliorate the diabetes associated complications. This can subsequently be standardized and be used as drug for the treatment of the DM.

Furthermore, in many continents such as Africa, herbs and natural products form an integral component of the health care delivery system (Cragg and Newman, 2013). This has been further supported by the World Health Organization (WHO) report that 80% of the population in Africa depends almost entirely on traditional medicines, herbal medicines in particular, for their primary health care needs (WHO, 2001). This is attributed to the proven effectiveness of the plant-based therapies as well as the availability of these medicinal plants. Because, the African continent accounts for about 25% of the total number of higher plants in the world where more than 5400 medicinal plants were reported to have over 16300 medicinal uses (van Wyk et al. 2008). Fortunately, some plant products either in the form of crude extracts, fractions or isolated compounds have been screened or investigated for possible anti-diabetic remedy in Africa (Mohammed et al. 2014). However, the number of plants
and/or isolated bioactive compounds with potential anti-diabetic actions is very limited and many of their anti-diabetic effects have not yet been scientifically validated.

1.1 Literature Review

1.2 Diabetes Mellitus

Diabetes mellitus (DM) is a disorder that causes elevation of blood glucose, otherwise known as hyperglycemia (fasting blood glucose level: ≥126 mg/dL or 7.0 mmol/L; or postprandial hyperglycemia: ≥200 mg/dL or 11.1 mmol/L) due to either decrease in insulin secretion and/or insulin sensitivity of target tissues (Panini, 2013; ADA, 2015).

1.2.1 Types of diabetes mellitus

Originally, diabetes has been classified into two major classes: (1) type 1 or insulin dependent diabetes mellitus (IDDM) and (2) type 2 or non-insulin dependent diabetes mellitus (NIDDM) (WHO, 1980). However, rapidly changing pathogenesis of diabetes has been taken into account for the new classification of DM. The recent classification by the American Diabetes Association (ADA), diabetes is categorized into four types: type 1 diabetes, type 2 diabetes, gestational diabetes and the secondary form of diabetes which encompasses all types of diabetes due to other causes, for instance, monogenic diabetes syndromes, diseases of the exocrine pancreas and drug- or chemical-associated diabetes (ADA, 2015).

1.2.2 Type 1 diabetes mellitus

This form of diabetes is due to autoimmune-mediated destruction of the pancreatic β-cells as a result of production of humoral auto antibodies (ADA, 2015; Canivell and Gomis, 2014). Although the cause of type 1 diabetes (T1D) remains elusive, it is strongly linked to interplay between genetic predisposition and environmental factors that possibly triggers an autoimmune destruction of the pancreatic β-cells leading to absolute insulin deficiency (Patterson et al. 2014). The environmental factors include infectious agents such as viruses (coxsackie B virus, rubella virus) and food toxins. The destruction of pancreatic β-cells is gradual and variable, being rapid in infants and children and slower in adults (Joslin and Kahn, 2005). The mechanism involves on selective destruction of pancreatic β-cells in T1D is poorly understood due to the dissimilarities of pancreatic lesions (Ozougwu et al. 2013). The proposed mechanism involves the infiltration of lymphocytes (innate immune cells) or insulitis due to co-interaction of genetic and environmental factors. The infiltration of innate immune cells produces cytokines such as glutamic acid decarboxylase antibodies (GAD-65), islet cell antibodies (ICA512A/ICA) and insulin antibodies (IAA), which promote pancreatic β-cell apoptosis and increase
infiltration of islet reactive T cells that ultimately attack and destroys pancreatic β-cells (Szablewski, 2014).

Similarly, other form of T1D categorized as “idiopathic diabetes” which includes all forms of T1D with no known etiology and is mostly found among individuals from Asian or African regions. Individuals with this type of diabetes demonstrate no evidence of autoimmunity and exhibit insulinopenia and are prone to ketoacidosis (ADA, 2015; Canivell and Gomis, 2014). Apart from above, a brief summary of the pathogenesis of T1D is presented in Figure 1.1.

**Figure 1.1:** Pathogenesis of type 1 diabetes (copied without permission from Atkinson and Eisenbarth, 2001). FPIP, first phase of insulin response; GADA, glutamic acid decarboxylase antibodies; ICA512A/ICA, islet cell antibodies; IAA, insulin antibodies.

### 1.2.3 Type 2 diabetes mellitus

Type 2 diabetes (T2D) is a heterogeneous disorder characterized by insulin resistance and partially dysfunctional pancreatic β-cells which cannot properly secrete insulin in response to hyperglycemia (Hui et al. 2007). It is the most prevalent type of diabetes, accounting for more than 90% of all reported diabetes cases in the world (IDF, 2014). The insulin deficiency is relative rather than absolute and usually no insulin treatment (unless special cases) is required for T2D (ADA, 2015). The pathogenesis of insulin resistance in T2D is complex and involves genetic (defect on insulin and its receptor genes etc.) and environmental (obesity, sedentary life, age and physical inactivity) factors (Tuomilehto et al. 2001). Furthermore, inadequate insulin secretion by pancreatic β-cell in type 2 diabetic individuals disrupts the regulation of hepatic gluconeogenesis, muscles glucose uptake and lipolysis in adipose tissues (Gastaldelli, 2011). The consequence is postprandial hyperglycemia which results in to T2D. The summary of the pathogenesis for T2D is presented in Figure 1.2.
1.2.4 Gestational diabetes mellitus

Gestational diabetes mellitus (GDM) have been defined as heterogeneous group of disorders associated with any glucose intolerance diagnosed usually in the third trimester of the pregnancy (Ashwal and Hod, 2015) when in many cases disorder may improve or disappear after the delivery of baby. Similarly, it has been reported that about 3% to 65% of women with a history of GDM are at high risk of developing T2D in the later part their lives (Lee et al. 2008). In genetic predisposed women (during pregnancy), the alterations in glucose metabolism may lead to mix insulin resistance and impaired insulin secretion (Whitelaw and Gayle, 2010). This condition usually exaggerates as the pregnancy period increases which ultimately result in to hyperglycemia. The pathogenesis of GDM is summarized in Figure 1.3.
1.2.5 Specific types of diabetes due to other causes (secondary type)

This is further sub-divided in to the following:

a) Maturity-onset diabetes of the young (MODY)

This form of diabetes is due to the defect on DNA methylation of genes for pancreatic β-cell development (Thomas and Philipson, 2015). It is mainly categorized into neonatal diabetes and maturity-onset diabetes of the young (MODY). The onset of elevated blood glucose as a result of pancreatic β-cell failure usually occurs at an early stage of life (within the first 6 months). Furthermore, in MODY, there is a defect in insulin secretion with less or no reported alterations in insulin function (ADA, 2015; Thomas and Philipson, 2015).

b) Cystic fibrosis related diabetes

The cystic fibrosis–related diabetes is frequently occurs in individuals with cystic fibrosis. The disorder is due to impair insulin secretion as a result of partial fibrotic reduction of the pancreatic β-cell mass (ADA, 2015). Insulin sensitivity is usually normal or may be partially impaired in this kind of diabetes (Ode and Moran, 2013). Other disorders associated with pancreas include chronic pancreatitis, hereditary hemochromatosis and pancreatic neoplasia.
c) Drug associated diabetes

The medications of some diseases were reported to interfere with complex metabolic processes, thereby alters the secretion and/or function of insulin in physiological system (Thomas and Philipson, 2015). This may likely triggers diabetes or insulin resistance in susceptible individuals. For instance, glucocorticoids were reported to cause insulin resistance, hyperglycemia, and do interfere with some stages in the insulin-signaling pathways via multiple mechanisms (Ferris and Kahn, 2012). Antiretroviral drugs (HIV protease inhibitors) were also reported to induce insulin resistance and impaired glucose tolerance by inhibiting glucose disposal via GLUT4 in cellular system (Koster et al. 2003). Hence, it is clear that the pathogenesis of diabetes changing rapidly to create newer type of diabetes which directly affecting the total prevalence of diabetes mellitus.

1.2.6 Prevalence of diabetes mellitus

The global prevalence of DM is increasing exponentially. Recent data from the International Diabetes Federation (IDF) indicates that DM affects over 387 million people globally and this figure is likely to rise to 592 million by 2035 (IDF, 2014; Guariguata et al. 2014). The disease affects nearly 8.3% of adult (20-79 years) which are mostly live in the low- and middle-income countries. In Africa, more than 22 million people have diabetes, accounting for about 5.1% of adults (mostly < 60 years) in the region (Peer et al. 2014; IDF, 2014). In addition, multiple factors contribute to this rising prevalence of diabetes including population growth, urbanization, dietary change, nutritional transition, physical inactivity and so on (Guariguata et al. 2014; Hirst et al. 2013).

1.2.7 Diabetes associated complications

Although the pathogenesis of T1D and T2D are different, the consequences of their resulting complications are almost similar (van Dijk and Berl, 2004). These complications are categorized as either acute or chronic, which partly or solely depend on the uncontrolled hyperglycemia (Monnier et al. 2006). Acute complications include ketoacidosis, hyperosmolar non-ketotic coma and hypoglycemia (Fishbein and Palumbo, 1995). The chronic crises comprise of microvascular (retinopathy, neuropathy and nephropathy) and macro vascular (myocardial infarction, atherosclerosis and peripheral vascular diseases) complications (Heydari et al. 2010; Rahman et al. 2007). The over production of reactive oxygen species (ROS) via metabolic processes and due to hyperglycemia have been considered as the major hallmarks of diabetes-associated complications (Pazdro and Burgess, 2010). In chronic uncontrolled hyperglycemia, there is an increased production of ROS and a declined of *in vivo* antioxidant defense system, a term referred as oxidative stress. The ROS are derived from the normal physiological processes and become highly deleterious if the levels increases and not arrested by complex anti-oxidant systems in the body (Chang and Chuang, 2010). Furthermore, major contributors of hyperglycemia-induced oxidative stress include glucose toxicity, protein glycosylation, increased
production of glycation end products and mitochondrial ROS, the polyol, hexosamine and affected protein kinase C pathways (Giacco and Brownlee, 2010; Chang and Chuang, 2010; Chung et al. 2003; Maritim et al. 2003). The schematic diagram indicating hyperglycemia-induced oxidative damages is presented in Figure 1.4. The mechanisms involve in micro-vascular and macro-vascular complications can be linked to hyperglycemia-induced oxidative stress. For instance, in diabetic retinopathy, the increased oxidative stress due to inability of the cells to convert glucose to sorbitol caused cellular injury and increased the accumulation of advanced glycated end products (Jiang, 2000).

Figure 1.4: Hyperglycemia-induced oxidative damages in diabetes mellitus (copied without permission from Brownlee, 2005).

1.2.8 Treatment and management of diabetes mellitus

The importance of protecting or delaying hyperglycemia cannot be overemphasized. The acute as well as chronic complications of diabetes are the major causes of morbidity and mortality in all types of diabetes (Fowler, 2008). Fortunately, in the last 2 decades, concerted research efforts by diabetologists and other relevant research scientists highlighted two main strategies to control DM which can be used either alone or in combination of the two, depending on the type and severity of diabetic condition. These include pharmacological and non-pharmacological approaches (Stolar et al. 2008).
1.2.9 Non-pharmacological therapy

The non-pharmacological option refers to the use of lifestyle intervention strategies in patients with established risk factors for T2D in order to delay the progression and development of DM. Lifestyle changes such as weight loss, increased physical exercise and some dietary manipulations are effective in preventing and controlling diabetes (Tuomilehto, 2009) and this was demonstrated in a number of randomized controlled clinical trials such as Swedish Malmo feasibility study (Eriksson and Lindgarde, 1991), Chinese Da Qing study (Pan et al. 1997), Finnish diabetes prevention study (Tuomilehto et al. 2001), U.S diabetes prevention program (DPP, 2002), Japanese lifestyle intervention study (Kosaka et al. 2005) and the Indian diabetes prevention program (Ramachandran et al. 2006). It has been reported that lifestyle modification by diabetic patients significantly improved hepatic and muscle insulin sensitivity, muscle glucose uptake and utilization, and the overall glycemic control in addition to decrease lipids and blood pressure levels (Hayes, 2008).

1.2.10 Pharmacological therapy

Pharmacological intervention to prevent diabetes involves the use of drugs or any agents to treat patients with established risk factors. The basic risk factors are impaired glucose tolerance (IGT) or impaired fasting blood glucose (IFG) because approximately 50% of IGT and IFG individuals progress to diabetes (T2D) over their lifetime (De Fronzo and Abdul-Ghani, 2011).

- **Insulin**

  It has been well established that insulin therapy reduce hyperglycemia and micro- and macrovascular complications associated with diabetes in several randomized clinical trials (Holman et al. 2008; UKPDS, 1998; Ohkubo et al. 1995). However, insulin therapy is associated with two major limitations; hypoglycemia and weight gain, arose due to intensive insulin treatment (Swinnen et al. 2009).

- **Mode of insulin action**

  Insulin is a polypeptide hormone produced by the pancreatic β-cells of the islets of Langerhans in response to hyperglycemia (Harvey and Ferrier, 2011). The β-cells are freely permeable to glucose via type 2 glucose transporter (GLUT 2) which are immediately phosphorylated to glucose 6-phosphate by glucokinase enzyme. This stimulates the rise in metabolic flux through glycolysis, the citric acid cycle, and the generation of ATP (Murray et al. 2003). Elevation of ATP concentration down regulates the ATP-sensitive K+ channel, leading to depolarization of the pancreatic β-cell membrane. This may eventually increases Ca2+ influx via voltage-sensitive Ca2+ channels and stimulates the exocytosis of insulin (Kieffer et al. 1997). Hence, the level of insulin in the blood is proportionate to that of the blood glucose. Therefore, insulin stimulates glucose transport into adipose tissue and skeletal muscles via type
4 glucose transporter (GLUT 4) which are utilized as metabolic fuel and stored as well (Kieffer et al. 1997).

- **Conventional oral hypoglycemic drugs**

  Several classes of pharmacological agents have shown to reduce the risk of developing DM in double blind randomized controlled clinical trials (Phung et al. 2011). These include biguanides (phenformin), α-glycosidase inhibitors (acarbose), sulfonylureas (glibenclamide), thiazolidinediones (rosiglitazone) and dipeptidyl peptidase-4 inhibitors (vildagliptin) (Phung et al. 2011; Padwal et al. 2005).

  Moreover, these classes of drugs demonstrate their anti-diabetic actions via diverse mechanisms (Figure 1.5). Sulfonylureas, also called insulin secretagogues, improve insulin release from the pancreatic β-cells (Aguilar-Bryan et al. 1995). Biguanides improve sensitivity of insulin to the liver and muscle cells and inhibit hepatic glucose production (De Fronzo, 1999). Some enzyme inhibitors also reduce blood glucose by inhibiting the activities of carbohydrates digestive enzymes (α-amylase and α-glucosidase) located at the intestinal brush border (Krentz et al. 1994). Furthermore, thiazolidinediones also referred as peroxisome proliferator activated receptor (PPAR)-γ agonist which improves insulin sensitivity in the liver, muscle and adipose tissues. They do achieve such effects by binding to PPARγ receptor, which eventually increase the expression of glucose transporters (Kintscher and Law, 2005; Lehrke and Lazar, 2005). Dipeptidyl peptidase-4 (DPP-4) inhibitors cause reduction on blood glucose levels via inhibiting an enzyme involve in the breakdown of incretin like peptides such as glucagon-like peptide (GLP)-1 and glucose-dependent insulinotropic peptide (GIP) (Hung et al. 2012).

  However, these conventional oral drugs stated above have many unwanted adverse effects (hypoglycemia, weight gain, gastrointestinal discomfort, nausea, hypersensitivity, liver and heart failure, and diarrhea) and are not cost effective for the majority of the people, particularly for those who are from the developing countries (Michael et al. 2005). Therefore, safer and more effective treatment options with fewer side effects are required in order to control the progression DM. Thus, with the increasing interest in alternatives to the present conventional oral drugs in the treatment of diabetes, the use of plant-derived products is emerging and receiving much attention by many researchers.
1.3 Medicinal plants

Medicinal plants are a plants of which one or more parts contain substances that can be used for therapeutic purposes or which are precursors of the useful drugs (Ebadi, 2006; Lewis, 1981). Medicinal plants have formed the basis of health care system worldwide for several years and are still widely used as good sources for the present modern drugs. Recognition of their therapeutic and economic value is emerging and receiving much attention. Plants are important for pharmacological research and drug discovery, not only when bioactive compounds are used directly for treatment of ailments, but also as template for the synthesis of modern conventional drugs (Mendonça-Filho, 2006; Geldenhuys and Mitchell, 2006).

1.3.1 Medicinal plants in future drug discovery

Medicinal plants are natural products endowed with tremendous capacities to treat wide arrays of diseases. The use of plant-based formulation to treat diseases also known as herbal medicine is the oldest form of medicine since around 2600 BCE (Cragg and Newman, 2013; Soladoye et al. 2012). Fortunately, this area has documented the traditional uses of more than 1000 plant-derived formulations which are still being used to treat both communicable and non-communicable diseases. Studies conducted previously have demonstrated that about 80% of isolated compounds, which are used in the modern medicines, are derived from medicinal plants and traditionally these medicinal plants were used for the same or similar purposes (Cragg and Newman, 2013; Farnsworth et al. 1985). For instance, the anti-diabetic agent galegine (a template for synthesis of metformin) was isolated from *Galega officinalis* L., which plant was used to treat diabetes traditionally (Heinrich, 2010). Similarly, the anti-malarial drug, artemisinin and anti-HIV drug, calanolide A were derived from the plant *Artemisia annua* L. and *Calophyllum lanigerum* var. *austrocoriaceum* (Whitmore) P.F. Stevens, respectively. These plants have
also been widely used in the traditional medicines for the treatment of fevers and some microbial infections (Gurib-Fakim, 2006).

Moreover, search for plant-derived drugs is a multidisciplinary work incorporating research, financial and technical supports, and hence needs carefully planned strategy (Rates, 2001) and has been a difficult and expensive task (Borris, 1996; Turner, 1996). For instance, a huge amount of money (US$ 100-360 million) is estimated for the development of newer drug with a minimum of 10 years of work, with about 0.01% probability of getting promising one (Williamson et al. 1996). The schematic flow chart pattern of choosing plant for drug discovery is presented in Figure 1.6.

![Figure 1.6: Flow chart for plant-derived drug discovery (modified from Balunas and Kinghorn, 2005).](image)

**1.3.2 Selection of medicinal plant for drug discovery**

In order to select medicinal plant for target drug discovery, various approaches were map out by scientists to evaluate plant materials for their pharmacological properties (Heinrich et al. 2004; Fabricant and Farnsworth, 2001). Ethnobotanical uses play a significant role in selecting plant as a candidate for drug discovery. In this approach, information on the local usage of plant is the key-driven factor which is normally obtained through ethnobotanical studies (Shizen and Xiwen, 2004). The phytochemistry-based selection mainly focused on the isolated ingredients responsible for the pharmacological uses of the plant under study, with no prior information on the target biomolecule (Brusotti et al. 2014). Other
approaches include random and/or target selection of plants followed by some biological studies and follow-up bioactivity reports (Heinrich et al. 2004; Harborne, 1998).

1.3.3 Collection, authentication and preparation of plant material

Collection of individual plant materials and/or whole plant samples depends on the parts with rich bioactive constituents and is greatly affected by some environmental factors such as nature of the soil, rainfall, weather and temperature (Harborne, 1998). The collected plant parts need to be scientifically and correctly authenticated by either taxonomist or a botanist (Satyajit et al. 2006). The voucher specimen number is usually deposited in the herbarium for subsequent reference purposes for an unlimited period of time (Harborne, 1998).

Furthermore, extraction of the plant samples is an integral part on the way of plant-derived drug discovery (Brusotti et al. 2014). Several methods are employed to achieve successful extraction of the bioactive compounds from the plant materials. The conventional solid-liquid solvent extraction procedure depends mainly on the polarity and solubility of the target bioactive compounds in the extracting solvent (Hostettmann et al. 1991). Such conventional methods include but not limited to maceration, infusion, decoction, percolation, steam distillation, sequential solvent extraction and soxhlet extraction (Harborne, 1998). This is in addition to some modern techniques currently employed for sample extraction which include the microwave-assisted extraction, ultrasound assisted extraction, supercritical fluid extraction and pressurized liquid extraction (Brusotti et al. 2014). Before subjecting the sample to extraction procedure, the plant materials must be dried, usually under shade in a well ventilated space to avoid microbial contamination and loss of target compounds. After drying, the samples are grounded into a fine powder for proper extraction of the bioactive metabolites. Then the extracts can be used either for in vitro, ex-vivo or in vivo study.

1.3.4 Preliminary in vitro studies

Once the extracts are obtained, the next step is to subject the extracts to some in vitro bioassay protocols in order to examine whether the extracts are active or not. In vitro assays are usually faster, specific and not much amount of the extracts are used (mostly in micro or milligram amount) (Cos et al. 2006). Furthermore, some of the in vitro methods employed include chemical and enzymatic procedures, which depend on spectrophotometric analysis (Beretta and Facino, 2010). For instance in evaluating plants as possible anti-diabetic drugs, several in vitro models are used to assess the anti-diabetic effects and mode of actions as well. These models include enzyme inhibition-based assays (e.g. α-amylase, α-glucosidase and glucose 6-phosphatase inhibitions), glucose uptake bioassays (using cell lines such as C2C12 myocytes, 3T3-L1 pre-adipocytes and human Chang liver cells) and stimulation of insulin release.
which is usually conducted in perfused pancreas, isolated pancreatic islets cells or clonal pancreatic β-cell-lines (van de Venter et al. 2008; Bhandari et al. 2008; Hannan et al. 2007).

1.3.5 Bioassay-guided fractionation of the active principles

Bioassay-guided fractionation is an important method employed to identify the plant derived bioactive compounds. It involves repetitive fractionation (column chromatography and thin layer chromatography) and evaluation of bioactivity of the fractions up to the isolation of pure target principles with the selected biological activity (Gurib-Fakim, 2006). As soon as the isolation of a pure compound is achieved, the next step is the structural elucidation of the compound isolated which requires the use of Nuclear Magnetic Resonance Spectroscopy (NMR) and Mass Spectroscopy (MS) (Verpoorte, 1989).

1.3.6 In vivo, clinical and toxicological studies

In in vivo approach, animals are used to investigate the efficacy, mode of action and side effects of the plant extracts, fractions and their active principles (Eddouks et al. 2012). In vivo screening is usually conducted based on the results of the in vitro study and its require a large scale production and/or extraction of the target compound from the extract. In some cases structural modification is employed to ascertain or improve the bioactivity of the target substance (Rates, 2001). This approach otherwise called pre-clinical investigation can take longer time and demand a lot of resources.

Furthermore, in some diseases such as DM, several models are employed in order to establish the potency of the target compounds, due to the complex heterogeneity of human body system in disease conditions. These include chemically induced animal models such as alloxan and streptozotocin-induced diabetic animals that are mostly used for the induction of T1D (Wang et al. 2009). The animal models of T2D include genetically induced or spontaneous (e.g. Zucker diabetic fatty model) and experimentally induced or nonspontaneous (e.g. high-fats diet-fed and fructose-fed/streptozotocin) models (Eddouks et al. 2012; Islam and Loots, 2009). Due to the easy induction of diabetes, lower maintenance cost and wider availability the experimentally induced model has been recommended as a better option over genetically induced spontaneous model, particularly for the researchers and scientists from the developing countries (Eddouks et al. 2012; Islam and Loots, 2009). Additionally, if the target drug candidate was observed to cause some adverse effects in the animal model, structural modification of the compound is carried out to reduce the severity of the consequences (Lin and Lu, 1997). Preliminary toxicological effects of the plant derived compounds are conducted both in vitro (cell line) and at in vivo level to ascertain their safety for clinical use.

Clinical trials are usually carried out to confirm the safety of potential bioactive compounds in human subjects. Before the commencement of such investigation, a careful protocol has to be prepared due to a strict ethical procedures involved in using humans as subjects in various studies. For instance,
in USA or Europe, an investigational new drug application is usually applied to the relevant authorities such as Food and Drug Administration (FDA) or European Medicines Agency (EMA) (Mishra and Tiwari, 2011). Once the investigation is successful, the candidate drug is now ready for marketing and will subsequently be available to the general people.

1.3.7 Medicinal plants for the treatment of diabetes

The practice of using plants to treat DM by humans has been employed by different cultural settings worldwide since immemorial time (Gurib-Fakim, 2006; Bnouham et al. 2006; Samuelsson, 2004). With the recent escalation of the prevalence of DM, high cost and inaccessibility to modern drugs, the popularity of using medicinal plants derived medicines to treat DM has been increased especially in the developing countries (Rachid et al. 2012). On the other side, reports available have also showed a dramatic increase on the use of plant-derived formulations to manage DM in developed countries (Achaya and Shrivastava, 2008; Gilani, 2005; Cordell and Colvard, 2005). This increase has been attributed due to the undesirable adverse effects of synthetic anti-diabetic drugs (Shu, 1998).

Recently, Hung et al. (2012) highlighted that during 2005 to 2010; about 100 plant-derived preparations have been investigated for possible anti-diabetic actions. Interestingly, some of these preparations are now formulated and available in the market as anti-diabetic remedy. Additionally, 85 bioactive compounds have demonstrated anti-diabetic potentials within the same periods of time. In a similar study reported earlier, about 106 plant-derived compounds were reported to show possible anti-diabetic effects in various studies published within the period of 2000-2005 (Jung et al. 2006). However, the exert mode of actions of plant-derived formulations and/or compounds used in diabetes treatment is still remain elusive and speculative. Some of the mechanisms proposed include insulin-like action (Broadhurst et al. 2000), inhibition of carbohydrate digestive enzymes (Bhandari et al. 2008), decreasing the absorption of glucose at the gut (Gallagher et al. 2003), stimulation of hepatic and muscle glucose uptake (Eid et al. 2010), anti-oxidative effects in preventing β-cell damage (Sef et al. 2011), complement the action of insulin (Donga et al. 2011).

1.3.8 African medicinal plants with anti-diabetic potentials

African region is among the regions endowed with the richest biodiversity in the world, with an abundance of plants used for therapeutic purposes. According to the World Health Organization (WHO) report, more than 80% of the population in Africa depends almost entirely on plant-derived preparations to treat various types of diseases (Zhang, 2001). However, many of these plants still await proper scientific investigation.

Similarly, the undesirable adverse effects and high cost of conventional oral synthetic drugs coupled with an exponential increase in the prevalence of DM motivate researchers to scientifically
validate the folkloric use of a number of African medicinal plants as possible alternative therapies. The anti-diabetic potentials of a number of these medicinal plants have been reported and some bioactive compounds were isolated and screened as well. In review, we have reported that a total number of 185 plants species from 75 families were reported to be investigated for anti-diabetic effects in Africa during 2000 to 2013 (Mohammed et al. 2014). Plants such as *Anacardium occidentale* L. (Anacardiaceae), *Gongronema latifolium* Benth (Asclepiadaceae), *Sclerocarya birrea* (A. Rich) Hochst. (Anacardiaceae), *Sutherlandia frutescens* (L.) R.Br. (Fabaceae), *Trigonella foenum graecum* L. (Leguminosae) *Vernonia amygdalina* Del. (Asteraceae) and *Zizyphus spina-christi* (L.) Desf (Rhamnaceae) are some of the African medicinal plants that received much attention due to the content of possible anti-diabetic agents (Table 1.1).

### Table 1.1: Most studied anti-diabetic medicinal plants in the African continent.

<table>
<thead>
<tr>
<th>Scientific name</th>
<th>Part used (s)</th>
<th>Phytochemicals</th>
<th>Efficacy</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Anacardium occidentale</em> L.</td>
<td>Stem bark</td>
<td>Polyphenols</td>
<td>• Stimulated muscles glucose uptake</td>
<td>Tedong et al. 2010</td>
</tr>
<tr>
<td></td>
<td>L.</td>
<td></td>
<td>• Antihyperglycemic in type 1 &amp; 2 diabetic animals</td>
<td>Abdullahi and Olatunji, 2010</td>
</tr>
<tr>
<td><em>Gongronema latifolium</em> Benth.</td>
<td>Leaf/ Stem</td>
<td>α- and β-Amyrin Cinnamates, Lupenyl cinnamates, Lupenyl acetate, Triterpenoids</td>
<td>• Mimic insulin action</td>
<td>Adebajo et al. 2013</td>
</tr>
<tr>
<td>(Asclepiadaceae)</td>
<td>bark</td>
<td></td>
<td>• Decreased blood glucose level in diabetic animals/ Anti-oxidative</td>
<td>Akah et al. 2011</td>
</tr>
<tr>
<td><em>Sclerocarya birrea</em> (A. Rich)</td>
<td>Stem bark</td>
<td>(−)-Epicatechin-3-galloyl ester</td>
<td>• Stimulated insulin release</td>
<td>Galvez et al. 1992</td>
</tr>
<tr>
<td>Hochst. (Anacardiaceae)</td>
<td></td>
<td></td>
<td>• Enhanced glucose-stimulated insulin secretion in INS-1E cells</td>
<td>Ndifossap et al. 2005</td>
</tr>
<tr>
<td><em>Sutherlandia frutescens</em> (L.)</td>
<td>Leaf/ Shoot/ Whole plant</td>
<td>Flavonoid</td>
<td>• Decreased blood glucose level in diabetic animals</td>
<td>Ojewole, 2004</td>
</tr>
<tr>
<td><em>Trigonella foenum graecum</em> L.</td>
<td>Seed</td>
<td>Diosgenin</td>
<td>• Inhibited α-glucosidase action</td>
<td>Gad et al. 2006</td>
</tr>
</tbody>
</table>
**Vernonia amygdalina** Leaf Sesquiterpene lactones • Improved postprandial glucose utilization in human subjects Okolie et al. 2008

**Ziziphus spina-christi** Leaf Chritinin-A • Decreased blood glucose level in diabetic animals Atangwo et al. 2007

• Stimulated insulin release Abdel-Zahera et al. 2005

### 1.3.9 Bioactive compounds from African medicinal plants with anti-diabetic potentials

The major target of screening medicinal plants as anti-diabetic agent is to isolate pure bioactive compounds that could be responsible for the observed activities of the plant under study. Such compounds can be further standardized and used as alternative anti-diabetic drugs. In Africa, just like other continents, some bioactive compounds were isolated and screened as possible anti-diabetic agents. In our current search, a total of 53 compounds isolated from African medicinal plants which were reported to be investigated as possible anti-diabetic agents include 3 phenolic compounds from marine algae *Ecklonia maxima* (Osbeck) Papenfuss. Interestingly, 45 compounds were found to be active in the models used in the reported studies (Table 1.2). However, numerous other plants and plant-based natural products from the African continent are still need to be evaluated scientifically to identify better alternative of existing anti-diabetic medicines or natural products. Some of these plants are included in this study which is described below.
<table>
<thead>
<tr>
<th>Type</th>
<th>Compounds</th>
<th>Plant species</th>
<th>Families</th>
<th>Parts/Dose</th>
<th>Bioactivity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaloids</td>
<td>Akuammicine</td>
<td><em>Picralima nitida</em></td>
<td>Apocynaceae</td>
<td>Seed (10 µg/ml)</td>
<td>Stimulate glucose uptake in fully differentiated 3T3-L1 adipocytes (200% basal)</td>
<td>Shittu et al. 2010</td>
</tr>
<tr>
<td></td>
<td>3-Formylcarbazole</td>
<td><em>Clausena lansium</em></td>
<td>Rutaceae</td>
<td>Stem bark DCM extract (0.1 mg/ml)</td>
<td>Inactive to stimulate insulin secretion in INS-1 cell</td>
<td>Adebajo et al. 2009</td>
</tr>
<tr>
<td>Coumarins</td>
<td>Imperatorin</td>
<td><em>Clausena lansium</em></td>
<td>Rutaceae</td>
<td>Stem bark DCM extract (0.1 mg/ml)</td>
<td>Insulin secretion in INS-1 cell</td>
<td>Adebajo et al. 2009</td>
</tr>
<tr>
<td></td>
<td>Chalepin</td>
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<tr>
<td>Flavonoids/Phenolics</td>
<td>Azaleatin</td>
<td><em>Carya illinoiensis</em></td>
<td>Juglandaceae</td>
<td>Bark (10 mg/kg bw) (i.p.)</td>
<td>Anti-hyperglycemic, Anti-oxidative</td>
<td>Abdallah et al. 2011</td>
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<tr>
<td></td>
<td>Caryatin-3'methyl ether</td>
<td></td>
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<tr>
<td></td>
<td>Caryatin-3'methyl ether-7-O-β-D-glucoside</td>
<td>Protocatechuic acid</td>
<td></td>
<td></td>
<td>Anti-hyperglycemic, Inhibit aldose reductase, Anti-oxidant</td>
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<tr>
<td></td>
<td>Protocatechuic acid</td>
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<tr>
<td></td>
<td>Isorhamnetin-3-O-β-D-glucoside</td>
<td>Quercetin</td>
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<tr>
<td></td>
<td>Kaempferol-4'-methoxy-3,7-dirhamnoside</td>
<td><em>Cleome drosferifolia</em> (Forssk.) Del.</td>
<td>Cleomaceae</td>
<td>Aerial part (50 µM)</td>
<td>Increase glucose uptake in C2C12 skeletal muscle cells and 3T3-L1 adipocyte</td>
<td>Abdel Motaal et al. 2011</td>
</tr>
<tr>
<td>Compound</td>
<td>Species</td>
<td>Family</td>
<td>Part</td>
<td>Action</td>
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<tr>
<td>Kolaviron</td>
<td><em>Garcinia kola</em></td>
<td>Clusiaceae</td>
<td>Seed (100 mg/kg bw)</td>
<td>Inhibited rat lens aldose reductase activity ($IC_{50}$: 5.4 µM)</td>
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<tr>
<td>Epicatechin</td>
<td><em>Euclea undulata</em></td>
<td>Ebenaceae</td>
<td>Root bark (200 µg ml⁻¹)</td>
<td>Anti-hyperglycemic, Anti-oxidative Inhibition of $\alpha$-glucosidase ($IC_{50}$: 5.86 ± 4.28 µg/ml) Increase glucose uptake C2C12 myocytes</td>
<td></td>
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<tr>
<td>Dibenzo [1,4] dioxine-2,4,7,9-tetraol</td>
<td><em>Ecklonia maxima</em></td>
<td>Laminariaceae</td>
<td>Brown alga</td>
<td>Inhibition of $\alpha$-glucosidase ($IC_{50}$: 33.693 ± 0.61 µM); DPPH ($EC_{50}$: 0.012 ± 0.001 µM)</td>
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<tr>
<td>Hexahydroxyphenoxydibenzodioxine</td>
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<td>1,3,5-Trihydroxybenezene</td>
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<tr>
<td>Vanillic acid</td>
<td><em>Fagara tesselmannii</em></td>
<td>Rutaceae</td>
<td>Stem bark (800 µM)</td>
<td>Inhibition of $\alpha$-glucosidase ($IC_{50}$: 69.4 ± 0.8 µM)</td>
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</tr>
<tr>
<td>Brevipsidone A</td>
<td><em>Garcinia brevipedicellata</em></td>
<td>Clusiaceae</td>
<td>Leaf</td>
<td>Inhibition of $\alpha$-glucosidase ($IC_{50}$: 21.22 ± 0.45 µM)</td>
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<tr>
<td>Brevipsidone B</td>
<td></td>
<td></td>
<td></td>
<td>Inhibition of $\alpha$-glucosidase ($IC_{50}$: 27.80 ± 0.76 µM)</td>
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<tr>
<td>Compounds</td>
<td>Inhibitor</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; (μM)</td>
<td>Source</td>
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<tr>
<td>Brevipsidone C</td>
<td></td>
<td>59.67 ± 0.41</td>
<td>Wansi et al., 2007</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Brevipsidone D</td>
<td></td>
<td>7.04 ± 0.26</td>
<td></td>
<td></td>
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<tr>
<td>Gallic acid</td>
<td><em>Terminalia superba</em></td>
<td>5.2 ± 0.2</td>
<td>Wansi et al., 2007</td>
<td></td>
<td></td>
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<tr>
<td>Methyl gallate</td>
<td></td>
<td>11.5 ± 0.1</td>
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<tr>
<td>Tannins Ellagic acid</td>
<td><em>Terminalia superba</em></td>
<td>194.1 ± 0.2</td>
<td>Tabopda et al., 2008</td>
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</tr>
<tr>
<td>Tannins Ellagic acid 3,3'-dimethyl ether</td>
<td></td>
<td>184.6 ± 0.9</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Tannins Ellagic acid 3,3'-dimethyl ether 3,4'-Di-O-methylellagic acid 3'-O-β-D-xylopyranoside 4'-Galloy-3,3'-di-O-methylellagic acid 4-O-β-D-xylopyranoside</td>
<td></td>
<td>118.7 ± 0.1</td>
<td>Wansi et al., 2007</td>
<td></td>
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<tr>
<td>Tannins Ellagic acid 3,3'-dimethyl ether</td>
<td></td>
<td>7.95 ± 0.33</td>
<td>Tabopda et al., 2008</td>
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<tr>
<td>Tannins Ellagic acid 3,3'-dimethyl ether 3,4'-Di-O-methylellagic acid 3'-O-β-D-xylopyranoside 4'-Galloy-3,3'-di-O-methylellagic acid 4-O-β-D-xylopyranoside</td>
<td></td>
<td>21.21 ± 0.69</td>
<td>Tabopda et al., 2008</td>
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<tr>
<td>Terpenoids Oleanolic acid</td>
<td><em>Olea europaea</em> subsp. africana (Mill.) P.S.Green</td>
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<tr>
<td>Terpenoids Oleanolic acid</td>
<td>Syzygium aromaticum</td>
<td></td>
<td>Hypoglycemic, Anti-hyperlipidemic, Anti-hypertensive, Anti-oxidant</td>
<td>Somova et al., 2003a</td>
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<tr>
<td>Plant Name</td>
<td>Family</td>
<td>Plant Part</td>
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<tr>
<td>Ursolic acid Olea europaea subsp. africana</td>
<td>Oleaceae</td>
<td>Leaf (100 mg/kg bw) (p.o.)</td>
<td>Anti-hyperglycemic, Inhibit aldose reductase, Anti-oxidant</td>
<td>Merr. &amp; L.M. Perry 2003b</td>
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<tr>
<td>Thymus vulgaris L.</td>
<td>Lamiaceae</td>
<td>Leaf (100 mg/kg bw) (p.o.)</td>
<td>Hypoglycemic, Anti-hyperlipidemic, Cardioprotective, Anti-oxidant</td>
<td>P.S.Green Somova et al. 2003b</td>
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<tr>
<td>Christinin-A Zizyphus spinachristi (L.) Desf.</td>
<td>Rhamnaceae</td>
<td>Leaf (100 mg/kg bw) (p.o.)</td>
<td>Anti-hyperglycemic, Insulinotropic</td>
<td>Abdel-Zahera et al. 2005</td>
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<tr>
<td>Foetidin Momordica foetida Schumach.</td>
<td>Cucurbitaceae</td>
<td>Fruit (1 mg/kg bw)</td>
<td>Anti-hyperglycemic</td>
<td>Marquis et al. 1977</td>
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<tr>
<td>α-Amyrin-3-O-β-(5-hydroxy) ferulic acid</td>
<td>Ebenaceae</td>
<td>Root bark (200 µg/ml)</td>
<td>Inhibition of α-glucosidase (IC₅₀: 4.79 ± 2.54 µg/ml)</td>
<td>Deutschländer et al. 2011</td>
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<td>Lupeol</td>
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<td></td>
<td>Inhibition of α-glucosidase (IC₅₀: 6.27 ± 4.75 µg/ml)</td>
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<td>Betulin</td>
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<td></td>
<td>Inhibition of α-glucosidase (IC₅₀: 32.04 ± 2.79 µg/ml)</td>
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<tr>
<td>3β-Acetoxy-16β-hydroxy betulinic acid</td>
<td>Rutaceae</td>
<td>Stem bark (800 µM)</td>
<td>Inhibition of α-glucosidase (IC₅₀: 7.6 ± 0.6 µM)</td>
<td>Mbaze et al. 2007</td>
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<tr>
<td>(24 E) Stigmasta-5,8-dien-3β-Ol Teucladiol</td>
<td>Cleomaceae</td>
<td>Aerial part (50 µM)</td>
<td>Increase glucose uptake in C2C12 skeletal muscle cells and 3T3-L1 adipocyte</td>
<td>Abdel Motaal et al. 2011</td>
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<td>1-Hydroxy-guai-3,10 (14)-diene</td>
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<td>Compound</td>
<td>Family</td>
<td>Part of Plant</td>
<td>Concentration</td>
<td>Effect</td>
<td>Reference</td>
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<tr>
<td>18-Hydroxy-dollabela-8 (17)-ene Guai-7(11), 8-diene Buchariol</td>
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<tr>
<td>2,6-Dimethoxy-1,4-benzoquinone</td>
<td>Rutaceae</td>
<td>Stem bark (800 μM)</td>
<td>Inhibition of α-glucosidase (IC_{50}: 900.0 ± 3.5 μM)</td>
<td>Mbaze et al. 2007</td>
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<tr>
<td>D-3-O-Methylchiroinositol</td>
<td>Fabaceae</td>
<td>Stem bark (2-8 mg/kg bw) (p.o.)</td>
<td>Anti-hyperglycemic, Anti-hyperlipidemic</td>
<td>Asuzu and Nwaehujor, 2013</td>
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</table>
1.4 Aframomum melegueta K. Schum.

Figure 1.7: Aframomum melegueta plant, whole plant (A), fruits (B).

1.4.1 Description

Aframomum melegueta K. Schum. commonly known as alligator pepper, grains of paradise or guinea pepper belongs to Zingiberaceae family (Iwu, 2014). It is a perennial herbaceous plant with aromatic spicy fruits and grows up to 4 m height. The fruits are indehiscent with many tiny seeds integrated in tasting axils. The seeds are strongly aromatic and pungent, usually golden or red-brown in color when fresh but darken on drying. The flowers are pinkish-orange and develop separately from the leafy stem. The leaves are large, parallel pinnate nerves and appear with open or closed sheath. It has short, branched and leafy stem with adventitious root located at the lower end. It also possessed tuberous rhizome with scaly leaves and occasional buds in axils (Figure 1.7) (Iwu, 2014; Irvine, 1961).

1.4.2 Distribution

A. melegueta is an indigenous spice widely available throughout the tropical Africa, but mostly cultivated in West and Central African regions (Iwu, 2014).

1.4.3 Phytochemistry

Many of the phytochemical investigations reported on A. melegueta were carried out on the seed part, with very scanty or no information on the other parts (fruit, leaf and stem) of this plant. Available studies indicated that, A. melegueta seed aqueous extract is rich in phenolic compounds and minerals (Adefegha and Oboh, 2012a; Gbadamosi et al. 2011; Okwu, 2005). It was also reported that gingerols, shogaols and paradols as are the active constituents of A. melegueta seed (Tackie et al. 1975; Connelland McLachlan, 1972; Connell, 1970). Other compounds isolated from this plant include gingerdione, 5-oxo-l-(4-hydroxy-3-methoxyphenyl) decan-3-one, [6]-paradol, 1-(4-hydroxy-3-methoxyphenyl) decan-3-one, [6]-gingerol, 5-hydroxy-l-(4-hydroxy-3-methoxyphenyl) decan-3-one
and [6]-shogaol, 1-(4-hydroxy-3-methoxyphenyl) dec-5-en-3-one (Ilic et al. 2014; El-Halawany et al. 2014; Escoubas et al. 1995). The oil derived from the seed was reported to contain volatile compounds such as α-humulene, β-caryophyllene, δ-cadinene and dibutyl phthalate (Markson et al. 2011; Ukeh et al. 2009; Ajaiyeoba and Ekundayo, 1999; Menut et al. 1991). Similarly, labdane dieterpenoids was recently isolated from the organic extracts of *A. melegueta* rhizome (Ngwoke et al. 2014). In our most recent study, the fruit and leaf ethanolic extracts were found to have eugenol, gingerol, capsaicin, 3-decanone, 1-(4-hydroxy-3-methoxyphenyl), ethyl homovallinate, oleic acid and other fatty acid derivatives (Mohammed et. al. 2015).

### 1.4.4 Ethnobotanical uses

*A. melegueta* has been widely used in food preparation as a spice and also locally in the treatment of various ailments. Various seed decoctions are used traditionally against bacterial infections, diarrhea, abdominal pain, snakebite and wounds healing in most parts of Africa (Akendengue and Louis, 1994; Kokwaro, 1993). Similarly, fruit, seed and leaf were also reported to be used in the treatment of DM and other metabolic diseases in Nigeria (Soladoye et al. 2012; Gbolade, 2012; 2009). It was also reported that leaf and stem of *A. melegueta* are used for wounds and bacterial infections (Akendengue and Louis, 1994). Additionally, the root has been a good remedy against snakebite and dysentery (Kokwaro, 1993).

### 1.4.5 Pharmacological importance

The *A. melegueta* seed extracts have been reported to demonstrate broad anti-microbial activities (Nneka and Jude, 2013; Doherty et al. 2010). Doherty et al. (2010) reported that seed ethanolic extract (5-50 mg/mL) exhibited higher anti-microbial (*Salmonella* spp, *Escherichia coli*, *Shigella* spp and *klebsiella* spp) action compared to the aqueous extract. In another study, the seed methanolic extract (5%-30%) has also shown anti-fungal activity against *Helminthosporium solani*, *Aspergillus niger*, *Penicillium digitatum* and *Mucor piriformis* (Nneka and Jude, 2013). In a more recent study, the dieterpenoids isolated from the organic extracts (0.97-250 mg/mL) of *A. melegueta* rhizome showed bactericidal activity in vitro against *Escherichia coli*, *Listeria moncyogenes* and *Staphylococcus aureus* strains (Ngwoke et al. 2014). The seed diethyl ether extracts (0.6 mg/mL) was reported to exhibit potent insect repellant activity against the maize weevil, *Sitophilus zeamais* (Ukeh et al. 2009).

Furthermore, the various extracts from *A. melegueta* seed showed anti-oxidative and potent inhibitory effects against α-glucosidase activity in vitro (Adefegha and Oboh, 2012a; Kazeem et al. 2012; Adefegha and Oboh, 2011; Etoundi et al. 2010). In another study by Adefegha and Oboh (2012a), the seed aqueous extract (0-3 mg/mL) demonstrated moderate radical scavenging ability (IC$_{50}$: 17.38 mg/mL) and the inhibition of the carbohydrate hydrolyzing enzymes (IC$_{50}$ values: α-glucosidase: 2.14
mg/mL; \( \alpha \)-amylase: 4.83 mg/mL). The same extract at the concentration of 0.2 g/mL was also reported to inhibit the \( \alpha \)-amylase (30.61\%) and lipase (8.91\%) activities in vitro (Etoundi et al. 2010). It was also reported previously that the seed aqueous extract (0-3 mg/mL) attenuated the \( \text{Fe}^{2+} \)-induced lipid peroxidation in normal rat’s brain (Adefegha and Oboh, 2011). Kazeem et al. (2012) have reported that seed polyphenolic-rich extract (0.1-1 mg/ml) exhibited good anti-oxidant action (IC\(_{50}\) values: DPPH: 0.11 mg/mL; anti-glycation: 0.125 mg/mL; Superoxide anion: 0.105 mg/mL). In a more recent study, treatment of seed ethanolic extract (100-400 mg/kg bw) daily for 4 weeks was reported to improve in vivo anti-oxidative status in normal rats (Onoja et al. 2014).

Similarly, the seed aqueous extract (4.8 g/kg bw/day) demonstrated hepato-protective effect in ethanol-induced toxic animals after 16 days oral intervention (Nwozo and Oyinloye, 2011). The seed ethanolic extract (0.5-1 g/kg bw) including paradol, gingerol, and shogaol isolated from the seed (0.15 g/kg bw) have showed anti-inflammatory activities in a rats paw edema model after 3 hour post-administration period (Ilic et al. 2014). Umukoro and Ashorobi (2008) had earlier reported that treatment of aqueous extract (50-200 mg/kg bw) daily for 4 days exhibited anti-inflammatory action in rats. Some previous findings have showed the potential of the seed extracts in ameliorating problems associated with reproductive function. Kamtchouing et al. (2002) have shown that treatment of aqueous extract (115 mg/kg bw/day) improved penile erection in rats. Subsequently, after 55 days treatment, the extract at the same dose increased the secretions of epididymis and seminal vesicle in animals (Mbongue et al. 2012). In another study, seed aqueous extract (25-200 mg/kg bw) showed anti-stress as well as anti-nociceptive potentials in mice after 30 minutes post-treatment period (Umukoro and Ashorobi, 2007; 2005).

Sugita et al. (2013) have recently reported that daily administration of \textit{A. melegueta} capsule (10 mg) for 4 weeks activates brown adipose tissue and increases whole-body energy expenditure in healthy human subjects. In a preliminary study, Adesokan et al. (2010) reported that daily treatment of \textit{A. melegueta} seed aqueous extract (200, 400 mg/kg bw) demonstrated blood glucose lowering ability in alloxan-induced diabetic rats. Similarly, the leaf extract (50-200 mg/kg bw) was also reported to cause reduction of blood glucose level in alloxanized animals after 5 day post-treatment period (Mojekwu et al. 2011). Additionally, the seed ethanolic extract was reported safe up to a dose of 300 mg/kg bw (Akpanabiatu et al. 2013; Ilic et al. 2010). In another study, paradol, gingerol, and shogaol isolated from the seed have showed anti-inflammatory activity in a rats paw edema model (Ilic et al. 2014). El-Halawany et al. (2014) have also reported that diarylheptanoid, 8-dehydrogingerdione, 6-dehydroparadol, dihydrogingerenone, 6-gingerol, dihydroparadol, paradol and 6-shogaol demonstrated hepato-protective and anti-oxidative actions in CCl\(_4\) induced acute liver injury in rats.
1.5 *Xylopia aethiopica* (Dunal) A. Rich

![Image of Xylopia aethiopica](image)

**Figure 1.8:** *Xylopia aethiopica* whole plant (A), fruits (B).

### 1.5.1 Description

*Xylopia aethiopica* (Dunal) A. Rich (Anonaceae) also known as Ethiopian pepper or African pepper is an evergreen plant, 20 m height, the peppery fruits are in carpels, forming dense cluster, twisted bean-like pods, dark brown, cylindrical, 1.5-6 cm long and 4-7 mm thick. It has short roots and smooth grey bark and usually scented when fresh. The leaves are broad (5-15 cm long and 2.5-6 cm wide), while flowers are greenish-white, usually in clusters of about 3-5 (Iwu, 2014; Orwa et al. 2009; Burkill, 1985).

### 1.5.2 Distribution

*Xylopia aethiopica* is an African indigenous spices located in savannah rain forest and widely distributed in West, Central and Southern parts of Africa (Iwu, 2014).

### 1.5.3 Phytochemistry

Active principles of *X. aethiopica* fruit include xylopic acid (a kaurene diterpene) (Biney et al. 2014; Woode et al. 2012), kaurenoic, 15-oxo-kaurenoic acid, and kauran-16-α-ol (Iwu, 2014; Ekong and Ogan, 1968). The oils derived from the fruit were reported to contain α- and β-pipenes, careen, cymene, α-phellandrene, limonene, terpinolene, cineole, bisabolone, linoolool, terpinen-4-ol, terpineal, cuminyl alcohol, and cumminicaldyhyde (Harrigan et al. 1994).

### 1.5.4 Ethnobotanical uses

The fruit is popularly used as condiment in many local dishes by different traditions in Africa and Asia (Kingsley, 2012). In addition, *X. aethiopica* fruit is widely used locally in treatment of various
ailments and also as an excipient to many other medicines as well (Freiesleben et al. 2015; Fall et al. 2003). In Nigerian, Guinean, Togolese and Senegalese traditional medicines, the fruit decoction is widely used in the treatment of diabetes (Soladoye et al. 2012; Diallo et al. 2012; Karou et al. 2011; Diéye et al. 2008). The fruit and/or stem bark are also used as a pain reliever and in the treatment of stomach aches, dysentery, ulceration, skin infections and coughs (Ezeikwesili et al. 2010). The seed is employed by women after childbirth to promote healing, lactation and/or promote fertility (Iwu, 2014). The leaf or root decoction is used as tonic and in the treatment of constipation (Ndukwu and Ben-Nwadibia, 2005).

1.5.5 Pharmacological importance

The *X. aethiopica* fruit methanolic extract (20 µg/mL) was reported to inhibit more than 50% of the proliferation of the tested cancer cells; human pancreatic cancer cell line MiaPaCa-2, leukemia CCRF-CEM cells and their multidrug resistant (MDR) subline CEM/ADR5000, and the normal human umbilical vein endothelial cells (HUVECs) (Kuete et al. 2013; 2011). Additionally, Choumessi et al. (2012) have reported that the fruit ethanolic extract (100 mg/mL) demonstrated anti-proliferative activity against HCT116 colon cancer cells, U937 and KG1a leukemia cells.

In another study, fruit aqueous extract (100 mg/mL) exhibited moderate anti-microbial activity against *Escherichia coli* and *Staphylococcus aureus* (Esekhiagbe et al. 2009). Tekwu et al. (2012) have reported that the fruit methanolic extract (0.002-2 mg/mL) showed anti-mycobacterial activity against *Mycobacterium tuberculosis* strains (H$_{37}$Rv (ATCC27294) and H$_{37}$Ra (ATCC25177)).

Furthermore, various extracts from *X. aethiopica* fruit have shown good anti-oxidative action *in vitro* in a number of previously reported studies. The fruit aqueous and methanolic extracts (0-3 mg/mL) have demonstrated excellent radical scavenging ability (Adefegha and Oboh, 2012a,b; 2011; George and Osima, 2011; Etoundi et al. 2010; Odukoya et al. 2005). In addition, dietary inclusion of *X. aethiopica* fruit (2% and 4%) for 2 weeks improved the *in vivo* anti-oxidant status of normal rats (Adefegha and Oboh, 2012b). Moreover, fruit aqueous extract (0-3 mg/mL) demonstrated moderate inhibition of the carbohydrate hydrolyzing enzymes (IC$_{50}$ values: $\alpha$-glucosidase: 2.57 mg/mL; $\alpha$-amylase: 2.81 mg/mL) (Adefegha and Oboh, 2012a; Etoundi et al. 2010). In another study, fruit ethanolic extract (30-300 mg/kg bw/day) showed anti-anaphylactic and anti-inflammatory actions in mice after 7 days treatment period (Obiri and Osafo, 2013). Furthermore, oral treatment of fruit ethanolic extract (100-400 mg/kg bw) for 2 weeks did not cause any toxicological effect in the treated animals (Johnkennedy et al. 2011; Taiwo et al. 2009).

More recently, Etoundi et al. (2013) have reported that the blood glucose tolerance ability after 2 hour post-administration period was improved in high-sucrose fed rats treated with 400 mg/kg bw of fruit ethanolic extract. Additionally, no significant anti-hyperlipidemic action was observed after 2-day post-treatment period of the extract in hyperlipidemic rats (Etoundi et al. 2013). Conversely, in another
study, administration of *X. aethiopica* aqueous extract (250 mg/kg bw) for 8 weeks demonstrated potent anti-hyperlipidemic effect in hypercholesterolemic rats (Nwozo et al. 2011). Nwangwa (2012) has reported that the fruit ethanolic extract (750 mg/kg bw/day) treatment for 4 weeks demonstrated decrease in the semen count and motility, indicating anti-fertility action. Furthermore, the aqueous, ethanol and methanol extracts of fruit (10-100 mg/kg bw) exhibited anti-helminthic potential after 30 minute post-treatment period (Ekeanyanwu and Etienjirhevwe, 2012).

1.6 *Capsicum annuum* L.

Figure 1.9: *Capsicum annuum* whole plant (A), fruits (B).

1.6.1 Description

*Capsicum annuum* L. (*Solanaceae*) popularly known as red pepper is a spice that is readily available worldwide. The fruit is widely consumed in vast quantities as flavoring agent in various traditional foods. The plant is an annual or biennial of about 1 m in height. The green leaves are oval in shape while flowers are greenish-white and borne on axillary bunches. Fruit are usually globular, ovoid, or oblong in shape; green colored and yellow to red when ripe (Griffiths, 1959).

1.6.2 Distribution

The plant is widely distributed in almost all part of the world, from tropical Africa to Europe, Asia, South America and Middle East, with over 30 different varieties (Iwu, 2014).

1.6.3 Phytochemistry

*C. annuum* fruit has been consistently reported to be the rich source of phenolics, vitamins and carotenoids (Hernández-Ortega et al. 2012; Herver-Hernández et al. 2010; Zimmer et al. 2012; Park et al. 2010). The active phytochemicals isolated from the fruit extracts include capsaicin, dihydrocapsaincin, carotenoids, vitamin A, C, and E, feruloyl O-glucosides, kaempferol O-
pentosylhexoses, dihydroxyflavone O-hexoses and quercetin-3-O-L-rhamnoside (Santos et al. 2012; Hervert-Hernández et al. 2010; Materska and Perucka, 2005). In another study, capsiate, dihydrocapsiate and vanillyl alcohol were also isolated from C. annuum fruit (Kobata et al. 1998). Furthermore, some amide derivative compounds were also isolated from the stem of C. annuum (Chen et al. 2011). These compounds include N-p-trans-coumaroyl-tyramine, N-trans-cafeoyltyramine, β-sitostenone, ferulic acid, hydroferulic acid, 5-hydroxy-3,4-dimethoxycinamic acid, vatic acid, vanillic acid, isovanillic acid, syringic acid, (+)-syringaresinol, pheophorbide, 7'(4'-hydroxyphenyl)-N-[(4-methoxyphenyl) ethyl] propanamide, 7'(3',4'-dihydroxyphenyl)-N-[(4-methoxyphenyl) ethyl] propanamide (Chen et al. 2011).

1.6.4 Ethnobotanical uses

The fruit is locally used as a blood tonic, stimulant and in the treatment of nerve weakness due to diabetes in Africa and Asia (Iwu, 2014; Jayakumar et al. 2010). In most part of Africa, the fruit is used in the treatment of skin infections and other bacterial infections (Iwu, 2014). Additionally, the fruit and seed are also used in the remedy of cold, fever, dysentery, malaria and gonorrhea (Ndukwu and Ben-Nwadibia, 2005).

1.6.5 Pharmacological importance

The radical scavenging ability of C. annuum fruit has been well documented (Tundis et al. 2013; Tundis et al. 2012; Chen et al. 2012; Ghasemnezhad et al. 2011; Kim et al. 2011; Kumar et al. 2010; Deepa et al. 2007; Kwon et al. 2007; Perucka and Materska, 2001). In these studies, the aqueous and ethanolic extracts (0.01-1 mg/mL) have demonstrated excellent electron quenching ability (IC$_{50}$: 0.1-0.5 mg/mL). Kim et al. (2011) have also reported the potent anti-oxidative actions (DPPH: IC$_{50}$: 0.2-3 mg/mL; ABTS: 0.05-0.3 mg/mL) of C. annuum fruit and leaf ethanolic extracts (0.01 mg/mL) in vitro. Additionally, the fruit aqueous extract (3.3-16.7 mg/mL) was reported to prevent Fe$^{3+}$-induced lipid peroxidation in rat’s brain homogenate in vitro (Oboh et al. 2007).

In some other studies, C. annuum fruit ethanolic extract (0.005-1 mg/mL) was reported to inhibit the activities of α-amylase (IC$_{50}$: 0.05-0.5 mg/mL) and α-glucosidase (IC$_{50}$: 0.1-1 mg/mL) in vitro in addition to its anti-oxidative actions (Tundis et al. 2013; Kwon et al. 2007). Capsaicin and dihydrocapsaicin (0.005-1 mg/mL) have also demonstrated α-amylase (IC$_{50}$: capsaicin: 0.083 mg/mL; dihydrocapsaicin: 0.092 mg/mL) and α-glucosidase (IC$_{50}$: capsaicin > 0.5 mg/mL; dihydrocapsaicin > 0.5 mg/mL) inhibitory action (Tundis et al. 2013). Moreover, the volatile oil (0.3-7.8%) derived from C. annuum fruit has shown anti-parasitic effect against Anopheles gambiae. (Dadji et al. 2011). In another study, fruit ethanolic extract (0.004-0.3%) was also exhibited potent larvicidal efficacy against Anopheles stephensi and Culex quinquefasciatus (Madhumathy et al. 2007).
Furthermore, fruit ethanolic extract (0.1 mg/mL) showed anti-nociceptive and anti-inflammatory actions in mice (Hernández-Ortega et al. 2012). Cichewicz and Thorpe (1996) have earlier reported that the fruit juice extract (100 µL) showed anti-microbial action against Bacillus spp., Clostridium spp., Enterobacter aerogenes, Salmonella typhimurium and Staphylococcus aureus. In another study, the fruit ethyl acetate extracts (5-40 mg/mL) and the capsaicinoids (2.5-10 µg/mL) demonstrated excellent anti-microbial activity against Streptococcus mutans (Santos et al. 2012). Careaga et al. (2003) have also reported that the fruit juice (0.02-5 mL/100g) showed anti-bacterial activity against Salmonella typhimurium and Pseudomonas aeruginosa inoculated in raw beef meat. In a preliminary study, Monsereenusorn (1980) has reported that the intraperitoneal injection of fruit extract (500-700 mg/kg bw) showed a significant hypoglycemic action after 30 minutes post-treatment period in normal rats.

Conversely, Babu and Srinivasan (1997) have reported that diabetic animals maintained on capsaicin (0.015%) in diet, for 8 weeks have shown no any significant reduction on blood glucose levels. In another study, consumption dietary C. annuum (0.9 g/day) for 2 days increases energy expenditure, decreases appetite and food intake in human subjects (Westerterp-Plantenga et al. 2004). Similarly, C. annuum and its pungent principle capsaicin has been reported to play a beneficial role on lipid metabolism in a number of studies reported previously. Capsaicin (0.015%) included in diet for 8 weeks was reported to ameliorate dyslipidemia in diabetic and high-fat diet-fed animals (Kempaiah and Srinivasan, 2006; Babu and Srinivasan, 1997). Additionally, oral treatment of capsaicin (50 mg/kg bw) or whole fruit extract (500 mg/kg bw) for 2 months demonstrated hypolipidemic actions in normal rats (Monsereenusorn, 1983).

1.7 Statement of the problem

The epidemic of DM is among the major cause of morbidity and mortality worldwide and has been on the rise particularly in developing countries such as Africa. It has been estimated that about 80% of diabetic patients live in low- and middle- income countries (ADA, 2015). In Africa, more than 22 million people have diabetes, accounting for about 5.1% of adults and it is responsible for about 8.6% deaths in the region (IDF, 2014). Furthermore, the high cost and unwanted adverse effect of most of the modern synthetic drugs for the treatment of DM is also an issue of concern. Similarly, treatment and control of DM is still a major challenge to medical community and requires safer and cheaper alternative to effectively curb the problem of this disease. However, the use of natural products for the treatment of DM is getting more popular due to the lower side effects, availability and cost effectiveness compared to conventional synthetic drugs. However, the number of plants with potential anti-diabetic actions is very limited and many of their anti-diabetic effects have not yet been scientifically validated. Additionally, mechanisms of actions as well as active principles from these natural products are still unknown.
1.8 Justification and significance of the research work

With rapid advancement in medicine, novel treatment options with fewer side effects have become feasible for the long-term control of diabetic complications. Recent scientific investigations have confirmed the efficacy of many plant-derived formulations (extracts and fractions) and their isolated bioactive compounds in the treatment and control of diabetes (Hung et al. 2012; Ponnusamy et al. 2010). The recommendation of the WHO committee on DM encouraged research on hypoglycemic agents from natural products such as plant (WHO, 1980). Therefore, there is a need to investigate and establish the anti-diabetic effects of natural products. This will prove and display their anti-diabetic potentials for the treatment of DM. Subsequently, a chemical lead for the development of newer classes of drugs that can be used in the treatment of DM could be derived from this research work as well. Moreover, previous ethnobotanical studies have shown that the fruits and leaves from the selected plants (Aframomum melegueta, Xylopia aethiopica, and Capsicum annuum) are used locally in the treatment of diabetes. This is in addition to some preliminary studies that demonstrated the blood glucose lowering potentials of A. melegueta (Adesokan et al., 2010), X. aethiopica (Adefegha and Oboh, 2012a,b; Etoundi et al. (2013) and C. annuum (Monserereusorn, 1980). Thus, this study will prove and establish the detail scientific evidences to support the anti-diabetic potentials of these plants.

1.9 Objective of the study

1.9.1 General Objective

This research work is aimed at screening and isolation of some potent anti-diabetic compounds from some African medicinal plants [Aframomum melegueta K. Schum., Xylopia aethiopica (Dunal.) A. Rich. and Capsicum annuum L.] by using several in vitro and in vivo models.

1.9.2 Specific Objectives

The research work specifically focused on:

(i) Screening the various solvent extracts from different parts of the selected medicinal plants for in vitro anti-oxidative and anti-diabetic activities using several in vitro models.

(ii) Screening the in vitro anti-oxidant and anti-diabetic activities of various solvent fractions from the most active extracts from the selected parts of the medicinal plants.

(iii) Preliminary phytochemical studies of the most active extracts and fractions from the selected natural products using Gas Chromatography-Mass Spectrophotometry (GC-MS) technique.
(iv) Investigating the *in vivo* anti-diabetic and anti-oxidative effects of the most active fractions (from the *in vitro* studies in (ii) above) from the selected natural in an experimentally-induced type 2 diabetes model of rats.

(v) Based on the results of above *in vitro* and *in vivo* studies, isolation and structural elucidation of possible bioactive components from the most active fractions of the selected medicinal plant parts by thin layer chromatography (TLC), column chromatography and Nuclear Magnetic Resonance (NMR) spectroscopy.

(vi) Investigating the $\alpha$-glucosidase and $\alpha$-amylase inhibitory effects of the isolated compounds *in vitro*. 
CHAPTER 2

Materials and methods

2.1 Chemicals and reagents

Ascorbic acid, quercetin, hemoglobin (human lyophilized powder), gallic acid, aluminium chloride, citric acid, sodium citrate, sodium phosphate dibasic (Na$_2$HPO$_4$), sodium phosphate monobasic (NaH$_2$PO$_4$), dimethyl sulfoxide (DMSO), potassium hydroxide, Streptozotocin (STZ), metformin, formalin, α-amylase from porcine pancreas, α-glucosidase from Saccharomyces cerevisiae and 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), xylene, hematoxylin dye, absolute ethanol, hexane, ethyl acetate, dichloromethane and methanol were purchased from Sigma-Aldrich through Capital Lab Supplies, New Germany, South Africa. Folin Ciocalteau reagent was purchased from Merck Chemical Company, South Africa. Gentamycin was purchased from EMD Chemicals, San Diego, CA, USA. Ultrasensitive rat insulin ELISA kit was purchased from Mercodia, Uppsala, Sweden.

2.2 Equipment

Glucometer (Glucoplus Inc., Saint-Laurent, Quebec, Canada), rotary evaporator (Buchi Rotavapor II, Buchi, Germany), Whatmann filter paper (No. 1), multiplate reader (MR-96A, Vacutec Pvt. Ltd., Durban, South Africa), Automated Chemistry Analyzer (Labmax Plenno, Labtest Co. Ltd., Lagoa Santa, Brazil), Leica slide scanner (SCN 4000, Leica Biosystems, Wetzlar, Germany), Nuclear Magnetic Resonance (NMR) spectroscopy (Bruker BioSpin, Rheinstetten, Germany), spectrophotometer (Shimadzu Corporation, Kyoto, Japan).

2.3 Plant materials

The various parts of Aframomum melegueta (Rosc.) K. Schum. and Xylopia aethiopica (Dunal) A. Rich. were freshly collected in December, 2012 from Ibadan, Oyo State, Nigeria. Capsicum annuum L. parts were collected in December, 2012 from Zaria, Kaduna State, Nigeria. They were identified and authenticated at the herbarium unit of the Biological Science Department, Ahmadu Bello University, Zaria, Nigeria by Mr. Umar Gallah and a voucher specimen number for each plant was deposited accordingly [A. melegueta (1511), X. aethiopica (1025) and C. annuum (1755)]. The various plants samples (fruits, leaves, stems and roots) were immediately washed and shade-dried for two weeks to constant weights. The dried samples were ground to a fine powder, and then stored individually in airtight containers for transport to the University of KwaZulu-Natal, Westville campus, Durban, South Africa for further investigations.
2.3.1 Preparation of the plant extracts

Three (3) kilograms of each of the finely powdered plant parts were separately defatted with 10 L of hexane. The defatted material was sequentially extracted with ethyl acetate, ethanol and water by soaking for 48 hour in 200 mL of the relevant solvent followed by a 2 hour orbital shaking at 200 rpm. After filtration through Whatmann filter paper (No. 1), respective solvents were evaporated under vacuum, using a rotary evaporator (Buchi Rotavapor II, Buchi, Germany) at 40°C under reduced pressure to obtain the different solvent extracts with the exception of the aqueous extracts which were dried on a water bath at 45°C. The extracts in each case were weighed, transferred to 50 mL falcon tubes and stored in a refrigerator at 4-8°C until further analysis.

2.3.2 Fractionation of the crude extracts

Forty (40) grams of the crude extracts of the selected plant parts were dissolved in 500 mL of distilled water: methanol (9:1) and successively partitioned with hexane (2 x 500 mL), dichloromethane (2 x 500 mL), ethyl acetate (2 x 500 mL) and acetone (2 x 500 mL). The fractions were evaporated to dryness in vacuum at 40°C under reduced pressure by using afore-mentioned rotary evaporator. The dried fractions were transferred to tubes and stored at 4°C until further analysis.

2.4 In vitro studies

2.4.1 Estimation of total polyphenol content

The total polyphenol content of each extracts and/or fraction was determined (as gallic acid equivalent) according to the method described by McDonald et al. (2001) with slight modifications. Briefly, a 200 µL of the extract (240 µg/mL) was incubated with 1 mL of 10 times diluted Folin Ciocalteau reagent and 800 µL of 0.7 M Na$_2$CO$_3$ for 30 minutes at room temperature. The absorbance values were then determined at 765 nm in a Shimadzu UV mini 1240 spectrophotometer (Shimadzu Corporation, Kyoto, Japan). All measurements were done in triplicate.

Quantification was done on the basis of a standard curve of gallic acid. Results were expressed mg/g (GAE) and calculated using following formula,

\[
\text{Total polyphenol content (mg/g GAE)} = [\text{GAE (mg/mL)} \times \text{V (mL)}]/\text{W (g)}
\]

Where GAE (mg/mL) = (y-c)/m

GAE: Gallic acid equivalent (mg/mL), V: Total volume of sample (mL), W: Sample weight (g), y: absorbance of the sample, c: intercept at the y-axis, m: slope of the standard curve
2.4.2 Determination of total flavonoid content

The total flavonoid content of the plant extracts and/or fractions was determined using a method reported by Chang et al. (2002) with slight modification. Briefly, a 500 µL (240 µg/mL) of each sample was mixed with 500 µL methanol, 50 µL of 10% AlCl₃, 50 µL of 1 mol/L potassium acetate and 1.4 mL water, and allowed to incubate at room temperature for 30 minutes. The absorbance of the reaction mixture was subsequently measured at 415 nm using the spectrophotometer mentioned above. The total flavonoid content was calculated as quercetin equivalent (QE) in mg per g of dry extract.

Quantification was done on the basis of a standard curve of quercetin. Results were expressed mg/g (QE) and calculated using following formula,

\[
\text{Total polyphenol content (mg/g QE) = \left[\frac{\text{QE (mg/mL) \times V (mL)}}{W (g)}\right]}
\]

Where QE (mg/mL) = \(\frac{y-c}{m}\)

QE: quercetin equivalent (mg/mL), V: Total volume of sample (mL), W: Sample weight (g), y: absorbance of the sample, c: intercept at the y-axis, m: slope of the standard curve.

2.4.3 DPPH radical scavenging activity

The total free radical scavenging activity of the extracts and/or fractions was determined and compared to that of ascorbic and gallic acids by using a slightly modified method described by Tuba and Gulcin (2008). An aliquot of 500 µL of a 0.3 mM solution of 1,1-diphenyl-2-picryl-hydrazyl (DPPH) in methanol was added to 1 mL of the extracts and/or fractions at different concentrations (30, 60, 120 and 240 µg/mL). These solutions were mixed and incubated in the dark for 30 minutes at room temperature. The absorbance was measured at 517 nm against blank samples lacking the free radical scavengers.

The results of the DPPH were expressed as a percentage of the control (blank) according to the following formula:

\[
\% \text{ Inhibition} = \left[\frac{\text{Abs of control} - \text{Abs of sample}}{\text{Abs of control}}\right] \times 100
\]

2.4.4 Ferric (Fe³⁺) reducing anti-oxidant power assay

The ferric reducing anti-oxidant power method of Oyaizu (1986) was used with slight modifications to measure the reducing capacity of the samples. To perform this assay, 1 mL of each extract and/or fractions (30, 60, 120 and 240 µg/mL) were incubated with 1 mL of 0.2 M sodium phosphate buffer (pH 6.6) and 1% potassium ferricyanide at 50°C for 30 minutes. After 30 minutes incubation, the reaction mixtures were acidified with 1 mL of 10% trichloroacetic acid. Thereafter, 1 mL of the acidified sample of this solution was mixed with 1 mL of distilled water and 200 µL of FeCl₃ (0.1%). The absorbance of the resulting solution was measured at 700 nm in a spectrophotometer.
Increased absorbance of the reaction mixture indicated the greater reductive capability of the extracts (Gulcin et al. 2004).

The results of the ferric reducing anti-oxidant power assay was expressed as a gallic acid equivalent according to the following formula:

\[
\% \text{ Inhibition} = \left( \frac{\text{Abs of sample}}{\text{Abs of Gallic acid}} \right) \times 100
\]

### 2.4.5 Inhibition of hemoglobin glycation

Inhibition of non-enzymatic glycosylation of hemoglobin by various extracts and/or fractions was measured by the modified method of Pal and Dutta (2006). Glucose (2%), hemoglobin (0.06%) and gentamycin (0.02%) solutions were prepared in phosphate buffer 0.01 M, pH 7.4. An aliquot of 1 mL of each of the solution above was mixed with 1 mL of extracts with different concentrations (30, 60, 120 and 240 µg/mL) in dimethyl sulfoxide (DMSO). These mixtures were incubated in the dark at room temperature for 72 hour. The percentage inhibition of glycosylation of hemoglobin was calculated from the absorbance measured at 520 nm. Gallic acid was used as a standard.

The results of the inhibition of hemoglobin glycation were expressed as a percentage of the control (blank) according to the following formula:

\[
\% \text{ Inhibition} = \left[ \frac{\text{Abs of control} - \text{Abs of sample}}{\text{Abs of control}} \right] \times 100
\]

### 2.4.6 α-Amylase (E.C. 3.2.1.1) inhibitory effect

The α-amylase inhibitory effect of the extracts and/or fractions was carried out using a modified method of McCue and Shetty (2004). Briefly, a 250 µL aliquot of extracts, fractions and/or compounds at different concentrations (30, 60, 120 and 240 µg/mL) was placed in a tube and 250 µL of 0.02 M sodium phosphate buffer (pH 6.9) containing α-amylase (2.0 U/mL) solution was added. This solution was pre-incubated at 25°C for 10 minutes, after which 250 µL of 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9) was added at a time interval of 10 seconds and then further incubated at 25°C for 10 minutes. The reaction was terminated after incubation by adding 1 mL of dinitrosalicylic acid (DNS) reagent. The tube was then boiled for 10 minutes and cooled to room temperature. The reaction mixture was diluted with 5 mL distilled water and the absorbance was measured at 540 nm using a Shimadzu UV mini 1240 spectrophotometer. A control was prepared using the same procedure, replacing the extract with distilled water.

The results of the α-amylase assay were expressed as a percentage of the control (blank) according to the following formula:

\[
\% \text{ Inhibition} = \left[ \frac{\text{Abs of control} - \text{Abs of sample}}{\text{Abs of control}} \right] \times 100
\]
2.4.7 \( \alpha \)-Glucosidase (E.C. 3.2.1.20) inhibitory effect

The inhibitory effect of the plant extracts and/or fractions on \( \alpha \)-glucosidase activity was determined according to the method described by Kim et al. (2005) using \( \alpha \)-glucosidase from \textit{Saccharomyces cerevisiae}. The substrate solution 5.0 mM \( p \)-nitrophenylglucopyranoside (\( p \)NPG) was prepared in 20 mM phosphate buffer, pH 6.9. An aliquot of 500 \( \mu \)L of \( \alpha \)-glucosidase (1.0 U/mL) was then pre-incubated with 250 \( \mu \)L of the different concentrations of the extracts, fractions and/or compounds (30, 60, 120 and 240 \( \mu \)g/mL) for 10 minutes. Thereafter, a 250 \( \mu \)L of 5.0 mM \( p \)NPG was dissolved in 20 mM phosphate buffer (pH 6.9) as a substrate to start the reaction. The reaction mixture was incubated at 37°C for 30 minutes. The \( \alpha \)-glucosidase activity was determined by measuring the yellow coloured \( p \)-nitrophenol released from \( p \)NPG at 405 nm.

The results of the \( \alpha \)-glucosidase assay were expressed as a percentage of the control (blank) according to the following formula:

\[
\text{% Inhibition} = \frac{\text{Abs of control} - \text{Abs of sample}}{\text{Abs of control}} \times 100
\]

2.4.8 Calculation of IC\textsubscript{50} values

Concentrations of extracts, fractions or compounds resulting in 50\% inhibition of radical or enzyme activities (IC\textsubscript{50}) were calculated from the plot of percentage inhibition against log(concentration of the samples).

\[
\text{IC}_{50} = 10^x
\]

Where \( x \) (mg/mL) = (y-c)/m

\( y \): 50\%, \( c \): intercept at the y-axis, \( m \): slope of the graph of log(concentration of sample used) against the percentage inhibitions.

2.4.9 Mechanism of \( \alpha \)-glucosidase and \( \alpha \)-amylase inhibitions

The most active fractions or compounds were subjected to kinetic experiments to determine the type of inhibition exerted on \( \alpha \)-glucosidase and \( \alpha \)-amylase. The experiment was conducted according to the protocols as described above at a constant concentration of the fractions or compounds (30 \( \mu \)g/mL) with a variable concentration of substrate. For the \( \alpha \)-glucosidase inhibition assay, 0.313-5.0 mmol/L of \( p \)NPG was used and 0.063-1.0\% of starch was used for the \( \alpha \)-amylase inhibition assay. The initial rates of reactions were determined from calibration curves constructed using varying concentrations of \( p \)-nitrophenol (0.313-5.0 mmol/L) and maltose (0.063-1.0\%) for the \( \alpha \)-glucosidase and \( \alpha \)-amylase inhibition assays, respectively. The initial velocity data obtained were used to construct Lineweaver-Burke’s plot to determine the \( K_M \) (Michaelis constant) and \( v_{\text{max}} \) (maximum velocity) of the enzyme as well as the \( K_I \) (inhibition binding constant as a measure of affinity of the inhibitor to the enzyme) and the type of inhibition for both enzymes.
2.4.10 Gas Chromatography-Mass Spectroscopic (GC-MS) analysis

Based on the results of *in vitro* anti-oxidative and anti-diabetic studies, the most active extracts and/or fractions were subjected to GC-MS analysis. The GC-MS analysis was conducted with an Agilent technology 6890 GC coupled with an Agilent 5973 Mass Selective Detector and driven by Agilent Chemstation software. Compounds were identified by direct comparison of the retention times and mass spectral data with those in the National Institute of Standards and Technology (NIST) library.

2.5 *In vivo* studies

2.5.1 Experimental animals

Six-week-old male Sprague-Dawley (SD) rats were obtained from the Biomedical Resource Unit (BRU) located at the University of KwaZulu-Natal (Westville Campus), South Africa with initial mean body weight (bw) 163.25 ± 13.67 g. Animals were housed as two in one medium size poly-carbonated cage in a temperature and humidity controlled room with a 12 hour light-dark cycle. A standard rat pellet diet was supplied *ad libitum* to all animals during the entire experimental period. Animals were maintained according to the rules and regulations of the Experimental Animal Research Ethics Committee of the University of KwaZulu-Natal, South Africa (Ethical approval number: 018/14/Animal).

2.5.2 Animal grouping

Animals were randomly divided into twelve groups of 5 (non-diabetic groups) or 8 (diabetic groups) animals namely; NC: Normal Control, DBC: Diabetic Control, DAML: Diabetic + low dose (150 mg/kg bw) of AMEF, DAMH: Diabetic + high dose (300 mg/kg bw) of AMEF, DCAL: Diabetic + low dose (150 mg/kg bw) of CAAF, DCAH: Diabetic + high dose (300 mg/kg bw) of CAAF, DXAL: Diabetic + low dose (150 mg/kg bw) of XAAF, DXAH: Diabetic + high dose (300 mg/kg bw) of XAAF, DMF: Diabetic + metformin (300 mg/kg bw), NAMH: Non-diabetic + high dose (300 mg/kg bw) of AMEF, NCAH: Non-diabetic + high dose (300 mg/kg bw) of CAAF, NXAH: Non-diabetic + high dose (300 mg/kg bw) of XAAF. The animals were allowed to acclimatize for two weeks before starting the experiment. The animal grouping was summarized in Figure 2.1 as well.
2.5.3 Induction of Type 2 diabetes (T2D)

In order to induce the two major pathogeneses of T2D, insulin resistance and partial pancreatic \( \beta \)-cell dysfunction, during the first two weeks of the experiment, the animals in the DBC, DAML, DAMH, DCAL, DCAH, DXAL, DXAH and DMF groups were supplied with a 10% fructose solution *ad libitum* for the induction of insulin resistance when the animals in the NC, NAMH, NCAH and NXAH groups were supplied with normal drinking water. After this period, a low dose of STZ (40 mg/kg bw) dissolved in citrate buffer (pH 4.5) were intraperitoneally injected to the animals in the DBC, DAML, DAMH, DCAL, DCAH, DXAL, DXAH and DMF groups to induce partial pancreatic \( \beta \)-cell dysfunction, whereas the animals in the NC and NAMH, NCAH and NXAH groups were injected with a similar volume of vehicle buffer only (Wilson and Islam, 2012). One week after the STZ injection, the non-fasting blood glucose (NFBG) of all animals were measured in the blood collected from the tail vein by using a portable glucometer (Glucoplus Inc., Saint-Laurent, Quebec, Canada). Animals with a NFBG level \( \geq 200 \) mg/dL were considered as diabetic (Davidson et al. 2011) while animals with a NFBG level \( < 200 \) mg/dL were excluded from the study.
2.5.4 Intervention period

After the confirmation of diabetes, a respective dose of the various fractions were orally administered once daily for four weeks using a gastric gavage needle to the animals in DAML, DAMH, DCAL, DCAH, DXAL, DXAH, NAMH, NCAH and NXAH groups while the animals in controls (NC and DBC) and DMF groups were treated with similar volume of the vehicle and metformin, respectively. Throughout the experimental period, feed and fluid intake were measured every morning by subtracting the remaining amount of feed and fluid respectively from the amount given on the previous morning. Moreover, the weekly bw and NFBG levels were measured in all animal groups during the entire intervention period.

2.5.5 Oral glucose tolerance test (OGTT)

To determine the glucose tolerance ability of each animal, the OGTT was performed in the last week of the four-week intervention period. To perform this test, a single dose of glucose solution (2 g/kg bw) was orally administered to each animal and the subsequent levels of blood glucose were measured at 0 (just before glucose ingestion), 30, 60, 90 and 120 minutes after the ingestion of glucose.

2.5.6 Collection and preparation of blood and organs

At the end of the experimental period, animals were euthanized with halothane anesthesia and blood and organ samples were collected. The whole blood of each animal was collected via cardiac puncture and immediately preserved in a refrigerator until further processing. The blood samples were centrifuged at 3000 rpm for 15 minutes and serum from each blood sample was separated and preserved at -30°C for further analysis. The liver, kidney, heart and pancreas were collected from each animal, washed with normal saline, wiped with filter paper, weighed and preserved at -30°C until subsequent analysis. A small piece of pancreatic tissue from each animal was cut and placed in a 10% neutral buffered formalin solution and preserved at room temperature for histopathological study. The neutral buffered formalin of each pancreatic tissue sample was replaced weekly during the entire preservation period.

For the organs, about 0.5 g each organ (liver, pancreas, heart and kidney) was homogenized in 4 mL of homogenization buffer (50 mM sodium phosphate buffer with triton X-100, pH 7.5) using a portable tissue homogenizer. Thereafter, the mixtures were transferred to micro tubes and spun for 15 minutes at 15000 rpm in a microcentrifuge at 4°C. The supernatants were then collected in other micro tubes and stored at -20°C for the analysis of in vivo anti-oxidative parameters.
2.5.7 Analytical methods

The serum insulin concentrations were measured by an enzyme-linked immunosorbent assay (ELISA) method using an ultrasensitive rat insulin ELISA kit (Mercodia, Uppsala, Sweden) in a multiplate reader (MR-96A, Vacutec Pvt. Ltd., Durban, South Africa). The serum lipid profile, fructosamine, urea, uric acid, total protein, lactate dehydrogenase (LDH), creatine kinase (CK-MB) and creatinine concentrations as well as liver function enzymes; aspartate and alanine aminotransferases (AST and ALT) and alkaline phosphatase (ALP) were measured using an Automated Chemistry Analyzer (Labmax Plenno, Labtest Co. Ltd., Lagoa Santa, Brazil) with commercial assay kits from the same company. Homeostatic model assessment (HOMA-IR and HOMA-β) scores were calculated at the end of the intervention according to the following formula:

\[
\text{HOMA-IR} = \left[\frac{\text{Fasting serum insulin in U/l x Fasting blood glucose in mmol/L}}{22.5}\right]
\]

\[
\text{HOMA-β} = \left(\frac{\text{Fasting serum insulin in U/l} \times 20}{\text{Fasting blood glucose in mmol/L} - 3.5}\right)
\]

Conversion factor: insulin (1 U/l = 7.174 pmol/L).

LDL-cholesterol was calculated according to Friedewald et al. (1972) equation as shown below:

\[
\text{LDL-Cholesterol (mg/dL)} = \text{TC} - (\text{HDL} + \frac{\text{TG}}{5})
\]

Where, TG/5 is equivalent to the concentration of VLDL-cholesterol.

Atherogenic index (AI) was calculated according to the method described by Liu et al. (1999) and expressed as:

\[
\text{AI} = \frac{\text{TC} - \text{HDL-cholesterol}}{\text{HDL-cholesterol}}.
\]

Coronary artery risk index (CRI) was also calculated using the following formula (Boers et al., 2003).

\[
\text{Coronary artery risk index (CRI): TC} / \text{HDL-cholesterol}
\]

Liver glycogen concentrations were measured by phenol-sulfuric acid method as described by Lo et al. (1970).

2.5.8 Histopathological examination of pancreatic tissue

A standard laboratory protocol for paraffin embedding was used to treat the formalin preserved pancreatic tissues. Tissue sections were cut into a size of 4 mm before fixing on the slides. Then, the slides were deparaffinized in p-xylene and rehydrated in ethanol gradient (100, 80, 70 and 50%) and rinsed with water. Slides were stained in hematoxylin for 5 minutes and rinsed with water, and were
counter stained in eosin, mounted in DPX, cover-slipped and viewed with Leica slide scanner (SCN 4000, Leica Biosystems, Wetzlar, Germany).

2.5.9 Determination of reduced glutathione (GSH)

The reduced glutathione levels were determined using the procedure described by Ellman (1959) with slight modifications. A 0.5 mL of the serum or tissue homogenate was added to 0.5 ml of 10% TCA and centrifuged at 5000 rpm for 10 minutes. Thereafter, 0.5 mL of the supernatant was mixed with 0.5 mL of Ellman’s reagent (19.80 mg of DTNB dissolved in 100 mL of 0.1% NaNO₃) and 3 mL of phosphate buffer (200 mM, pH 8.0). The mixture was incubated for 30 minutes at room temperature and the absorbance was measured at 412 nm. The concentration of the reduced glutathione in the samples was calculated from a standard glutathione curve.

2.5.10 Determination of thiobarbituric acid reactive substance (TBARS) concentration as malondialdehyde (MDA) equivalent

Thiobarbituric acid reactive substances, expressed as MDA equivalent, were measured to determine the extent of lipid peroxidation using the protocol described by Fraga et al. (1988). A 200 μL of the sample or MDA standards were thoroughly mixed with 200 μL of 8.1% sodium dodecyl sulphate (SDS) solution, 750 μL of 20% acetic acid, 2 mL of 0.25% TBA and 850 μL of distilled water. Subsequently, the resulting mixture was heated in a water bath at 95°C for 1 hour and allowed to cool to room temperature. The absorbance of the resulting solution was then read at 532 nm and the concentration of TBARS in the samples was calculated from the MDA standard curve.

2.5.11 Determination of catalase activity

This assay was carried out as described by Aebi (1984). A 340 μL of 50 mM sodium phosphate buffer (pH 7.0) was mixed with 150 μL of 2 M H₂O₂. Thereafter, a 10 μL of the sample was added and the decrease in absorbance at 240 nm was monitored for 3 min at 1 minute interval.

Activity (μmol/min/µg protein) = [(Abs₁-Abs₂)/ε] x 0.5/μmol/min/µg protein. Where ε= 0.00394 mM⁻¹cm⁻¹; Abs₁= Initial absorbance; Abs₂= Final absorbance

2.5.12 Determination of superoxide dismutase (SOD) activity

This assay was carried out by transferring 1 ml of 0.1 mM diethylthiocarbamoylaminepentaaetic acid (DETPAC) and 90 μl of the sample into a quartz cuvette. Then a 90 μl of 1.6 mM 6-hydroxydopamine (6-HD) was added and the mixture was quickly mixed. Absorbance of the resulting mixture was recorded at 492 nm for 3 minutes at 1 minute interval (Kakkar et al. 1984).
Activity (µmol/min/µg protein)= [(Abs\text{1}-Abs\text{2})/ε] x 0.5/µg protein. Where ε= 1.742 mM\text{−1} cm\text{−1}; Abs\text{1}= Initial absorbance; Abs\text{2}= Final absorbance

2.5.13 Determination of glutathione peroxidase (GPx) activity

GPx was assayed by the method of Paglia and Valentine (1967). The reaction mixture contained 0.1 M phosphate buffer (pH 7.0), 1 mM EDTA, 10 mM glutathione, 1 mM NaN\text{3}, 1 unit of glutathione reductase, 1.5 mM NADPH and 0.1 ml of sample. After incubating for 10 minutes at 37\textdegree C, H\textsubscript{2}O\textsubscript{2} was added to each sample to a final concentration of 1 mM. GPx activity was measured as the rate of NADPH oxidation at 340 nm in a spectrophotometer for 3 minutes at 1 minute intervals.

Activity (µmol/min/µg protein)= [(Abs\text{1}-Abs\text{2})/ε] x 0.5/µg protein. Where ε= 6.22 mM\text{−1} cm\text{−1}; Abs\text{1}= Initial absorbance; Abs\text{2}= Final absorbance

2.5.14 Determination of glutathione reductase (GR)

Glutathione reductase (GR) activity was determined according to the method described by Foyer and Halliwell (1976). About 800 μL of 25 mM sodium phosphate buffer (pH 7.8) was added to the sample and blank tubes respectively. This was followed by the addition of 100 μL of each oxidized glutathione (GSSG) and NADPH in both blank and the sample tubes. A 100 μL of the sample is finally added into the sample tube. The oxidation of NADPH was recorded by reading the absorbance at 340 nm continuously for 180 seconds.

Activity (µmol/min/µg protein)= [(Abs\text{1}-Abs\text{2})/ε] x 0.5/µg protein. Where ε= 6.22 mM\text{−1} cm\text{−1}; Abs\text{1}= Initial absorbance; Abs\text{2}= Final absorbance

2.6 Isolation of the bioactive anti-diabetic compounds from the fractions

All NMR data, \textsuperscript{1}H, \textsuperscript{13}C and 2D experiments were recorded on a Bruker Avance III 400 MHz spectrometer. Samples were acquired with deuterated chloroform (CDCl\textsubscript{3}). The spectra were referenced according to the deuteriocloroform signal at δ\text{H} 7.24 (for \textsuperscript{1}H NMR spectra) and δ\text{C} 77.0 (for \textsuperscript{13}C NMR spectra) for CDCl\textsubscript{3}. The HRESIMS spectra were obtained from a Bruker Micro TOF-QII instrument.

A bioassay guided isolation protocol was used to isolate the pure bioactive anti-diabetic agent(s) from each of the most active fraction (from in vitro studies described in sub chapter 2.4) but the approach slightly differs from one fraction to another.
2.6.1 Isolation of the bioactive compounds from the ethyl acetate fraction of ethanolic extract of *Aframomum melegueta* fruit

15.5 g of the ethyl acetate (EtOAc) crude extract of *A. melegueta* was subjected to column chromatography on a silica gel column using gradient solvent systems of hex: EtOAc and EtOAc: MeOH with increments of 10% in each step to afford 84 fractions. After monitoring with TLC, similar fractions were pooled together to afford seven major fractions (A: 11-13; B: 14-17; C: 20-25; D: 26-32; E: 33-34; F: 35-47 and G: 48-55). Compound 1 (210.0 mg), was obtained as an oil from fraction C (2.51 g) after further purification on a 2.0 cm diameter column using hex: EtOAc (17:3) as eluent. Fraction D (990.3 mg) was further purified on a column (1.5 cm in diameter) by elution with hex: EtOAc (8:2) to obtain compound 2 (490.0 mg) as an oil. Compound 3 (990.9 mg) was obtained as an oil by purification of fraction F (4.13 g) on a silica column and eluted with hex: EtOAc (3:2). Fraction E (100.9 mg) was further purified with a hex: EtOAc (7:3) solvent system, which yielded compound 4 (60.0 mg). All the isolated bioactive compounds demonstrated considerable α-amylase and α-glucosidase inhibitory actions.

2.6.2 Isolation of the bioactive compound from the acetone fraction of ethanolic extract of *Xylopia aethiopica* fruit

A part of the acetone crude extract of *X. aethiopica* (16 g) was fractionated on a 3.5 cm diameter column over silica gel (0.040-0.063 mm) using a gradient elution of n-hexane:EtOAc (with 10% increments of EtOAc) and then MeOH (100%) resulting in 64 fractions. The fractions were pooled together on the basis of TLC to afford six main fractions (A: 6-8; B: 9-13; C: 14-16; D: 18-22; E: 27-32 and F: 33-54). Compound 4 (440.7 mg) was purified with hex: EtOAc (7:3) from fraction C (1.55 g) by CC (2 cm column) where 4 eluted again in fractions 16-43. Fraction E (1.92 g) was separated by CC on a 2.0 cm column using Hex: EtOAc (1:1) which yielded 3 (350.6 mg) again as a brownish oil in fractions 16-44. Additionally, these compounds showed considerable α-amylase and α-glucosidase inhibitory actions.

2.6.3 Isolation of the bioactive compound from the acetone fraction of ethanolic extract of *Capsicum annuum* fruit

A 15.13 g sample of the acetone crude extract of *C. annuum* was fractionated by column chromatography using Hex: EtOAc and then EtOAc: MeOH in a stepwise gradient elution by 10% increments using a 3.5 cm diameter column. After TLC, the 4 main fractions were combined as follows: A: 12-18; B: 19-23; C: 24-45 and D: 46-49. Repeated purifications by column chromatography of
fraction C (3.03g) yielded a mixture of compounds 5, which could not be separated despite several attempts and exhibited considerable \( \alpha \)-amylase and \( \alpha \)-glucosidase inhibitory actions.

2.7 Statistical analysis

For all the \textit{in vitro} studies, data were presented as the mean \pm SD of triplicates determination while in the \textit{in vivo} studies, the data were presented as the mean \pm SD of 5 to 7 animals. Data were analyzed by using a statistical software package (SPSS for Windows, version 23, IBM Corporation, NY, USA) using Tukey's-HSD multiple range \textit{post-hoc} test. Values were considered significantly different at \( p < 0.05 \).
CHAPTER 3

3.0 Anti-diabetic and anti-oxidative actions of various extracts and fractions from different parts of Aframomum melegueta: an in vitro and in vivo approach

3.1 Anti-oxidative activity, phytochemistry, and inhibition of key enzymes linked to type 2 diabetes by various parts of Aframomum melegueta in vitro

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3.1.1 Abstract

Objective: Aframomum melegueta K. Schum. (Zingiberaceae) fruits, leaf or seeds are used for treatment of different diseases such as diabetes, microbial infections, stomach ache, snakebite and diarrhea in Africa. This study investigated and compared the anti-oxidative, anti-diabetic effects and possible compounds present in various solvent extracts of fruit, leaf and stem of A. melegueta.

Methods: Samples were sequentially extracted using solvents of increasing polarity. They were subjected and investigated for 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging activity, ferric reducing power, inhibition of hemoglobin glycosylation, α-amylase and α-glucosidase activities as markers of in vitro anti-diabetic effects at various doses (30-240 µg/mL). Possible compounds were analyzed using Gas Chromatography-Mass Spectrometry (GC-MS) analysis.

Results: Fruit ethanolic (EtOH) extract showed higher total polyphenol (12.52 ± 0.13 mg/gGAE) and flavonoid (4.92 ± 0.12 mg/gQE) contents compared to other extracts. Similarly for all the in vitro models used in this study, fruit EtOH extract exhibited lower IC₅₀ values compared to other extracts and comparable to standards used accordingly (DPPH 0.04 ± 0.01 mg/mL; Ascorbic acid: 0.03 ± 0.02 mg/mL; Gallic acid: 0.05 ± 0.01 mg/mL; Hemoglobin glycosylation: 0.72 ± 0.03 mg/mL; Gallic acid: 0.20 ± 0.01 mg/mL; α-amylase: 0.13 ± 0.01 mg/mL; Acarbose: 0.37 ± 0.03 mg/mL; α-glucosidase: 0.06...
± 0.01 mg/mL; Acarbose: 0.21 ± 0.01 mg/mL). The GC-MS analysis of the most active extracts (EtOH fruit and leaf) revealed the presence of some phenolics and other fatty acids derivatives as possible compounds present.

**Conclusion:** Fruit EtOH extract exhibited higher anti-oxidative and anti-diabetic effects compared to other solvent extracts *in vitro* and thus require further work to fully validate these effects *in vivo*.

**Keywords:** *Aframomum melegueta*, Anti-oxidative, α-amylase, α-glucosidase, Type 2 diabetes

### 3.1.2 Introduction

Globally, the prevalence of diabetes mellitus (DM) is increasing exponentially. Presently, over 387 million people are diabetic and this figure is likely to rise to 592 million by 2035 (IDF, 2014). Among the major types of diabetes (type 1 and type 2), type 2 is the most prevalent one and about 90-95% of diabetics suffer from type 2 diabetes (T2D) (van-Dam et al. 2002). T2D is a heterogeneous disorder characterized by insulin resistance and partially dysfunctional pancreatic β-cell to properly secrete insulin in response to hyperglycemia (Hui et al. 2007). The hyperglycemia increases the generation of reactive oxygen species (ROS) that bind to carbohydrates, proteins, lipids or DNA, thereby cause cellular and tissue damages, leading to oxidative stress in T2D (Devasagayam et al. 2007; Rosen et al. 2001). Hence, oxidative stress is considered as the major causal factor of T2D associated complications. Moreover, anti-oxidants are molecules that can inhibit or terminate the initiation or propagation of oxidative chain reactions, and can therefore prevent or repair the damage caused by ROS (Halliwell et al. 1995). However, the increasing risk associated with the use of existing synthetic anti-oxidants has been an issue of concern. Therefore, focus has been shifted to the use of natural anti-oxidants in preventing oxidative damage either in disease condition or food preservation.

Furthermore, the major treatment options for T2D include the use oral hypoglycemic drugs in addition to lifestyle modification. However due to the number of short- and long-term side effects associated with the use of the synthetic drugs, their popularity are decreasing gradually (Stephen, 2006; Suba et al. 2004). This has paves way for a search of newer alternative agents such as plant-derived products to control T2D that are known to pose fewer side effects. Furthermore, the inhibition of carbohydrate digesting enzymes such as α-glucosidase is now regarded as another alternative approach to control T2D. This caused a reduction of postprandial hyperglycemia via decrease in the intestinal glucose absorption (Imam, 2015). Additionally, in a number of recently published data, plant-derived extracts or fractions have shown potent anti-oxidant and demonstrated abilities to inhibit α-glucosidase activities (Ibrahim et al. 2014; Kong et al. 2014; Gulati et al. 2012; Adefegha and Oboh, 2012a).

*Aframomum melegueta* K. Schum. (Zingiberaceae) commonly known as guinea or alligator pepper is abundantly found in central and western part of Africa (Iwu, 2014). The seeds, leaf or fruit are consumed as flavoring agent in various traditional foods and are used locally to treat diseases such
as diabetes, microbial infections, diarrhea, abdominal pain, snakebite and wounds healing in Nigeria, Cameroon, Kenya and Gabon (Iwu, 2014; Soladoye et al. 2012; Gbolade, 2009; Akendengue and Louis, 1994; Kokwaro, 1993). Recently, the dipterpenoids isolated from the organic seed extract of \textit{A. melegueta} has showed bactericidal activity \textit{in vitro} against \textit{Escherichia coli}, \textit{Listeria monocytogenes} and \textit{Staphylococcus aureus} strains (Ngwoke et al. 2014). In another study, Sugita et al. (2013) have reported that \textit{A. melegueta} alcohol extract activates brown adipose tissue and increases whole-body energy expenditure in human subjects which is directly linked to the pathogenesis of T2D. Moreover, in a number of studies reported earlier, various extracts from \textit{A. melegueta} seed possessed anti-oxidative as well as anti-diabetic effects \textit{in vitro} (Kazeem et al. 2012; Adefegha and Oboh, 2012a; Adefegha and Oboh, 2011; Etoundi et al. 2010) and \textit{in vivo} (Onoja et al. 2014; Adesokan et al. 2010) when the anti-oxidative or anti-diabetic effects of fruit, leaf and stem are still unknown. Additionally, to our knowledge, no study is available that investigated and compared anti-oxidative and anti-diabetic effects of \textit{A. melegueta} fruit, leaf and stem in a single study either \textit{in vitro} or \textit{in vivo}.

Therefore, this study was designed to investigate and compare the anti-oxidative and inhibition of key enzymes linked to T2D by various extracts from fruit, leaf and stem of \textit{A. melegueta} using several \textit{in vitro} models. Additionally, possible bioactive compounds on most active extracts were analyzed using GC-MS.

### 3.1.3 Materials and methods

For sample collection, identification and preparation of the extracts, including the detail experimental protocols, please refer to chapter 2, sub-sections 2.3 and 2.4.

### 3.1.4 Results

The yields recovered, total polyphenols and flavonoids contents from different solvent extracts of various parts are presented in Table 3.1. The aqueous leaf extract showed higher yield compared to other solvent extracts, while stem ethyl acetate (EtOAc) extract was the least recovered. Moreover, significantly ($p < 0.05$) higher total polyphenols and flavonoids contents were observed in the fruit ethanolic (EtOH) extract compared to other solvent extracts of the same or different parts of \textit{A. melegueta} (Table 3.1). Interestingly, the contents of leaf EtOH extract were also higher compared to the EtOAc and aqueous extracts of this part. However, in the stem part, the EtOAc extract showed significantly ($p < 0.05$) higher polyphenols with no detectable flavonoid content (Table 3.1).
Table 3.1: Percentage yield, total polyphenol and flavonoid contents of various solvent extracts of A. melegueta parts

<table>
<thead>
<tr>
<th>Samples</th>
<th>Yield (%)</th>
<th>Total polyphenols content (mg/g GAE)</th>
<th>Total flavonoids content (mg/g QE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fruit</td>
<td>Ethyl acetate</td>
<td>0.73</td>
<td>9.03 ± 0.21&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Ethanol</td>
<td>0.53</td>
<td>12.52 ± 0.13&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Aqueous</td>
<td>1.63</td>
<td>2.97 ± 0.03&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Leaf</td>
<td>Ethyl acetate</td>
<td>1.05</td>
<td>5.49 ± 0.51&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Ethanol</td>
<td>2.63</td>
<td>9.43 ± 0.03&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Aqueous</td>
<td>3.00</td>
<td>1.84 ± 0.44&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Stem</td>
<td>Ethyl acetate</td>
<td>0.25</td>
<td>1.58 ± 0.54&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Ethanol</td>
<td>1.13</td>
<td>0.66 ± 0.09&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Aqueous</td>
<td>2.38</td>
<td>0.18 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SD values of triplicate determinations. Different superscripted letters for a given value within a column are significantly different from each other (Tukey’s-HSD multiple range post hoc test, p < 0.05). ND, Not determined.

The results of ferric (Fe<sup>3+</sup>) reducing anti-oxidant power (expressed as gallic acid equivalents) of various extracts are presented in Figure 3.1. According to the data, various solvent extracts from fruit and leaf parts of A. melegueta showed considerable reducing abilities, irrespective of the solvent used. The EtOH extracts had demonstrated significantly (p < 0.05) higher activities compared to other solvent extracts. In addition, fruit EtOH extract showed higher reducing power compared to other solvent extracts of the same or different parts and was comparable to ascorbic acid used as a standard (Figure 3.1). Moreover, various solvent extracts from stem showed no Fe<sup>3+</sup> to Fe<sup>2+</sup> reducing abilities.
Figure 3.1. Ferric reducing power (relative to gallic acid) of fruit (A) and leaf (B) extracts of *A. melegueta*. Data are presented as mean ± SD of triplicate determinations. *a-c* Different letters presented over the bars for a given concentration of each extract are significantly different from each other (Tukey’s-HSD multiple range post hoc test, *p* < 0.05).

The data of DPPH radical scavenging activities and the calculated IC₅₀ values of various extracts from different parts of *A. melegueta* are presented in Figure 3.2 and Table 3.2, respectively. The IC₅₀ values recorded for various solvent extracts from fruit ranges from 0.04 to 0.16 mg/mL (Table 3.2). The EtOH extract has demonstrated significantly (*p* < 0.05) lower IC₅₀ value (0.04 ± 0.01 mg/mL) compared to other extracts and is comparable to the standards used in this study (ascorbic acid: 0.03 ± 0.02 mg/mL; gallic acid: 0.05 ± 0.01 mg/mL). Similarly, leaf EtOH extract exhibited lower IC₅₀ value (0.07 ± 0.02 mg/mL) compared to other extracts of this part which did not differ significantly with
those of the standards. Various extracts from stem showed weak scavenging effect as the calculated IC$_{50}$ values were significantly higher compared to other solvent extracts from the other parts and the standards as well (Table 3.2). In addition, EtOH extracts from different parts exhibited the least IC$_{50}$ values for DPPH scavenging activity compared to other extracts from the respective parts of A. melegueta.

**Table 3.2:** IC$_{50}$ values of various extracts of A. melegueta parts in different anti-oxidative and anti-diabetic models.

<table>
<thead>
<tr>
<th>Samples</th>
<th>DPPH scavenging activity</th>
<th>Non-enzymatic glycation of hemoglobin</th>
<th>α-amylase inhibition</th>
<th>α-glucosidase inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fruit</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>0.16 ± 0.01$^a$</td>
<td>1.55 ± 0.32$^a$</td>
<td>3.59 ± 0.12$^c$</td>
<td>2.73 ± 0.08$^e$</td>
</tr>
<tr>
<td>Ethanol</td>
<td>0.04 ± 0.01$^a$</td>
<td>0.72 ± 0.03$^a$</td>
<td>0.13 ± 0.01$^a$</td>
<td>0.06 ± 0.01$^a$</td>
</tr>
<tr>
<td>Aqueous</td>
<td>0.06 ± 0.01$^a$</td>
<td>3.34 ± 0.32$^b$</td>
<td>3.62 ± 0.03$^c$</td>
<td>0.32 ± 0.01$^b$</td>
</tr>
<tr>
<td><strong>Leaf</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>0.17 ± 0.01$^a$</td>
<td>16.75 ± 0.39$^d$</td>
<td>25.99 ± 0.26$^f$</td>
<td>1.05 ± 0.01$^c$</td>
</tr>
<tr>
<td>Ethanol</td>
<td>0.07 ± 0.02$^a$</td>
<td>5.12 ± 1.21$^c$</td>
<td>8.79 ± 0.44$^d$</td>
<td>0.06 ± 0.01$^a$</td>
</tr>
<tr>
<td>Aqueous</td>
<td>1.78 ± 0.10$^b$</td>
<td>57.57 ± 0.82$^a$</td>
<td>15.10 ± 0.35$^e$</td>
<td>2.65 ± 0.40$^e$</td>
</tr>
<tr>
<td><strong>Stem</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>4.45 ± 0.16$^d$</td>
<td>ND</td>
<td>ND</td>
<td>2.31 ± 0.18$^{d,e}$</td>
</tr>
<tr>
<td>Ethanol</td>
<td>2.46 ± 0.01$^c$</td>
<td>ND</td>
<td>ND</td>
<td>1.93 ± 0.02$^d$</td>
</tr>
<tr>
<td>Aqueous</td>
<td>4.70 ± 0.21$^e$</td>
<td>ND</td>
<td>ND</td>
<td>81.29 ± 0.90$^f$</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>0.03 ± 0.02$^a$</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>0.05 ± 0.01$^a$</td>
<td>0.20 ± 0.01$^a$</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Acarbose</td>
<td>ND</td>
<td>ND</td>
<td>0.37 ± 0.03$^b$</td>
<td>0.21 ± 0.01$^b$</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SD values of triplicate determinations. $^{a-f}$ Different superscript letters presented within a column for a given parameter are significantly different from each other (Tukey’s-HSD multiple range *post hoc* test, $p < 0.05$). ND, Not determined.
Figure 3.2. DPPH Radical scavenging activity (%) of fruit (A), leaf (B) and stem (C) extracts of *A. melegueta*. Data are presented as mean ± SD of triplicate determinations. Different letters presented over the bars for a given concentration of each extract are significantly different from each other (Tukey's HSD multiple range *post hoc* test, *p* < 0.05).
The results of the inhibition of hemoglobin glycation by the extracts are presented in Figure 3.3 and Table 3.2. Fruit EtOH extract has exhibited a significantly ($p < 0.05$) lower IC$_{50}$ value (0.72 ± 0.03 mg/mL) compared to other solvent extracts from the same or different parts, which again was comparable to the gallic acid (0.20 ± 0.01 mg/mL) used as a standard (Table 3.2). Moreover, leaf EtOH extract exhibited lower IC$_{50}$ value (5.12 ± 1.21 mg/mL) compared to the EtOAc and aqueous extracts. It was also observed that all the extracts from the stem part showed no inhibitory activity towards hemoglobin glycosylation.

Figure 3.3. Inhibition of hemoglobin glycosylation (%) of fruit (A) and leaf (B) extracts of *A. melegueta*. Data are presented as mean ± SD of triplicate determinations. *Different letters presented over the bars for a given concentration of each extract are significantly different from each other (Tukey’s HSD multiple range post hoc test, $p < 0.05$).
The results of inhibition of α-amylase action by various extracts from *A. melegueta* are presented in Figure 3.4 and Table 3.2. It was observed from the data that, fruit EtOH extract exhibited significantly (*p* < 0.05) lower IC$_{50}$ value (0.13 ± 0.01 mg/mL) compared to other solvent extracts of this part (EtOAc: 3.59 ± 0.12 mg/mL; aqueous: 3.62 ± 0.03 mg/mL) and comparable to acarbose (0.37 ± 0.03 mg/mL). The degree of inhibitions was in the increasing order: aqueous < EtOAc < EtOH (Table 3.2). On the other hand, the calculated IC$_{50}$ value of leaf EtOH extract (8.79 ± 0.44 mg/mL) was significantly (*p* < 0.05) higher compared to acarbose (0.37 ± 0.03 mg/mL). Furthermore, none of the extracts from the stem part the showed α-amylase inhibitory actions.

![Figure 3.4](image)

**Figure 3.4.** α-Amylase inhibition (%) of fruit (A) and leaf (B) extracts of *A. melegueta*. Data are presented as mean ± SD of triplicate determinations. *a-d* Different letters presented over the bars for a given concentration of each extract are significantly different from each other (Tukey’s HSD multiple range *post hoc* test, *p* < 0.05).
The data of the \(\alpha\)-glucosidase inhibitory action by various solvent extracts from \textit{A. melegueta} are presented in \textbf{Figure 3.5} and \textbf{Table 3.2}. According to the data, fruit and leaf EtOH extracts have demonstrated potent \(\alpha\)-glucosidase inhibitory effects with the same IC\(50\) value (0.06 ± 0.01 mg/mL) which is significantly lower compared to that of acarbose (0.21 ± 0.01 mg/mL). Stem EtOH extract which shows no \(\alpha\)-amylase inhibitory effect exhibited moderate \(\alpha\)-glucosidase (IC\(50\) = 1.93 ± 0.02 mg/mL) inhibition (\textbf{Table 3.2}).

\textbf{Figure 3.5.} \(\alpha\)-Glucosidase inhibition (%) of fruit (A), leaf (B) and stem (C) extracts of \textit{A. melegueta}. Data are presented as mean ± SD of triplicate determinations. \textit{a-d} Different letters presented over the bars for a given concentration of each extract are significantly different from each other (Tukey’s HSD multiple range post hoc test, \(p < 0.05\)).
Figures 3.6 and 3.7 as well as Table 3.3 present the data of the phytochemical analysis of the most active extracts (EtOH fruit and leaf) from *A. melegueta*. Several peaks appeared in the chromatogram of the fruit EtOH extract and were identified and compared with standards from the NIST library (Figure 3.6A). The available constituents (1-5 and 8-10) in fruit EtOH extract were phenolics and other long chain fatty derivatives (Figure 3.7 and Table 3.3). The phenolics include eugenol (1), gingerol (8) and capsaicin (9), whereas the fatty derivatives detected were oleic acid (4), hexadecanoic derivatives (2, 3, 5) and 13-docosenoic acid, methyl ester (10) (Figure 3.7 and Table 3.3). Furthermore peaks visible from the chromatogram of leaf EtOH extract (Figure 3.6B) lead to identification of some phenolics; gingerol (8), 3-decanone, 1-(4-hydroxy-3-methoxyphenyl) (10), ethyl homovallinate (6), with other fatty acid derivatives as oleic acid (4) and 13-docosenoic acid, methyl ester (10) (Figure 3.7 and Table 3.3). It is also evident from the result that all the aromatic compounds (1, 6, 8, 9) identified in both extracts are of 4-hydroxy-3-methoxyphenyl derivatives.

Figure 3.6. GC-MS Chromatograms of ethanolic extracts of fruit (A) and leaf (B) of *A. melegueta*
Figure 3.7. Structures of identified compounds from fruit and leaf of A. melegueta.
Table 3.3: Identified compounds from the EtOH fruit and leaf extracts of *A. melegueta* by GC-MS

<table>
<thead>
<tr>
<th>Extracts</th>
<th>Compounds</th>
<th>Retention time (min)</th>
<th>Mass/amu</th>
</tr>
</thead>
<tbody>
<tr>
<td>EtOH fruit</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Eugenol</td>
<td>6.68</td>
<td>164 [M]+</td>
</tr>
<tr>
<td>2</td>
<td>n-Hexadecanoic acid</td>
<td>10.22</td>
<td>256 [M]+</td>
</tr>
<tr>
<td>3</td>
<td>Hexadecanoic acid, ethyl ester</td>
<td>10.39</td>
<td>284 [M]+</td>
</tr>
<tr>
<td>4</td>
<td>Oleic acid</td>
<td>11.11⁹</td>
<td>282 [M]+</td>
</tr>
<tr>
<td>5</td>
<td>Z-9-Hexadec-1-ol acetate</td>
<td>11.24²</td>
<td>282 [M]+</td>
</tr>
<tr>
<td>6</td>
<td>Gingerol</td>
<td>12.20</td>
<td>294 [M]+</td>
</tr>
<tr>
<td>7</td>
<td>Capsaicin</td>
<td>12.49</td>
<td>239 [M]+</td>
</tr>
<tr>
<td>10</td>
<td>13-Docosenoic acid, methyl ester, (Z)</td>
<td>13.37</td>
<td>352 [M]+</td>
</tr>
<tr>
<td>EtOH leaf</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Oleic acid</td>
<td>11.11</td>
<td>282 [M]+</td>
</tr>
<tr>
<td>6</td>
<td>3-Decanone, 1-(4-hydroxy-3-methoxyphenyl)</td>
<td>11.63</td>
<td>278 [M]+</td>
</tr>
<tr>
<td>8</td>
<td>Ethyl homovanillate</td>
<td>12.15</td>
<td>210 [M]+</td>
</tr>
<tr>
<td>6</td>
<td>Gingerol</td>
<td>12.20</td>
<td>294 [M]+</td>
</tr>
<tr>
<td>10</td>
<td>13-Docosenoic acid, methyl ester, (Z)</td>
<td>13.37</td>
<td>352 [M]+</td>
</tr>
</tbody>
</table>

⁹ isomers with same mass/amu at the different retention time

3.1.5 Discussion

Natural products in the form of crude or plant preparations have been used in the treatment and control of wide array of diseases. In many of such plants, some parts usually receive much attention compared to others. For instance *A. melegueta*, where most of the studies previously reported focused on seed alone, rendering the other parts to almost wastage. In this regard, we intend to explore the antioxidative and inhibition of key enzymes linked to T2D by various solvent extracts from fruit, leaf and stem of *A. melegueta*. This is of great importance as this will shade more light on the medicinal potentials of other parts of *A. melegueta*.

In previous studies, Adefegha and Oboh (2012a; 2011) have reported that, the seed aqueous extract of *A. melegueta* possessed higher polyphenolic and flavonoid contents, which was further supported in another study by Etoundi et al. (2010). Our data is partly in line with above-mentioned studies (Table 3.1). The higher polyphenolic and flavonoid contents of fruit EtOH extract compared to aqueous extract could be due variation in the method of extraction used. Additionally, in this present
study the polyphenolic and flavonoid contents of the aqueous extract were relatively similar with that of the seed aqueous extract reported earlier (Adefegha and Oboh, 2012a; 2011). Interestingly, leaf EtOH extract was also found to be rich in polyphenols and flavonoids contents. This is the first study that highlighted the contents of these phytochemicals from either the leaf or the stem parts. Furthermore, it is important to notice that, the amount and distribution of plants products such as polyphenols varies greatly from one species of plants to another or among the parts of the same plants. The production and accumulation of polyphenols are influenced by many factors, such as genetic and environmental factors (nature of the soil, high temperature and rain fall) in addition to growth or maturation stages for most of the parts of the plants (Mamphiswana et al. 2010; Pandey and Rizvi, 2009). Hence, the higher polyphenols and flavonoids contents of fruits compared to other parts could be attributed to one or combination of the above-mentioned factors, which were linked to most of the anti-oxidant ability of various plant-derived extracts.

In this study, a number of in vitro models were used to investigate the anti-oxidative potentials of various solvent extracts from different parts of *A. melegueta*. Among such model is the reduction of Fe$^{3+}$ to Fe$^{2+}$ by plant extracts or compounds is another reliable index and a good indicator of their electron-quenching abilities (Chung et al. 2002). This reaction is monitored by the formation of Perl’s Prussian blue color at 700 nm. In an attempt to further support the anti-oxidative nature of *A. melegueta*, Adefegha and Oboh, (2012b) have earlier reported higher reducing potential of *A. melegueta* aqueous seed extract, which correlate well with the results of our present study (Figure 3.1). Moreover, although no previous study is available on the *A. melegueta* leaf, our data have shown that leaf could be a good source of anti-oxidant in addition to seed and fruit that are popularly utilized. The higher ferric iron (Fe$^{3+}$) reducing anti-oxidant power of various extracts from *A. melegueta* scavenging activity of EtOH extracts than other extracts from different parts of *A. melegueta* could be attributed to their rich polyphenols and flavonoids contents of these extracts, which has been further supported by the DPPH radical scavenging activity (Table 3.2).

The DPPH radical scavenging activity is a quick and reliable method for determining the anti-oxidative nature of plant-derived products. DPPH is a stable radical that usually delocalized by accepting electrons from the referenced anti-oxidant, thereby becoming a stable molecule (Lobo et al. 2010). The anti-oxidative effectiveness of various solvent extracts was assessed base on the calculated IC$_{50}$ values. The lower the IC$_{50}$ value, the higher the anti-oxidative potential of the extract. In some previous studies various extracts from the *A. melegueta* seed have shown good radical scavenging capacities. Kazeem et al. (2012) have reported an IC$_{50}$ value of 0.110 ± 0.010 mg/mL for seed acetone extract, whereas 17.38 ± 2.00 mg/mL was earlier reported for seed aqueous extract (Adefegha and Oboh, 2012a). However, these are by far higher compared to that of fruit EtOH and aqueous extracts (EtOH: 0.04 ± 0.01mg/mL; aqueous: 0.04 ± 0.01 mg/mL) as well observed in our present study (Table 3.2). This has indicated higher electron scavenging ability of fruit than the seed part (Table 3.2).
Additionally, this is the lowest IC$_{50}$ value reported for DPPH scavenging of any extracts derived from *A. melegueta*.

Hemoglobin glycation is a non-enzymatic reaction occurs when proteins are exposed to excess reducing sugars and contributes immensely to the formation of advanced glycation end products that lead to oxidative stress (Yamagishi et al. 2008; Rahbar and Figarola, 2003; Brownlee et al. 1984). Kazeem et al. (2012) have earlier reported an IC$_{50}$ value of 0.125 ± 0.02 mg/mL for acetone extract (0.25-1 mg/mL), which is obviously lower compared to that of fruit EtOH extract and other solvent extracts (*Table 3.2*). This could be due to the lower concentrations (30-240 µg/mL) used in our present study. The results were further supported by the α-amylase and α-glucosidase inhibitory actions of various extracts from *A. melegueta* (*Table 3.2*).

On the other hand, the control of postprandial hyperglycemia is crucial for the management of DM and prevention of its complications at early stage (Ali et al. 2006). This is achieved by delaying the absorption of glucose from the small intestine through the inhibition of carbohydrates metabolizing enzymes such as α-amylase and α-glucosidase, located at the intestinal tract. The α-amylase catalyzes the endo-hydrolysis of α-1, 4-glucosidic linkage releasing disaccharides and oligosaccharides which are further hydrolyzed at the small intestinal brush border by α-glucosidases to release glucose (Hua-Quang et al. 2012; Hanhineva et al. 2010). In a previous study, Adefegha and Oboh (2012a) have reported an IC$_{50}$ value of 4.83 ± 0.56 mg/mL for α-amylase inhibition by seed aqueous extract. However, according to our results all the extracts derived from fruit part have demonstrated lower IC$_{50}$ values compared to that of seed aqueous extract reported earlier, indicating better activity of the fruit than the seed part (*Table 3.2*). Similarly, the calculated IC$_{50}$ value of the seed aqueous extract (2.14 ± 1.08 mg/mL) for α-glucosidase inhibition reported earlier was again far higher compared to that of fruit EtOH extract (0.06 ± 0.01 mg/mL) observed in our present study (Adefegha and Oboh, 2012a). Also observed in our data is that the IC$_{50}$ value of the fruit aqueous extract was about 6-fold lower compared to that reported by Adefegha and Oboh (2012a) for seed aqueous extract. Furthermore, the relatively similar α-glucosidase inhibitory action of the leaf and fruit EtOH extracts has shown that leaf could be another potential source for α-glucosidase inhibitors (*Table 3.2*).

Based on the results of this study, fruit and leaf EtOH extracts that exhibited higher activities were subjected to GC-MS analysis to find the possible bioactive compounds that could be responsible for their activities. The study reported by Ilic et al. (2010) has indicated that, seed EtOH extract contains mainly the phenolic compounds such as gingerol, shogaol and paradol, which supports our present finding. It is also evident that eugenol (1), gingerol (6), capsaicin (7) 3-decanone, 1-(4-hydroxy-3-methoxyphenyl) (10) and ethyl homovallinate (11), detected in fruit and leaf EtOH extracts were of 4-hydroxy-3-methoxyphenyl derivatives. Therefore, it is propose that the presence of phenolics is regarded as the key feature that could be responsible for the higher anti-oxidative and anti-diabetic effects of the fruit and leaf EtOH extracts observed in this study in addition to the contribution of other compounds (*Figure 3.7*). Phenolics and other related compounds have the ability to act as anti-oxidants
due to their low reduction potential compared to highly reactive species such as hydroxyl (‘OH), superoxide (O$_2^-$), nitric oxide (NO$^\cdot$) radicals. Their ability to donate electron or proton from hydroxyl moieties result in stabilizing lipid peroxidation, neutralizing ROS and ultimately inhibit the initiation and propagation of chain reaction associated with oxidative damage (Brewer, 2011). Likewise, it has been proposed that phenolics interact with one or multiple sites of the $\alpha$-glucosidase or $\alpha$-amylase surface proteins and thus form a hydrophobic layer. This leads to aggregation and precipitation which changes the conformation of the enzyme structure and hence decrease in activity (Toda et al. 2001; Spencer et al. 1988).

3.1.6 Conclusions

In conclusion, various solvent extracts from fruit and leaf of $A$. melegueta have demonstrated anti-oxidative as well as anti-diabetic effects in vitro when stem extracts showed very low or no significant activities in these regards. The fruit EtOH extract exhibited higher actions compared to other extracts of the same or different parts. Thus, it is recommended for further bioassay-guided fractionation in order to fully investigate the in vivo anti-diabetic and anti-oxidative effects of this extract.
According to the in vitro anti-oxidative and anti-diabetic studies of the various solvent extracts derived from different parts of A. melegueta stated above, fruit ethanolic extract demonstrated higher activities compared to other extracts. Therefore it was selected for further studies.

3.2 Phytochemistry, anti-oxidative and anti-diabetic effects of various solvent fractions from fruit ethanolic extract of Aframomum melegueta in vitro

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3.2.1 Abstract

Objectives: This study was aimed to investigate the anti-oxidative potentials and α-glucosidase and α-amylase inhibitory effects of various fractions derived from crude ethanolic (EtOH) extract of A. melegueta fruit. Additionally, possible bioactive compounds from the fraction with higher activity were analyzed using GC-MS.

Methods: Fruit EtOH extract was fractionated using solvents of increasing polarity. They were subjected and investigated for 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging activity, ferric reducing power, inhibition of hemoglobin glycosylation, α-amylase and α-glucosidase activities as markers of in vitro anti-diabetic effects at various doses (30-240 µg/mL). Possible compounds were analyzed using Gas Chromatography-Mass Spectroscopic (GC-MS) analysis.

Results: Our findings revealed that the ethyl acetate (EtOAc) fraction had the highest anti-oxidative activities (IC₅₀ values, DPPH: 15.49 ± 4.38 µg/mL; Hemoglobin glycosylation: 111.33 ± 6.46 µg/mL) and inhibition of α-amylase (IC₅₀ values: 68.69 ± 6.05 µg/mL) and α-glucosidase (IC₅₀ values: 40.44 ± 5.77 µg/mL) actions compared to other fractions and the respective standards used in this study. GC-MS analysis revealed that the EtOAc fraction contained some compounds aromatic in nature.

Conclusion: Data obtained from the study suggest that the EtOAc fraction derived from the EtOH extract of A. melegueta fruit possessed potent anti-oxidative as well as α-glucosidase and α-amylase inhibitory activities than other solvent fractions.

Keywords: Aframomum melegueta, Anti-oxidative, Ethyl acetate fraction, Type 2 diabetes
3.2.2 Introduction

Type 2 diabetes (T2D) is a heterogeneous disorder characterized by insulin resistance and partially dysfunctional pancreatic β-cell to properly secrete insulin in response to elevation of blood glucose levels (Hui et al. 2007). The inability of pancreatic β-cell to secret insulin disrupts the regulation of hepatic gluconeogenesis, muscles glucose uptake and lipolysis in adipose tissues. The consequence is postprandial hyperglycemia which results into T2D (Gastaldelli, 2011). Moreover, in chronic uncontrolled hyperglycemia, there is an increased production of reactive oxygen species (ROS) and a declined of in vivo anti-oxidant defense system, a term referred as oxidative stress (Maritim et al. 2003). The ROS are derived from the normal physiological processes and become highly deleterious if the levels increases and not arrested by complex anti-oxidant systems in the body (Chang and Chuang, 2010). The most common ROS include superoxide (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), peroxyl (ROO·) and reactive hydroxyl (OH·) radicals (Niki, 2010). Furthermore, increase intake of anti-oxidants has good correlation in amelioration of complication caused by ROS. However, people are now aware of the high risk caused by the synthetic anti-oxidants due to their chemical instability and hence depend on plant-derived anti-oxidants for food preservations and disease controls such as T2D.

On the other hand, inhibition of the activities of carbohydrate hydrolysing enzymes such as α-glucosidase has been used as one of the recent alternatives for the treatment and control of T2D (Adefegha and Oboh, 2012). This reduces the rate of intestinal glucose absorption which apparently decreases the postprandial hyperglycemia (Bhadari et al. 2008). Currently, some synthetic α-glucosidase inhibitors such as acarbose and miglitol are available for the control of T2D. But the undesirable adverse effects with the use of these drugs cause a shift to look for options from natural sources such as plants that are to cause fewer or no adverse side effects.

Interestingly, in most of the recent studies, plant-derived extracts and/or fractions have shown potent anti-oxidant as well as inhibition of α-glucosidase activities and therefore could possibly be a potential candidates for the treatment and control of T2D (Ibrahim et al. 2014; Kong et al. 2014; Adamson and Oboh, 2012; Adefegha and Oboh, 2012a).

*Aframomum melegueta* K. Schum. (Zingiberaceae) commonly known as guinea or alligator pepper is a native to west and central parts of Africa (Iwu, 2014). The seeds, leaf or fruit are consumed as flavoring agent in various traditional foods and are used locally to treat diseases such as diabetes, microbial infections, diarrhea, abdominal pain, snakebite and wounds healing in Nigeria, Kenya and Gabon (Iwu, 2014; Soladoye et al. 2012; Gbolade, 2009; Akendengue and Louis, 1994; Kokwaro, 1993). Previously, Sugita et al. (2013) have reported that *A. melegueta* seed alcoholic extract stimulates brown adipose tissue and increases whole-body energy expenditure in human subjects which is directly linked with the pathogenesis of T2D. Additionally, in a number of recent studies reported that various solvent extracts from *A. melegueta* seed have demonstrated potent anti-oxidant as well as anti-diabetic actions in vitro (Adefegha and Oboh 2012; Kazeem et al. 2012; Adefegha and Oboh 2011; Etoundi et
al. 2010) and in vivo (Onoja et al. 2014). Furthermore, in our recent study, we have reported that fruit ethanolic (EtOH) extract demonstrated higher anti-oxidant and anti-diabetic actions in vitro compared to other solvent extracts from the various parts of A. melegueta (Mohammed et al. 2015a). Therefore, in our effort to find potent anti-diabetic plant-derived formulations, we further partitioned and fractionated the crude EtOH extract from A. melegueta fruit with the hope to obtain the best fraction with highest anti-diabetic activities.

Therefore, our present study was aimed to investigate the anti-oxidative potentials and inhibitory effects of various fractions derived from crude EtOH extract of A. melegueta fruit on the activities of α-glucosidase and α-amylase enzymes. Additionally, possible bioactive compounds from the fraction with highest activities was also analysed using GC-MS.

3.2.3 Materials and methods

For sample collection, identification and preparation of the fractions, including the detail experimental protocols, please refer to chapter 2, sub-sections 2.3 and 2.4.

3.2.4 Results

The results of the percentage yield recovered, total polyphenols and flavonoids contents of various fractions from the fruit EtOH extract are presented in Table 3.4. It was observed from the data that the least polar solvent (hexane) recovered higher yield whereas the acetone, which is the most polar recovered the least amount. Similarly, the most polar fractions (ethyl acetate (EtOAc) and acetone) have demonstrated higher polyphenols and flavonoids contents compared to the less polar solvent fractions (hexane and dichloromethane). Furthermore, within the most polar, the polyphenols and flavonoids contents of the EtOAc fraction were relatively higher compared to that of acetone fraction (Table 3.4). The total polyphenols content was in the order; EtOAc > acetone > dichloromethane > hexane.

Table 3.4. Percentage yield, total polyphenol and flavonoid contents of various fractions from ethanolic fruit extract of A. melegueta

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Yield (%)</th>
<th>Total polyphenols content (mg/gGAE)</th>
<th>Total flavonoids content (mg/gQE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexane</td>
<td>17.80</td>
<td>$1.46 \pm 0.16^a$</td>
<td>$0.53 \pm 0.41^a$</td>
</tr>
<tr>
<td>Dichloromethane</td>
<td>10.54</td>
<td>$3.64 \pm 0.41^b$</td>
<td>$0.52 \pm 0.14^a$</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>5.60</td>
<td>$18.98 \pm 0.06^c$</td>
<td>$6.06 \pm 0.06^b$</td>
</tr>
<tr>
<td>Acetone</td>
<td>1.70</td>
<td>$16.21 \pm 0.70^c$</td>
<td>$5.02 \pm 0.34^b$</td>
</tr>
</tbody>
</table>

Data are presented as mean±SD values of triplicate determinations. Different superscripted letters for a given value within a column are significantly different from each other (Tukey’s-HSD multiple range * post hoc test, $p < 0.05$).
The results of ferric (Fe$^{3+}$) reducing total anti-oxidant power (expressed as gallic acid equivalents) of the various fractions are presented in Figure 3.8. It was observed that EtOAc fraction showed a significantly ($p < 0.05$) higher and concentration dependent reducing power compared to other solvent fractions as well as the standard ascorbic acid (Figure 3.8). Hexane and acetone fractions demonstrated similar Fe$^{3+}$ to Fe$^{2+}$ reducing abilities while dichloromethane fraction was the least active among the fractions tested (Figure 3.8).

![Figure 3.8. Total reducing power (relative to gallic acid) of various fractions from fruit ethanolic extract of A. melegueta. Data are presented as mean±SD of triplicate determinations. a-4 Different letters presented over the bars for a given concentration of each extract are significantly different from each other (Tukey’s-HSD multiple range post hoc test, $p < 0.05$).]

The data of DPPH radical scavenging activities and the calculated IC$_{50}$ values of various fractions from the fruit EtOH extract are presented in Figure 3.9 and Table 3.5, respectively. All the solvent fractions have shown variable scavenging abilities. The EtOAc fraction exhibited significantly ($p < 0.05$) lower IC$_{50}$ value (15.49 ± 4.38 µg/mL) for DPPH scavenging activities compared to other solvent fractions and comparable to standards used in this study (Ascorbic acid: 25.34 ± 6.19 µg/mL; Gallic acid: 20.01 ± 4.42 µg/mL) (Table 3.5).
Figure 3.9: DPPH Radical scavenging activity (%) of various fractions from fruit ethanolic extract of *A. melegueta*. Data are presented as mean±SD of triplicate determinations. a–e Values with different letters presented for a given concentration for each extract are significantly different from each other (Tukey’s-HSD multiple range post hoc test, *p* < 0.05).

Table 3.5: IC$_{50}$ values of various solvent fractions from ethanolic fruit extract of *A. melegueta* in different anti-oxidative and anti-diabetic models.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>DPPH scavenging activity</th>
<th>Non-enzymatic glycation of hemoglobin</th>
<th>α-amylase inhibitory effect</th>
<th>α-glucosidase inhibitory effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexane</td>
<td>356.63 ± 28.02$^d$</td>
<td>1728.72 ± 714.10$^e$</td>
<td>1567.63 ± 71.46$^e$</td>
<td>6351.8 ± 606.74$^d$</td>
</tr>
<tr>
<td>Dichloromethane</td>
<td>262.60 ± 12.11$^c$</td>
<td>4054.78 ± 387.76$^d$</td>
<td>268.56 ± 2.79$^b$</td>
<td>133.99 ± 1.85$^b$</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>15.49 ± 4.38$^a$</td>
<td>111.33 ± 6.46$^a$</td>
<td>68.69 ± 6.05$^a$</td>
<td>40.44 ± 5.77$^a$</td>
</tr>
<tr>
<td>Acetone</td>
<td>69.88 ± 3.34$^b$</td>
<td>398.80 ± 44.24$^c$</td>
<td>678.14 ± 49.09$^d$</td>
<td>356.23 ± 4.50$^c$</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>25.34 ± 6.19$^a$</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>20.01 ± 4.42$^a$</td>
<td>199.09 ± 2.85$^b$</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Acarbose</td>
<td>ND</td>
<td>ND</td>
<td>374.95 ± 26.81$^c$</td>
<td>211.59 ± 3.39$^b$</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SD values of triplicate determinations. a–e Different superscripted letters for a given value within a column are significantly different from each other (Tukey’s-HSD multiple range post hoc test, *p* < 0.05). ND, Not determined.

The results of inhibition of hemoglobin glycation and the calculated IC$_{50}$ values of various fractions are presented in Figure 3.10 and Table 3.5, respectively. The EtOAc fraction demonstrated significantly (*p* < 0.05) lower IC$_{50}$ (111.33 ± 6.46 µg/mL) compared to hexane (1728.72 ± 714.10 µg/mL) and dichloromethane (4054.78 ± 387.76 µg/mL) fractions. Similarly, the IC$_{50}$ value for EtOAc fraction was again significantly lower compared to acetone fraction (398.80 ± 44.24 µg/mL) and the
standard gallic acid (199.09 ± 2.85 µg/mL), although did not differ significantly among the fractions (Table 3.5).

![Graph showing inhibition of hemoglobin glycation of various fractions from fruit ethanolic extract of A. melegueta.](image1)

Figure 3.10: Inhibition of hemoglobin glycation (%) of various fractions from fruit ethanolic extract of A. melegueta. Data are presented as mean±SD of triplicate determinations. **a-d** Values with different letters presented for a given concentration for each extract are significantly different from each other (Tukey’s-HSD multiple range post hoc test, p < 0.05).

The data of α-amylase inhibition by the various fractions and the calculated IC₅₀ values are presented in Figure 3.11 and Table 3.5, respectively. EtOAc fraction had exhibited a significantly (p < 0.05) lower calculated IC₅₀ value (68.69 ± 6.05 µg/mL) towards α-amylase inhibition compared to other solvent fractions and acarbose as well (Table 3.5). The activity of the acetone fraction did not differ significantly (p < 0.05) compared to the standard acarbose.

![Graph showing α-amylase inhibition of various fractions from fruit ethanolic extract of A. melegueta.](image2)

Figure 3.11: α-Amylase inhibition (%) of various fractions from fruit ethanolic extract of A. melegueta. Data are presented as mean±SD of triplicate determinations. **a-e** Values with different letters presented for a given concentration for each extract are significantly different from each other (Tukey’s-HSD multiple range post hoc test, p < 0.05).
Furthermore, results of \( \alpha \)-glucosidase inhibition by the various fractions and the calculated IC\(_{50} \) values are presented in Figure 3.12 and Table 3.5, respectively. The EtOAc fraction had showed a significantly \( (p < 0.05) \) lower IC\(_{50} \) value (40.44 ± 5.77 µg/mL) for \( \alpha \)-glucosidase inhibition compared to other solvent fractions and acarbose (211.59 ± 3.32 µg/mL) (Table 3.5). The inhibitory action exhibited by the EtOAc was almost 5-fold higher compared to the standard acarbose. Additionally, the order of inhibition by the various fractions was in the order; EtOAc > dichloromethane > acarbose >acetone > hexane.

![Figure 3.12: \( \alpha \)-Glucosidase inhibition (%) of various fractions from fruit ethanolic extract of A. melegueta. Data are presented as mean ± SD of triplicate determinations. Values with different letters presented for a given concentration for each extract are significantly different from each other (Tukey’s-HSD multiple range post hoc test, \( p < 0.05 \)).](image)

The data of the kinetics of the inhibitory mode of the \( \alpha \)-glucosidase and \( \alpha \)-amylase by the most active fraction are presented in Figure 3.13. According to the results, EtOAc fraction has exhibited a non-competitive inhibition towards \( \alpha \)-glucosidase action. The K\(_{M} \) value remains unchanged (1.10 mmol/L), whereas the V\(_{max} \) was found to decreases from 471.56 µmol/min to 114.45 µmol/min and the equilibrium constant for inhibitor binding (K\(_{i} \)) was calculated as 9.68 µg/mL (Figure 3.13A). On the other hand, the mode of inhibition of \( \alpha \)-amylase action by the EtOAc fraction was observed to be a mixed inhibition type. That is upon addition of the fraction, the K\(_{M} \) value (control: 0.06%; with EtOAc: 0.16%) was found to increase, when the value of V\(_{max} \) (control: 35.67 µmol/min; with EtOAc: 22.99 µmol/min) was observed to decrease and the K\(_{i} \) value for inhibiting \( \alpha \)-amylase was 54.34 µg/mL (Figure 3.13B).
Figure 3.13: Lineweaver-Burke’s plot of (A) α-glucosidase and (B) α-amylase catalyzed reactions in the presence and in the absence of the ethyl acetate (EtOAc) fraction derived from the A. melegueta fruit ethanolic extract.

In Figure 3.14 and Table 3.6, possible bioactive compounds detected from the most active fraction (EtOAc) including the chromatogram and their chemical structures are presented. Several peaks appeared in the chromatogram of the EtOAc fraction (Figure 3.14A) and were identified by their fragmentation pattern and in conjunction with the NIST library. The major peaks correspond to 4-methylphenol (1), 2,6-dimethylphenol (2), isobornyl propionate (3), eugenol (4), 5-ethyl-2-methyl thiazole (5), 4-hydroxy-3-methoxyphenyl butan-2-one (6), benzyl benzoate (7), 1,2-benzenedicarboxylic acid, butyl 2-methylpropyl ester (8), 4-hydroxy-3-methoxyphenyl decan-3-one (9) and 1,2-benzenedicarboxylic acid, mono(2-ethylhexyl) ester (10) (Table 3.6). Additionally, with the exception of compounds 3 and 5, the remaining compounds are aromatic in nature when compounds 1, 4, 6 and 9 are phenolic derivatives (Figure 3.14B).
Table 3.6: Identified compounds from the ethyl acetate fraction from fruit ethanolic extract of *A. melegueta* by GC-MS

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Compounds</th>
<th>Retention time (min)</th>
<th>Mass/amu</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethyl acetate</td>
<td>1-Methylphenol</td>
<td>4.44</td>
<td>107 [M]^+</td>
</tr>
<tr>
<td></td>
<td>2,6-Dimethylphenol</td>
<td>5.07</td>
<td>122 [M]^+</td>
</tr>
<tr>
<td></td>
<td>Isobornyl propionate</td>
<td>6.22</td>
<td>210 [M]^+</td>
</tr>
<tr>
<td></td>
<td>Eugenol</td>
<td>6.67</td>
<td>164 [M]^+</td>
</tr>
<tr>
<td></td>
<td>5-Ethyl-2-methyl thiazole</td>
<td>7.65</td>
<td>127 [M]^+</td>
</tr>
<tr>
<td></td>
<td>4-Hydroxy-3-methoxyphenyl butan-2-one</td>
<td>8.58, 12.43</td>
<td>194 [M]^+</td>
</tr>
<tr>
<td></td>
<td>Benzylational benzoate</td>
<td>9.36</td>
<td>212 [M]^+</td>
</tr>
<tr>
<td></td>
<td>1,2-Benzenedicarboxylic acid, butyl-2-methylpropyl ester</td>
<td>10.26</td>
<td>278 [M]^+</td>
</tr>
<tr>
<td></td>
<td>4-Hydroxy-3-methoxyphenyl decan-3-one</td>
<td>11.63</td>
<td>278 [M]^+</td>
</tr>
<tr>
<td></td>
<td>1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester</td>
<td>13.17</td>
<td>279 [M]^+</td>
</tr>
</tbody>
</table>
Figure 3.14. GC-MS Chromatogram (A) and the structures of compounds (B) identified from the ethyl acetate fraction from fruit ethanolic extract of *A. melegueta*
3.2.5 Discussion

T2D has been associated with a number of complications such as retinopathy, neuropathy, nephropathy, myocardial infarction, atherosclerosis and peripheral vascular diseases, which partly or completely depend on uncontrolled hyperglycemia (Heydari et al. 2010; Rahman et al. 2007; Monnier et al. 2006). The hyperglycemia initiate a sequence of oxidative processes that cause dysfunction and failure of organs such as liver, kidney, eyes and other peripheral tissues in type 2 diabetic patients. Several synthetic drugs were developed to control the hyperglycemia. But the associated adverse effects with the use of these drugs cause a shift to look for options from plant-derived products. This is because fewer side effects were associated with the use of plant-derived formulations (Singh, 2001). Therefore, our current study investigated the anti-oxidative potentials and inhibitory effects of various fractions derived from fruit EtOH extract of *A. melegueta* fruit on the activities of α-glucosidase and α-amylase actions in vitro.

In our recent study, we have reported higher polyphenols and flavonoids contents in the fruit EtOH extract compared to other extracts of the same or different parts of *A. melegueta* (Mohammed et al. 2015). In another studies, Adefegha and Oboh (2012; 2011) have reported higher polyphenols in the seed aqueous extract. These are in line with our present results, when higher contents were observed in the most polar fractions (EtOAc and acetone) (Table 3.4). This has signified that polarity of the solvent play a role in the availability of the polyphenols in the fractions. Polyphenols are anti-oxidants that can prevent the deleterious actions of reactive oxygen species (ROS) to cellular biomolecules thereby modulating the initiation and propagation of oxidative chain reactions, and thus attenuate oxidative stress (Alia et al. 2003). Furthermore, the higher polyphenolic content of the EtOAc fraction was further supported by the greater radical scavenging activities of the fraction than other solvent fractions (Figure 3.8).

The reduction of Fe$^{3+}$ to Fe$^{2+}$ is a reliable method of assessing the anti-oxidant nature of various plant-derived extracts, fractions and/or bioactive compounds (Chung et al. 2002). The higher reducing power of EtOAc fraction observed in our present study suggest that most of the anti-oxidant biomolecules in crude extract are available in this fraction (Figure 3.8). This might be attributed to the rich polyphenols of the EtOAc fraction and was further supported by the greater DPPH scavenging ability (Table 3.5).

According to the studies reported earlier, the fruit or seed extracts have demonstrated better radical scavenging ability compared to other extracts (Mohammed et al. 2015; Kazeem et al. 2012; Adefegha and Oboh, 2012: 2011). For instance, in our recent study, fruit EtOH extract has exhibited potent electron quenching ability and depicted the least IC$_{50}$ value for DPPH scavenging ability (0.04 ± 0.01 mg/mL) (Mohammed et al. 2015). However, based on our present data the IC$_{50}$ value for EtOAc fraction (15.49 ± 4.38 µg/mL) was lower and the least IC$_{50}$ value for DPPH scavenging ability reported
for any extract or fraction derived from *A. melegueta* Table 3.5). This was again supported by its capacity to inhibit hemoglobin glycosylation (Table 3.5).

Kazeem et al. (2012) have previously indicated that seed acetone extract possessed strong anti-glycosylation property. However, in our present study, such higher anti-glycosylation property was depicted by the EtOAc fraction derived from the fruit EtOH extract (Table 3.5). The higher inhibition of hemoglobin glycosylation by EtOAc fraction indicated the ability of this fraction to attenuate the hyperglycemia-induced oxidative stress, which may also be attributed the rich polyphenols of the EtOAc fraction compared to other fractions.

On the other hand, among the recent approach to control postprandial hyperglycemia involves the inhibition of **a**-amylase and **a**-glucosidase actions, which are key relevant enzymes linked to T2D located at the enterocyte brush border (Imam, 2015). They are known to be involved in the breakdown and absorption of complex carbohydrates biomolecules at intestinal tract (Hua-Quang et al. 2012; Hanhineva et al. 2010). Inhibitors of the activities of these enzymes such as acarbose and miglitol are currently utilized as potential anti-T2D agent. But the associated adverse effects such as diarrhea, bloating, flatulence, cramping and abdominal pain decrease their popularity (Fujisawa et al. 2005). This has shifted the search to plant-derived products such as *A. melegueta* for alternatives. In some previous studies, seed aqueous extract has exhibited good inhibitory effects toward the activities of **a**-amylase and **a**-glucosidase (Adefegha and Oboh, 2012: 2011). Moreover, in our comparative study, fruit EtOH extract was found to demonstrated higher inhibition of **a**-amylase and **a**-glucosidase actions compared to other solvent extract of the same or different parts of *A. melegueta* (Mohammed et al. 2015).

However, in our present study, when the crude EtOH extract was solvent-solvent fractionated, the inhibition of the EtOAc fraction was by far better compared to even the most polar fraction (acetone) and acarbose as well (Table 3.5). Therefore, we may suggest the presence of more active inhibitory compounds in EtOAc fraction that could delay the digestion of complex carbohydrates and subsequently intestinal glucose absorption, thereby reducing the postprandial hyperglycemia in T2D.

According to the kinetic parameters derived from the double reciprocal plot revealed that, the EtOAc fraction is a non-competitive inhibitor of **a**-glucosidase action (Figure 3.13A). Therefore, it is suggested that, EtOAc fraction binds to other site(s), apart from the active site of the enzyme, and induces some conformational changes in the three-dimensional structure of the enzyme. This slows down the activity of the enzyme. In addition, the affinity of the enzyme to the substrate is not affected since the *K*<sub>M</sub> value remains the same in the presence or absence of the inhibitor (EtOAc fraction). In the case of **a**-amylase action, it was observed that both the *V*<sub>max</sub> and *K*<sub>M</sub> are affected in the presence of the EtOAc fraction, which implied that the mode of inhibition of the fraction is a mixed inhibition type (Figure 3.13B). In this type of inhibition, both the *K*<sub>M</sub> and *V*<sub>max</sub> of the enzyme are greatly affected in the presence of the inhibitor. This tends to cause some conformational modifications at the active site of the enzyme that hinder the binding of the substrate and thus decreases the enzyme activity. It has been reported that, when the *K*<sub>M</sub> value increases and the *V*<sub>max</sub> decreases, then the inhibitor binds
favorably to the enzyme in free-form and thereby decrease the affinity of the enzyme for the substrate (Storey, 2004). Therefore, based on our present data, it seems that the EtOAc fraction binds to the α-amylase in a free state before binding to its substrate (starch) and results in decrease activity.

Based on the higher anti-oxidative and anti-diabetic actions exhibited by the EtOAc fraction, it was subjected to GC-MS analysis to detect possible bioactive compounds present in this fraction. It has been highlighted previously that *A. melegueta* fruit or seed extracts are rich in phenolic compounds such as gingerol, paradol, shagaol and eugenol (Mohammed et al. 2015; Ilic et al. 2010). This is in line with our present data and could possibly be attributed to the observed higher actions of EtOAc fraction than other solvent fractions. It is also possible from our results that, other compounds detected from the EtOAc fraction might further contribute significantly to the higher anti-oxidative and enzyme inhibitory actions of the EtOAc fraction (*Table 3.6* and *Figure 3.14B*). In a nutshell, these compounds may act individually or in synergy to achieve the strong activities by the EtOAc fraction reported in our present study.

### 3.2.6 Conclusions

In conclusion, various solvent fractions derived from the fruit ethanolic extract of *A. melegueta* possessed anti-oxidative as well as α-amylase and α-glucosidase inhibitory actions *in vitro*. The EtOAc fraction has exhibited higher activities compared to other fractions. Thus, it is recommended for further *in vivo* anti-diabetic and anti-oxidative actions to fully validate the efficacy of this fraction.
According to the anti-oxidative and anti-diabetic study of the various solvent fractions derived from ethanolic extract of *A. melegueta* fruit stated above, ethyl acetate fraction exhibited higher activities compared to other solvent fractions. Therefore it was selected for the *in vivo* studies.

### 3.3 Ethyl acetate fraction of *Aframomum melegueta* fruit ameliorates pancreatic β-cell dysfunction and major diabetes-related parameters in a type 2 diabetes model of rats

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**3.3.1 Abstract**

**Objectives:** In West Africa, various preparations of the fruit, seed and leaf of *Aframomum melegueta* K. Schum. are reputedly used for the management of diabetes mellitus (DM) and other metabolic disorders. The present study evaluated the anti-diabetic effects of *A. melegueta* ethyl acetate fraction (AMEF) from fruit ethanolic extract in a type 2 diabetes (T2D) model of rats.

**Methods:** T2D was induced in rats by feeding a 10% fructose solution *ad libitum* for two weeks followed by a single intraperitoneal injection of streptozotocin (40 mg/kg body weight) and the animals were orally treated with 150 or 300 mg/kg body weight (bw) of the AMEF once daily for four weeks.

**Results:** At the end of the intervention, diabetic untreated animals showed significantly higher serum glucose, serum fructosamine, LDH, CK-MB, serum lipids, liver glycogen, insulin resistance (HOMA-IR), AI, CRI and lower serum insulin, β-cell function (HOMA-β) and glucose tolerance ability compared to the normal animals. Histopathological examination of their pancreas revealed corresponding pathological changes in the islets and β-cells. These alterations were reverted to near-normal after the treatment of AMEF at 150 and 300 mg/kg bw when, the effects were more pronounced at 300 mg/kg bw compared to the 150 mg/kg bw.

**Conclusion:** The results of our study suggest that AMEF treatment at 300 mg/kg bw showed potent anti-diabetic effect in a T2D model of rats.

**Keywords:** *Aframomum melegueta*, Ethyl acetate fraction, Fruit ethanolic extract, Type 2 diabetes, Rats.
3.3.2 Introduction

Diabetes mellitus (DM) is a condition that causes hyperglycemia due to either decreased insulin secretion or insulin sensitivity of target tissues (Panni, 2013). Recent data indicate that more than 387 million people have DM and this figure is likely to be doubled by 2035 (IDF, 2014). Among the major types of diabetes (type 1 and type 2), type 2 is the most prevalent one, accounting for more than 90% of all diabetic cases. Type 2 diabetes (T2D) is a heterogeneous disorder characterized by insulin resistance and partially dysfunctional pancreatic β-cells which cannot properly secrete insulin in response to hyperglycemia (Hui et al. 2007). Insulin resistance usually occurs in the early stage of T2D after which a further decline of β-cells is induced, resulting in hyperglycemia and hyperlipidemia (Robertson et al. 2004).

The current treatment option for T2D include mainly oral anti-diabetic drugs, such as sulfonylureas, biguanides, α-glucosidase inhibitors, thiazolidinediones and dipeptidyl peptidase-4 (DPP-4) inhibitors among others. But these drugs do not adequately improve the consequences of T2D and have caused characteristic profiles of serious side effects, which include hypoglycemia, weight gain, hypersensitivity, gastrointestinal discomfort, nausea, liver and heart failure, and diarrhoea (Hung et al. 2012). Therefore, search for novel molecules has been extended to natural plant-based remedies, as they are considered more effective, safe, natural and cause less adverse effects compared to modern synthetic drugs.

Aframomum melegueta K. Schum. (Zingiberaceae) commonly known as guinea pepper is a herbaceous plant that grows up to 1500 cm height, with purple flowers that develop into 5-7 cm long pods containing small, reddish-brown aromatic and pungent seeds. The fruits are ovoid with reddish color and numerous small brownish angular seeds with a cardamorn flavor (Iwu, 2014). Fruit, seed and leaf of A. melegueta are commonly used as spices in various food preparations and have been locally used in the treatment of DM and other metabolic disorders in Nigeria (Gbolade, 2009). In a recent study, Sugita et al. (2013) reported that A. melegueta seed alcoholic extract stimulates brown adipose tissue and increases whole-body energy expenditure in human subjects which is directly linked with the pathogenesis of T2D. In a very recent study, we showed that different solvent extracts from the various parts (fruit, leaf and stem) of A. melegueta demonstrated anti-oxidative and anti-diabetic potentials in vitro (Mohammed et al. 2015a). Similarly, in vitro anti-diabetic as well as anti-oxidative potentials of the aqueous seed extracts of A. melegueta have been reported in many recent studies (Adefegha and Oboh 2012; Kazeem et al., 2012; Adefegha and Oboh 2011; Etoundi et al., 2010). In another study, Onoja et al. (2014) have reported the anti-oxidative potentials of seed methanolic extracts of A. melegueta both in vitro and in vivo. Additionally, phenolics such as gingerols, shogaols, paradols are compounds reported to be rich in the crude ethanolic extract of the seed which showed minimum toxicity after a four week oral treatment in rats (Ilic et al., 2010). In preliminary studies, seed (Adesokan et al., 2010) and leaf (Mojekwu et al., 2011) aqueous extracts of A. melegueta have demonstrated blood
glucose lowering ability in alloxan-induced diabetic rats. However, the detail in vivo anti-diabetic study on any part, extract or fraction of this plant has not yet been conducted either in humans or in experimental animals till today.

Therefore, the present study was designed to investigate the in vivo anti-diabetic effects of the ethyl acetate fraction derived from the fruit ethanolic extract of *A. melegueta* in a T2D model of rats.

### 3.3.3 Materials and methods

For sample collection, identification and preparation of the fraction, including the detail experimental protocols, please refer to chapter 2, sub-sections 2.3 and 2.5.1 to 2.5.8.

### 3.3.4 Results

The results of weekly mean body weight (bw) are presented in Figure 3.15. Although fructose feeding for two weeks did not significantly affect the mean bw of the treated animals compared to untreated animals, after STZ injection, the bw of DBC group was decreased for the entire intervention period. On the other hand, AMEF oral treatment ameliorated this reduction when significant (*p* < 0.05) amelioration was observed after the 2nd, 3rd and 4th week of intervention. Additionally, no significant difference of bw was observed between the AMEF treated groups (DAML and DAMH) and NC and DMF groups (Figure 3.15).

![Figure 3.15](image)

**Figure 3.15.** Mean body weight change in all animal groups during the entire study period. Data are presented as the mean ± SD of 5-7 animals. **Values with different letter near the lines for a given week 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; DAML, Diabetic *A. melegueta* low dose; DAMH, Diabetic *A. melegueta* high dose; DMF, Diabetic Metformin; NAMH, Normal *A. melegueta* high dose.

The results of feed and fluid intake in different group of animals are presented in Figure 3.16. The results of DBC group suggest that the induction of diabetes significantly (*p* < 0.05) increased feed
and fluid intake compared to NC group (Figure 3.16). Treatment with various dosages of AMEF for weeks to diabetic animals significantly \((p < 0.05)\) ameliorated the alterations of feed and fluid intake which are comparable to the DMF group (Figure 3.16).

![Figure 3.16](image.png)

**Figure 3.16.** Mean food and fluid intake of different animal groups during 4-week intervention period. Data are presented as the mean ± SD of 5-7 animals. \(^\text{a-d}\)Values with different letters over the bars for a given parameter 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; DAML, Diabetic *A. melegueta* low dose; DAMH, Diabetic *A. melegueta* high dose; DMF, Diabetic Metformin; NAMH, Normal *A. melegueta* high dose.

The results of weekly NFBG and FBG (last week only) are presented in Figure 3.17. The data showed that the induction of T2D significantly \((p < 0.05)\) increased NFBG in diabetic animals compared to the normal animals (NC). On the other hand, the oral administration of AMEF significantly \((p < 0.05)\) decreased NFBG in DAML and DAMH groups compared to the DBC group when no significant difference was observed between the AMEF (DAML and DAMH) and metformin (DMF) treated groups. This effect was more pronounced in the DAMH group compared to the DAML group. Additionally, no significant difference was observed between the last week FBG of DAMH and NC group (Figure 3.17).
Figure 3.17. Weekly NFBG of all animal groups during the entire experimental period. Data are presented as the mean ± SD of 5-7 animals. Values with different letters near the lines for a given week 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; DAML, Diabetic *A. melegueta* low dose; DAMH, Diabetic *A. melegueta* high dose; DMF, Diabetic Metformin; NAMH, Normal *A. melegueta* high dose; NFBG, Non-fasting blood glucose; FBG, Fasting blood glucose.

The data of oral glucose tolerance test (OGTT) are presented in Figure 3.18. According to the results, the glucose tolerance ability of the animals in AMEF treated groups was significantly better than the DBC group for the entire period of the test when the results were significantly better in the AMEF treated groups compared to the DMF group from 60-120 min of the test period. No significant difference was observed between the glucose tolerance ability of AMEF treated groups and NC group during this period as well (Figure 3.18). Furthermore, the calculated area under curve (AUC) for DBC group was significantly ($p<0.05$) higher compared to NC group when that of DAML and DAMH groups were significantly ($p<0.05$) lower compared to DBC groups and were comparable to the DMF group (Table 3.7).
Figure 3.18. Oral glucose tolerance test (OGTT) in all animal groups in the last week of the 4-week experimental period. Data are presented as the mean ± SD of 5-7 animals. *Values with different letters near the lines for a given parameter 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; DAML, Diabetic \textit{A. melegueta} low dose; DAMH, Diabetic \textit{A. melegueta} high dose; DMF, Diabetic Metformin; NAMH, Normal \textit{A. melegueta} high dose.

Table 3.7. Area under the curve (AUC) of different animal groups at the end of the experimental period

<table>
<thead>
<tr>
<th>Group</th>
<th>AUC ( \times 10^3 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td>15.75 ± 0.97(^a)</td>
</tr>
<tr>
<td>DBC</td>
<td>60.15 ± 7.50(^c)</td>
</tr>
<tr>
<td>DAML</td>
<td>24.54 ± 3.50(^b)</td>
</tr>
<tr>
<td>DAMH</td>
<td>25.32 ± 5.64(^b)</td>
</tr>
<tr>
<td>DMF</td>
<td>28.68 ± 7.21(^b)</td>
</tr>
<tr>
<td>NAMH</td>
<td>15.78 ± 0.97(^a)</td>
</tr>
</tbody>
</table>

Data are presented as the mean ± SD of 5-7 animals. \(^a-c\)Values with different letter along a row for a given parameter 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; DAML, Diabetic \textit{A. melegueta} low dose; DAMH, Diabetic \textit{A. melegueta} high dose; DMF, Diabetic Metformin; NAMH, Normal \textit{A. melegueta} high dose, AUC, Area under curve.

The data for serum insulin, fructosamine and calculated HOMA-IR and HOMA-\( \beta \) scores are presented in Table 3.8. Serum insulin level and the calculated HOMA-\( \beta \) scores were decreased significantly \( (p < 0.05) \) whereas serum fructosamine as well as HOMA-IR were increased significantly \( (p < 0.05) \) in the DBC group compared to the NC group (Table 3.8). Oral intervention of AMEF for four weeks to diabetic animals elevated serum insulin and HOMA-\( \beta \) scores with subsequent attenuation of serum fructosamine level and HOMA-IR scores in DAML and DAMH groups compared to the DBC group. These effects were found to be more pronounced in the DAMH group compared to the DAML group which is comparable with DMF group (Table 3.8).
**Table 3.8.** Serum insulin and fructosamine levels, 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>DAML</th>
<th>DAMH</th>
<th>DMF</th>
<th>NAMH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin (pmol/L)</td>
<td>76.50 ± 6.26&lt;sup&gt;b&lt;/sup&gt;</td>
<td>47.05 ± 9.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>59.86 ± 11.45&lt;sup&gt;b&lt;/sup&gt;</td>
<td>64.81 ± 8.77&lt;sup&gt;b&lt;/sup&gt;</td>
<td>75.88 ± 14.23&lt;sup&gt;b&lt;/sup&gt;</td>
<td>65.49 ± 11.87&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Fructosamine (μmol/L)</td>
<td>239.20 ± 47.49&lt;sup&gt;a&lt;/sup&gt;</td>
<td>365.43 ± 44.94&lt;sup&gt;b&lt;/sup&gt;</td>
<td>298.00 ± 23.39&lt;sup&gt;a&lt;/sup&gt;</td>
<td>247.86 ± 28.96&lt;sup&gt;a&lt;/sup&gt;</td>
<td>283.14 ± 32.46&lt;sup&gt;a&lt;/sup&gt;</td>
<td>274.00 ± 19.95&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>HOMA-IR *</td>
<td>2.50 ± 0.27&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.30 ± 1.64&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.75 ± 0.53&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.11 ± 0.61&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.82 ± 0.90&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.11 ± 0.44&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>HOMA-β **</td>
<td>127.66 ± 43.71&lt;sup&gt;d&lt;/sup&gt;</td>
<td>7.33 ± 1.26&lt;sup&gt;a&lt;/sup&gt;</td>
<td>26.17 ± 10.18&lt;sup&gt;b&lt;/sup&gt;</td>
<td>44.32 ± 9.86&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>52.57 ± 25.76&lt;sup&gt;c&lt;/sup&gt;</td>
<td>108.60 ± 19.24&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data are presented as the mean ± SD of 5-7 animals. *Values with different letters along the rows for a given parameter 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; DAML, Diabetic Annona melegueta low dose; DAMH, Diabetic Annona melegueta high dose; DMF, Diabetic Metformin; NAMH, Normal Annona melegueta high dose; HOMA-IR, Homeostasis model assessment-insulin resistance; HOMA-β, Homeostasis model assessment-β

* HOMA-IR = [(Fasting serum insulin in U/l x Fasting blood glucose in mmol/L)/22.5]. ** HOMA-β = (Fasting serum insulin in U/l x 20/Fasting blood glucose in mmol/L-3.5).

**Figure 3.19.** Histopathological examination of the pancreatic islets of different animal groups at the end of the intervention period. The NC had a larger islet with high number of β-cells while the DBC had a smaller islet and morphologically deformed β-cells. The DAML, DAMH and DMF groups had relatively larger islets with higher number of β-cells compared to the DBC group. NC, Normal Control; DBC, Diabetic Control; DAML, Diabetic Annona melegueta low dose; DAMH, Diabetic Annona melegueta high dose; DMF, Diabetic Metformin; NAMH, Normal Annona melegueta high dose.
The slides of pancreatic histopathology are presented in Figure 3.19. There was a significant degeneration of pancreatic islets with subsequent reduction on the number of β-cells in the DBC group compared to the NC group. However, treatment with the various dosages of AMEF as well as standard drug attenuated the diabetes induced pancreatic damage and restored the pancreatic morphology to near normal when the number of β-cells were significantly higher in the DAMH group compared to the DAML group (Figure 3.19).

The results of liver weight, relative liver weight and liver glycogen levels are presented in Table 3.9. The induction of T2D did not affect the absolute liver weight but significantly ($p < 0.05$) increase the relative liver weight and liver glycogen level in the DBC group compared to the NC group. A significant ($p < 0.05$) reduction of relative liver weight and liver glycogen levels was observed in the DAML and DAMH groups compared to the DBC group when the results were not significantly different compared to the NC and DMF groups (Table 3.9).

The data for serum lipid profile and calculated atherogenic index (AI) and coronary risk index (CRI) are presented in Table 3.10. Elevated serum concentrations of TC, TG and LDL-cholesterol levels as well as calculated AI and CRI with subsequent reduction on serum HDL-cholesterol were observed in the DBC group compared to the NC group (Table 3.10). Treatment with AMEF to diabetic animals significantly ($p < 0.05$) and dose-dependently reduced TC, TG and LDL-cholesterol, AI and CRI in DAML and DAMH groups compared to the DBC group. Although dose-dependent increase in serum HDL-cholesterol level was observed in the AMEF treated groups compared to the DBC and DMF groups, the data were not significantly different (Table 3.10).

The data for serum ALT, AST, ALP, urea, uric acid, creatinine, LDH and CK-MB are presented in Table 3.11. At the end of the experimental period, the concentrations of all of the above-mentioned serum parameters were increased in the DBC group compared to the NC group, when serum AST level was not affected by the induction of diabetes. On the other hand, oral administration of AMEF to diabetic animals significantly ($p < 0.05$) ameliorated these alterations in DAML and DAMH groups. The effects were more pronounced in the DAMH group compared to the DAML group which did not differ significantly ($p < 0.05$) compared to the DMF group regarding most of the parameters. Similarly, treatment of AMEF to non-diabetic animals demonstrated no significant ($p < 0.05$) effect on these serum parameters compared to the NC group (Table 3.11).
Table 3.9. Effect of AMEF on liver weights and liver glycogen concentrations in different animal groups as the end of the experimental period.

<table>
<thead>
<tr>
<th></th>
<th>NC</th>
<th>DBC</th>
<th>DAML</th>
<th>DAMH</th>
<th>DMF</th>
<th>NAMH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver weight (g)</td>
<td>9.25 ± 1.04</td>
<td>10.34 ± 0.31</td>
<td>9.79 ± 0.30</td>
<td>9.75 ± 0.40</td>
<td>9.10 ± 1.33</td>
<td>9.02 ± 0.70</td>
</tr>
<tr>
<td>Relative liver weight (%)*</td>
<td>2.89 ± 0.29a</td>
<td>4.55 ± 0.34b</td>
<td>3.36 ± 0.04a</td>
<td>3.31 ± 0.40a</td>
<td>3.10 ± 0.45a</td>
<td>2.90 ± 0.25a</td>
</tr>
<tr>
<td>Liver glycogen (mg/g tissue)</td>
<td>6.76 ± 0.81a</td>
<td>8.25 ± 0.83b</td>
<td>7.03 ± 0.53a</td>
<td>6.98 ± 0.63a</td>
<td>6.92 ± 0.56a</td>
<td>6.80 ± 0.57a</td>
</tr>
</tbody>
</table>

Data are presented as the mean ± SD of 5-7 animals. a-b Values with different letter along a row for a given parameter 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; DAML, Diabetic *A. melegueta* low dose; DAMH, Diabetic *A. melegueta* high dose; DMF, Diabetic Metformin; NAMH, Normal *A. melegueta* high dose.

* Relative liver weight (%) = [weight of the liver (g)/body weight (g)] x 100%
Table 3.10. Serum lipid profiles atherogenic and coronary risk indices of different animal groups at the end of the experimental period

<table>
<thead>
<tr>
<th></th>
<th>NC</th>
<th>DBC</th>
<th>DAML</th>
<th>DAMH</th>
<th>DMF</th>
<th>NAMH</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC (mg/dL)</td>
<td>76.20 ± 7.40(^a)</td>
<td>116.00 ± 12.92(^c)</td>
<td>94.86 ± 10.17(^b)</td>
<td>83.29 ± 8.40(^{a,b})</td>
<td>95.71 ± 7.74(^b)</td>
<td>78.00 ± 7.91(^a)</td>
</tr>
<tr>
<td>TG (mg/dL)</td>
<td>70.20 ± 18.10(^a)</td>
<td>150.86 ± 24.57(^b)</td>
<td>107.71 ± 38.13(^a)</td>
<td>81.14 ± 23.78(^a)</td>
<td>100.71 ± 32.47(^a)</td>
<td>68.80 ± 5.50(^a)</td>
</tr>
<tr>
<td>LDL-Cholesterol (mg/dL)(^*)</td>
<td>12.96 ± 4.16(^a)</td>
<td>57.54 ± 14.45(^c)</td>
<td>40.03 ± 11.21(^b)</td>
<td>30.34 ± 7.28(^{a,b})</td>
<td>42.86 ± 8.93(^{b,c})</td>
<td>22.84 ± 10.72(^a)</td>
</tr>
<tr>
<td>HDL-Cholesterol (mg/dL)</td>
<td>49.20 ± 8.35(^b)</td>
<td>28.29 ± 6.25(^a)</td>
<td>33.29 ± 6.99(^a)</td>
<td>36.71 ± 8.52(^{a,b})</td>
<td>32.71 ± 7.43(^a)</td>
<td>41.40 ± 6.62(^{a,b})</td>
</tr>
<tr>
<td>AI</td>
<td>0.57 ± 0.13(^a)</td>
<td>3.30 ± 1.08(^c)</td>
<td>2.00 ± 0.91(^b)</td>
<td>1.36 ± 0.54(^{a,b})</td>
<td>2.06 ± 0.69(^b)</td>
<td>0.93 ± 0.38(^{a,b})</td>
</tr>
<tr>
<td>CRI</td>
<td>1.57 ± 0.13(^a)</td>
<td>4.30 ± 1.08(^c)</td>
<td>3.00 ± 0.91(^b)</td>
<td>2.36 ± 0.54(^{a,b})</td>
<td>3.06 ± 0.69(^b)</td>
<td>1.93 ± 0.38(^{a,b})</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SD of 5-7 animals. * Values with different superscript letters along a row for a given parameter 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; DAML, Diabetic *A. melegueta* low dose; DAMH, Diabetic *A. melegueta* high dose; DMF, Diabetic Metformin; NAMH, Normal *A. melegueta* high dose; TC, Total cholesterol; TG, Triglyceride; LDL-cholesterol, Low density lipoprotein-cholesterol; HDL-cholesterol; High density lipoprotein-cholesterol; AI, Atherogenic index; CRI, Coronary risk index.

*LDL-Cholesterol (mg/dL)= [TC- HDL- (TG/5)]
**Table 3.11.** Serum ALT, AST, ALP and other biochemical parameters different animal groups at the end of the experimental period

<table>
<thead>
<tr>
<th>Parameter</th>
<th>NC</th>
<th>DBC</th>
<th>DAML</th>
<th>DAMH</th>
<th>DMF</th>
<th>NAMH</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT (U/L)</td>
<td>77.40 ± 20.38</td>
<td>116.14 ± 37.37</td>
<td>70.14 ± 10.07</td>
<td>74.14 ± 8.86</td>
<td>93.57 ± 6.86</td>
<td>58.80 ± 21.67</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>88.40 ± 11.41</td>
<td>83.29 ± 15.57</td>
<td>80.29 ± 4.19</td>
<td>85.57 ± 10.97</td>
<td>94.71 ± 9.39</td>
<td>82.20 ± 7.46</td>
</tr>
<tr>
<td>ALP (U/L)</td>
<td>149.40 ± 11.33</td>
<td>451.14 ± 35.10</td>
<td>230.14 ± 31.76</td>
<td>200.86 ± 26.82</td>
<td>208.86 ± 27.58</td>
<td>158.60 ± 22.55</td>
</tr>
<tr>
<td>Creatinine (mg/dL)</td>
<td>2.52 ± 0.78</td>
<td>3.12 ± 0.45</td>
<td>1.73 ± 0.52</td>
<td>1.53 ± 0.42</td>
<td>1.68 ± 0.27</td>
<td>2.42 ± 0.77</td>
</tr>
<tr>
<td>Urea (mg/dL)</td>
<td>22.00 ± 4.69</td>
<td>60.71 ± 15.71</td>
<td>21.29 ± 7.80</td>
<td>29.29 ± 7.11</td>
<td>26.00 ± 5.00</td>
<td>27.80 ± 3.90</td>
</tr>
<tr>
<td>Uric acid (mg/dL)</td>
<td>2.06 ± 0.67</td>
<td>2.85 ± 1.18</td>
<td>2.28 ± 0.84</td>
<td>2.77 ± 0.84</td>
<td>2.56 ± 1.36</td>
<td>1.69 ± 1.05</td>
</tr>
<tr>
<td>LDH (U/L)</td>
<td>284.00 ± 62.56</td>
<td>458.86 ± 68.05</td>
<td>419.14 ± 95.60</td>
<td>395.14 ± 57.94</td>
<td>361.57 ± 80.66</td>
<td>321.20 ± 78.44</td>
</tr>
<tr>
<td>CK-MB (U/L)</td>
<td>374.54 ± 82.56</td>
<td>1064.36 ± 537.18</td>
<td>692.53 ± 128.69</td>
<td>368.16 ± 83.06</td>
<td>743.97 ± 75.29</td>
<td>508.68 ± 127.34</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SD of 5-7 animals. *a-c* Values with different superscript letters along a row for a given parameter 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; DAML, Diabetic *A. melegueta* low dose; DAMH, Diabetic *A. melegueta* high dose; DMF, Diabetic Metformin; NAMH, Normal *A. melegueta* high dose; ALT, Alanine transaminase; AST, Alanine transaminase; ALP, Alkaline phosphate; LDH, Lactate dehydrogenase; CK-MB, Creatine kinase.
3.3.5 Discussion

In the present study, we have investigated the effect of oral administration of a low and a high dose of *A. melegueta* ethyl acetate fraction (AMEF) of fruit ethanolic extract in a T2D model of rats through four weeks post-treatment period. This is obviously the first study that comprehensively reported the anti-diabetic effect of *A. melegueta* despite the reported ethnobotanical potentials of *A. melegueta* in the management of diabetes.

It is evident from the results of this study that induction of T2D decreased bw and caused polyphagia and polydipsia, the classical symptoms of DM (*Figures 3.15 and 3.16*). The observed polydipsia could possibly be linked to polyuria due to the excessive fluid retention in uncontrolled diabetic condition (Kitabchi et al. 2009). Interestingly, post-treatment of AMEF for four weeks ameliorated these alterations even at a lower dose administered (150 mg/kg bw), indicating possible recovery from the diabetic state (*Figures 3.15 and 3.16*). The data were further supported by the significant reduction in hyperglycemia in AMEF treated groups (*Figure 3.17*).

It is well known that the successful treatment of T2D requires an effective control of hyperglycemia and hence search for active anti-hyperglycemic agents cannot be underscored (Bavarva and Narasimhacharya, 2008). In our study, the marked appearance of hyperglycemia in DBC group throughout the study period is an indication of successful induction of diabetes. On the other hand, the AMEF treated diabetic animals responded positively to the treatment and significantly reduced diabetes-induced hyperglycemia to near normal just after a four-week post-administration period (*Figure 3.17*). Similar reduction on blood glucose level was also reported previously when alloxan-induced diabetic rats were treated with seed aqueous extract (Adesokan et al. 2010). Moreover, in our present study, the more pronounced anti-hyperglycemic effect in the DAMH group compared to the DAML group suggests the dose-dependent effects AMEF, which has been further supported by the significantly better glucose tolerance ability of the animals in treated groups compared to the DBC group (*Table 3.7*). Additionally, the AMEF treatment at higher dose (300 mg/kg bw) to normal animals did not affect the blood glucose levels and therefore confirmed the anti-hyperglycemic ability of AMEF but not hypoglycemic effect (*Figure 3.17*), which has been again supported by the pancreatic β-cell ameliorating as well as insulin secreting effects of AMEF in this study (*Figure 3.19 and Table 3.7*).

T2D is a heterogeneous disorder characterized by insulin resistance and pancreatic β-cell dysfunction that cannot properly secrete insulin in response to hyperglycemia (Hui et al. 2007). In this study, decreased insulin levels and loss of β-cell integrity were observed in the DBC group compared to the NC group (*Table 3.8*). In the diabetic AMEF treated as well as DMF groups, these effects were significantly ameliorated and the morphology of pancreas was restored near to normal. The improved serum insulin levels in the AMEF treated diabetic animals (DAML and DAMH) (*Table 3.8*) could be due to the regeneration of pancreatic islets observed in the pancreatic histology (*Figure 3.19*), which has been additionally supported by the significantly higher HOMA-β index (β-cell function) in the
treated groups compared to the DBC group (Table 3.8). The significantly lower HOMA-IR index in the DAML and DAMH groups compared to the DBC group also confirmed the improvement of insulin sensitivity as well as the stimulation of peripheral glucose absorption in these groups (Table 3.8). Therefore, the anti-hyperglycemic effect of AMEF could partly be mediated via decreasing insulin resistance and not only by hyperinsulinemic mechanism. The anti-hyperglycemic effect of AMEF is further supported by the significantly lower serum fructosamine levels in the DAML and DAMH groups compared to the DBC group which did not differ significantly with the NC and DMF groups (Table 3.8). This could be attributed to some of the phenolic compounds such as gingerols, shogaols and paradols that were reported to possessed anti-oxidative potentials (Ilic et al. 2014; 2010).

On the other hand, increase liver weight as well as accumulations of liver glycogen are some common pathological features of metabolic disorders including T2D which apparently lead to hepatomegaly (Paschos and Paletas, 2009). In our study, the relative liver weight was found to decrease in AMEF treated diabetic rats whereas no significant effect was observed in the normal treated animals (Table 3.9), which is contradictory with an earlier report (Ilic et al. 2010). Ilic et al. (2010) highlighted an increase on the absolute and relative liver weight in normal animals after the supplementation of seed ethanolic extract, which could be attributed to the higher dosages (450 and 1500 mg/kg bw) used in the reported study when the lower dose (120 mg/kg bw) did not cause any significant increase on absolute and relative liver weight of normal treated animals (Ilic et al. 2010). Similarly, it was also observed a reduction on glycogen content in the liver of treated diabetic animals, which apparently indicates the modulation of hepatic glycogenesis. This could possibly be another anti-hyperglycemic mechanism exhibited by AMEF and has been a possible mode of action by several anti-diabetic plant-derived formulations and compounds (Bnouham et al. 2006; Grover et al. 2002) which was further supported by the anti-hyperlipidemic effect of AMEF (Table 3.10).

There has been a significant elevation on serum lipid profiles and calculated AI and CRI indices with a reduction on serum HDL-cholesterol in DBC group compared to the NC group (Table 3.10). This might be due to the effect of fructose feeding on DBC group. Fructose metabolism has been shown to be independent of insulin action, which is an important factor for the regulation of fats production as well as energy utilization by the cells (Basciano et al. 2005). In addition, fructose induced more hyperlipidemic condition by increasing the levels of LDL, VLDL and total cholesterol in experimental animals compared to other carbohydrates molecules (Benado et al. 2004). Oral intervention of AMEF at lower and higher dosages as well as the standard drug prevents diabetes-induced dyslipidemia, reduced the risk of atherogenesis and coronary artery disease and augmented serum HDL-cholesterol level (Table 3.10).

In addition to decreased dyslipidemia as well as the risk of atherogenesis and coronary artery diseases, diabetic AMEF treated animals demonstrated a lower serum LDH and CK-MB levels, suggesting the cardio-protective effects of AMEF (Table 3.11). The CK-MB occurs to a much greater extent in cardiac muscle than skeletal muscle and is thus a useful diagnostic marker in myocardial
infarction (Baird et al. 2012). Furthermore, the serum levels of ALT, ALP and urea were significantly elevated in DBC group compared to NC group (Table 3.11). In many disease conditions such as DM, hepatocellular damage increases the release of serum ALT, ALP and urea into circulation which is an indication of hepatic injuries, infarction and functional disturbance of hepatic cells (Abolfathi et al. 2012). It was observed from the result that serum AST level was not affected by the diabetes (Table 3.11). In comparison with ALT, AST is less specific for the detection of hepatic injury (Center, 2007). This is partly due to the fact that, the half-life of ALT is much longer compared to AST in experimental animals (Ramaiah, 2007; Meyer and Harvey, 2004). Therefore, in a condition of acute liver injury, there will be an increase in both serum ALT and AST levels. However, the serum AST level may likely return to normal more rapidly compared to the level of serum ALT, which make ALT more sensitive in assessing hepatic damage in disease conditions (Ramaiah, 2007; Meyer and Harvey, 2004). Therefore, the unaffected serum AST level in our present result could be link to above-stated factor. Hence, the hepato-protective ability of AMEF is supported by the reduced serum ALT, ALP and urea levels in the DAML and DAMH groups compared to the DBC group (Table 3.11). Furthermore, the moderate reduction on serum creatinine, urea and uric acid demonstrate the ability of AMEF to attenuate diabetic nephropathy (Fogo and Kon, 2001). The significantly lower serum creatinine, urea and uric acid in the AMEF treated groups compared to the DBC group suggest its possible effects on the amelioration of diabetic nephropathy (Table 3.11).

3.3.6 Conclusion

In conclusion, various doses of *A. melengueta* ethyl acetate fraction from fruit possess strong anti-T2D activity via improving bw gain, reducing food and fluid intake and hyperglycemia, improving glucose tolerance ability, insulin sensitivity, amelioration of pancreatic $\beta$-cell and $\beta$-cell functions, improving dyslipidemia and number of organ specific diabetic-complications related parameters. Hence, ethyl acetate fraction of *A. melengueta* fruit from crude ethanolic extract may be an excellent anti-diabetic natural product with no considerable side effects. Further clinical study is required to confirm its effects in human subjects. The isolation and partial characterization of the active principle from AMEF is currently underway to find the compounds responsible for this activity in addition to its molecular mechanisms behind the anti-diabetic activity.
3.4 Anti-oxidant potential of *Aframomum melegueta* fruit ethyl acetate fraction in a type 2 diabetes model of rats

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3.4.1 Abstract

Objectives: The present study evaluated the *in vivo* anti-oxidative actions of *Aframomum melegueta* ethyl acetate fraction (AMEF) from fruit ethanolic extract in a type 2 diabetes (T2D) model of rats.

Methods: T2D was induced in rats by feeding a 10% fructose solution *ad libitum* for two weeks followed by a single intraperitoneal injection of streptozotocin (40 mg/kg body weight (bw)) and the animals were orally treated with 150 or 300 mg/kg bw of the AMEF once daily for four weeks.

Results: After four weeks of intervention, diabetic untreated animals showed significantly higher levels of thiobarbituric acid reactive substances (TBARS) and lower levels of reduced glutathione (GSH) in the serum and organs (liver, kidney, heart and pancreas) compared to the normal animals. The activities of endogenous anti-oxidant enzymes (superoxide dismutase, catalase, glutathione peroxidase and reductase) were greatly altered in the serum and organs of diabetic untreated animals compared to the normal animals. These alterations were reverted to near-normal after the treatment of AMEF, especially at the dose of 300 mg/kg bw.

Conclusion: The results of our study suggest that AMEF treatment at 300 mg/kg bw showed potent anti-oxidative action in a T2D model of rats.

Keywords: *Aframomum melegueta*, Anti-oxidant, Ethyl acetate fraction, Fruit ethanolic extract, Type 2 diabetes, Rats.

3.4.2 Introduction

The progressive increase of DM is quite alarming and the disease affects people mostly living in the low- and middle-income countries. Recent data from the International Diabetes Federation (IDF) indicates that DM affects over 387 million people and this figure is likely to be increased to 592 million by 2035 (IDF, 2014; Guariguata et al. 2014). Between the two major types of diabetes, type 2 diabetes (T2D) is the most prevalent one and about 90-95% of diabetic patients suffer from T2D (van-Dam et al. 2002). T2D is a heterogeneous disorder characterized by insulin resistance and partial pancreatic β-
cell dysfunction, leading to hyperglycemia (Hui et al. 2007). As a result of the hyperglycemia in T2D, various macro- and micro-vascular complications develop which have been attributed to oxidative stress, a major contributor to pancreatic β-cell damage (Quilliot et al. 2005).

Oxidative stress is a condition due to an imbalance between the generations of reactive oxygen species (ROS) and the quenching of those free radicals by in vivo anti-oxidant system (AS), thereby shifting the ROS/AS balance in favor of stress (Rahimi et al. 2005). The ROS are derived from the normal physiological processes but become harmful when they are not quenched by the cascade of anti-oxidant defense systems. It has been highlighted that, the major contributors of hyperglycemia-induced oxidative stress include glucose toxicity, protein glycosylation, increased production of glycation end products and mitochondrial ROS, the polyol, hexosamine and protein kinase C pathways (Giacco and Brownlee, 2010; Chang and Chuang, 2010; Chung et al. 2003; Maritim et al. 2003). Therefore, the increased intake of anti-oxidants is necessary to prevent the oxidative damages due to oxidative stress. However, the use of synthetic anti-oxidants such as butylated hydroxy anisole (BHA), butylated hydroxy toluene (BHT), tertiary butylated hydroquinon and gallic acid esters in food protection and disease control have been associated with unwanted consequences. This has motivated researchers to look for options from plant-derived anti-oxidants that may offer better protection with lesser side effects (Michael et al. 2005).

*Aframomum melegueta* K. Schum. (Zingiberaceae) is a plant which fruits, seeds and leaves are commonly used as spices in various food preparations and have been locally used in the treatment of DM and other metabolic disorders in Nigeria (Gbolade, 2009). In our previous study, fruit EtOH extract of *A. melegueta* has demonstrated potent anti-oxidant action and anti-diabetic actions *in vitro* (unpublished data). Additionally several studies reported previously, various seed extracts of *A. melegueta* have shown interesting anti-oxidative as well as anti-oxidative actions *in vitro* (Adefegha and Oboh 2012; Kazeem et al. 2012; Adefegha and Oboh 2011; Etoundi et al. 2010). Our recent findings have shown that, *A. melegueta* ethyl acetate fraction (AMEF) fruit EtOH extract exhibited anti-hyperglycemic action, ameliorated pancreatic β-cell dysfunction and other major diabetes-related parameters in a T2D model of rats (Mohammed et al. 2015b). In a recent preliminary study, Onoja et al. (2014) reported that the seed EtOH extract of *A. melegueta* improved the serum anti-oxidant parameters in normal rats, when the detail in vivo anti-oxidant action of any part of the *A. melegueta* has not yet been conducted.

Therefore, in the present study we intend to comprehensively study the *in vivo* anti-oxidant potentials of the ethyl acetate fraction derived from the fruit ethanolic extract of *A. melegueta* in a T2D model of rats.
3.4.3 Materials and methods

For sample collection, identification and preparation of the fraction, including the detail experimental protocols, please refer to chapter 2, sub-sections 2.3 and 2.5.1 to 2.5.8.

3.4.4 Results

The results of thiobarbituric acid reactive substances (TBARS) expressed as malondialdehyde (MDA) concentration of serum and organs (liver, kidney, heart and pancreas) of all the groups are presented in Figure 3.20. According to the data, induction of diabetes significantly \( p < 0.05 \) elevated the serum, liver, heart and pancreas MDA levels in DBC group compared to the NC group. The increase in the kidney of the DBC group did not differ significantly \( p < 0.05 \) compared to the NC group. However, oral treatment of AMEF especially at the higher dose (300 mg/kg bw) for 4-weeks to diabetic animals significantly \( p < 0.05 \) decreased the of serum and organs MDA levels, which were comparable to the DMF group (Figure 3.20).

![Figure 3.20](image)

**Figure 3.20.** Serum and organs thiobarbituric acid reactive substances of all animal groups at the end of the intervention period. Data are presented as the mean ± SD of 5-7 animals. Values with different letter over 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; DAML, Diabetic A. melegueta low dose; DAMH, Diabetic A. melegueta high dose; DMF, Diabetic Metformin; NAMH, Normal A. melegueta high dose; MDA, Malondialdehyde.

The data of reduced glutathione (GSH) levels of serum and organs of all the groups are presented in Figure 3.21. The concentration of GSH in serum, liver and pancreas was significantly \( p < 0.05 \) decreased in DBC group compared to NC group. The reduction of GSH levels in the kidney and heart of the DBC group was not significantly \( p < 0.05 \) affected compared to the the NC group.
After 4 weeks of oral intervention, the levels of GSH of DAML and DAMH groups significantly ($p < 0.05$) improved in dose-dependent manner (Figure 3.21). Furthermore, the serum and organs GSH levels were not affected in NAMH group throughout the experimental period.

![Figure 3.21. Serum and organs reduced glutathione (GSH) content in all animal groups at the end of the study period. Data are presented as the mean ± SD of 5-7 animals. *Values with different letter over 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; DAML, Diabetic A. melegueta low dose; DAMH, Diabetic A. melegueta high dose; DMF, Diabetic Metformin; NAMH, Normal A. melegueta high dose; GSH, Reduced glutathione.

The results of the activity of superoxide dismutase (SOD) of serum and organs of all the groups are presented in Figure 3.22. It was observed from the results that the activity of SOD in serum, liver, heart and pancreas of DBC group was significantly ($p < 0.05$) decreased, when the reduction in the kidney of the DBC group was not reduced compared to the NC group. Treatment of AMEF to diabetic animals, especially the DAMH group showed a significant ($p < 0.05$) increased SOD activity which was comparable to the NC group (Figure 3.22). Additionally, the activity of SOD in the heart of NAMH group was significantly ($p < 0.05$) increased compared to NC group Figure 3.22.
Figure 3.22. Superoxide dismutase (SOD) activities in the serum and organs of different animal groups at the end of the study period. Data are presented as the mean ± SD of 5-7 animals. *Values with different letter over the bars for a sample week 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; DAML, Diabetic *A. melegueta* low dose; DAMH, Diabetic *A. melegueta* high dose; DMF, Diabetic Metformin; NAMH, Normal *A. melegueta* high dose.

The data of catalase activity in the serum and organs of all the groups are presented in Figure 3.23. According to the results, the activity of the catalase in the serum and organs was significantly ($p < 0.05$) decreased in the DBC group compared to the NC group. However, treatment of AMEF especially with high dose (300 mg/kg bw, DAMH group) significantly ($p < 0.05$) increased the activity of the enzyme in serum, liver and kidney when the increase in the heart and pancreas was not significantly ($p < 0.05$) difference from the DBC group (Figure 3.23).
Figure 3.23. Catalase activities in the serum and different organs of different animal groups at the end of the study period. Data are presented as the mean ± SD of 5-7 animals. Values with different letter over 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; DAML, Diabetic A. melegueta low dose; DAMH, Diabetic A. melegueta high dose; DMF, Diabetic Metformin; NAMH, Normal A. melegueta high dose.

The results of the activity of glutathione peroxidase (GPx) in serum and organs of all the groups are presented in Figure 3.24. The activity of the GPx in the serum and organs was significantly (p < 0.05) decreased in the DBC group compared to the NC group. Oral treatment with the fraction, significantly (p < 0.05) increased the GPx activity in the DAMH group which is comparable to the DMF group (Figure 3.24). It was also observed that the GPx activity in the serum and organs of NAMH group was increased although not statistically different from the NC group (Figure 3.24).

The data of glutathione reductase (GR) activity in the serum and organs of all the groups are presented in Figure 3.25. The activity of this enzyme was not affected by the induction of diabetes in the kidney and heart, whereas significantly (p < 0.05) decreased in the serum, liver and pancreas of the DBC group compared to the NC group (Figure 3.25). After treatment with AMEF for 4-weeks, the GR activity was significantly (p < 0.05) increased in the serum, liver and pancreas of the DAMH group, when the effect was not statistically different in the kidney and heart compared to the DBC group (Figure 3.25). In addition, the activity of GR in heart of NAMH group was significantly (p < 0.05) increased compared to the NC group (Figure 3.25).
Figure 3.24. Glutathione peroxidase activities in the serum and organs of different animal groups at the end of the study period. Data are presented as the mean ± SD of 5-7 animals. *Values with different letters over 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; DAML, Diabetic *A. melegueta* low dose; DAMH, Diabetic *A. melegueta* high dose; DMF, Diabetic Metformin; NAMH, Normal *A. melegueta* high dose.

Figure 3.25. Glutathione reductase activities in the serum and organs of different animal groups at the end of the study period. Data are presented as the mean ± SD of 5-7 animals. *Values with different letters over 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; DAML, Diabetic *A. melegueta* low dose; DAMH, Diabetic *A. melegueta* high dose; DMF, Diabetic Metformin; NAMH, Normal *A. melegueta* high dose.
3.4.5 Discussion

In our recent findings, EtOAc fraction derived from fruit EtOH extract of *A. melegueta* exhibited anti-hyperglycemic action, ameliorated pancreatic β-cell dysfunction and other major diabetes-related parameters in a T2D model of rats (unpublish data). Therefore we hypothesized that AMEF may attenuate serum and organs oxidative-stress induced by hyperglycemia in type 2 diabetic rats. The results of our present study are quite promising as oral treatment of the AMEF ameliorated most of the alterations caused by the induction of diabetes in the serum and various organs.

In a recent study, Onoja et al. (2014) reported that the treatment of *A. melegueta* seed extract (400 mg/kg bw) to normal rats decreased the serum MDA levels which is in line with our present study. According to the data of our present study, the treatment of AMEF even at a lower dose (300 mg/kg bw) has showed potent anti-oxidative action (Figure 3.20). It has been postulated that increase ROS production in T2D initiates peroxidation of lipids and stimulates hemoglobin and other protein glycation which in turn causes cellular and tissue damages (Sabu and Kuttan, 2002; Boynes, 1991). Therefore the reductions of MDA levels in the serum and organs by AMEF treatment supports the results of ours and many previous studies that showed the strong ability of extracts or fractions from *A. melegueta* in scavenging the deleterious effects of ROS *in vitro* (Mohammed et al. 2015; Adefegha and Oboh 2012; Kazeem et al. 2012; Adefegha and Oboh 2011; Etoundi et al. 2010). The reduction of MDA levels was further supported by the increase of reduced glutathione levels in the serum and organs as well (Figure 3.21).

Glutathione is a tripeptide that contributes immensely to the reduction of the deleterious cellular damages induced by lipid peroxidation (Lu, 1999). In our present study, the reduction of reduced glutathione (GSH) in the DBC group signifies the higher utilization of GSH due to diabetes-induced oxidative stress (Figure 3.21). Treatment of AMEF augmented the serum and organs GSH levels in dose-dependent manner (Figure 3.21). Therefore we suggest that AMEF may likely to improve the biosynthesis of GSH or attenuated the oxidative processes leading to GSH reduction, or exhibited both effects. Our results were further supported by the ability of the AMEF to increase the activity of superoxide dismutase (Figure 3.22).

There is a close relationship between hyperglycemia in T2D and the elevation of cellular superoxide radical production (Rahimi et al. 2005). An enzyme superoxide dismutase (SOD) scavenges the superoxide radical by converting the radical to hydrogen peroxide (H$_2$O$_2$) and molecular oxygen (Sati et al. 2010). In a recent study, Onoja et al. (2014) indicated that, a significant increase of serum SOD levels was observed in normal rats after 4-weeks treatment period of seed methanolic extract (400 mg/kg bw). This was similarly observed in our present data (Figure 3.22). Therefore, the anti-oxidative actions of *A. melegueta* may partly be mediated via an increase in the activities of SOD, which again was further supported by the increasing activities of catalase in the serum and other related organs (Figure 3.23).
The H₂O₂ release by the SOD is also harmful and can cause deleterious damages to cells. Catalase, an iron containing enzyme catalyzes the conversion of H₂O₂ to water and oxygen molecules and thus protects cellular and tissue oxidative damages. The catalase activity was reported to increase about 8-fold in normal animals treated with 400 mg/kg bw seed methanolic extract of *A. melegueta* (Onoja et al. 2014). However, according to our results, the catalase activity was greatly improved especially in the DAMH group which is comparable to the metformin treated groups (Figure 3.23). It was also observed that, the activity of catalase was less compared to that of SOD, which may be linked to the protective action of the SOD to catalase from the toxic effects of superoxide radical (Rajasekaran et al. 2005).

The activities of glutathione peroxidase (GPx) a selenium containing enzyme and glutathione reductase (GR) are entirely dependent on the GSH concentration. GPx in the presence of GSH catalyzes the reduction H₂O₂ to water and oxidized glutathione (GSSG). GR then converts GSSG to GSH, utilizing protons from NADPH molecules, which can also be used by GPx (Manonmani et al. 2005). According to the results of our study, oral intervention of AMEF for 4-weeks to diabetic animals especially in DAMH group attenuated the alterations in the GPx and GR activities, especially in DAMH group (Figures 3.24 & 3.25). Therefore, the anti-oxidant action of AMEF may partly be mediated via increased production of GPx and GR in the serum and organs as well.

### 3.4.6 Conclusion

In conclusion, oral treatment of AMEF especially at higher dose demonstrated a potent anti-oxidant activity via reduction of lipid peroxidation, improving glutathione levels and endogenous anti-oxidants enzymes in serum and organs mostly affected by diabetic-complications. Hence, AMEF from crude ethanolic extract *A. melegueta* fruit may be an excellent anti-oxidant natural product with no considerable side effects. Further clinical studies are required to confirm its effects in human subjects.
CHAPTER 4

4.0 In vitro and in vivo anti-diabetic and anti-oxidative effects of various extracts and fractions from different parts of Xylopia aethiopica

4.1 Phytochemistry, anti-oxidative and anti-diabetic effects of extracts from various parts of Xylopia aethiopica in vitro

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3Department of Biochemistry, Faculty of Science, Ahmadu Bello University, Zaria-Nigeria

4.1.1 Abstract

Objective: Various parts of Xylopia aethiopica (Dunal) A. Rich. are used for traditional treatment of different diseases such as diabetes, microbial infections and diarrhea in Africa. This study investigated and compared the in vitro anti-oxidative, anti-diabetic effects and possible compounds present in various solvent extracts of fruit, leaf, stem and root of X. aethiopica.

Methods: Samples were sequentially extracted using solvents of increasing polarity. They were subjected and investigated for 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging activity, ferric reducing power, inhibition of hemoglobin glycation, α-amylase and α-glucosidase activities as markers of in vitro anti-diabetic effects at various doses (30-240 µg/mL). Possible compounds were analyzed using Gas Chromatography-Mass Spectroscopic (GC-MS) analysis.

Results: The ethanolic (EtOH) extracts from the fruit and leaf have exhibited higher total polyphenol and flavonoid contents compared to other extracts. Furthermore, for all the in vitro models used in this study, EtOH extracts from the fruit and leaf demonstrated significantly lower IC_{50} values compared to other extracts, when fruit EtOH extract showed the lowest IC_{50} value (DPPH: 0.04 ± 0.01 mg/mL; inhibition of hemoglobin glycation: 0.16 ± 0.01 mg/mL; α-amylase: 0.21 ± 0.01 mg/mL; α-glucosidase: 0.08 ± 0.01 mg/mL). The GC-MS analysis of the most active extracts (EtOH fruit and leaf) revealed the presence of majorly terpenes and aromatic compounds and other fatty acids derivatives as possible compounds.

Conclusion: Fruit EtOH extract exhibited higher anti-oxidative and anti-diabetic effects compared to other solvent extracts in vitro and thus require further work to fully validate these effects in vivo.

Keywords: Anti-oxidative, α-amylase, α-glucosidase, Type 2 diabetes, Xylopia aethiopica
4.1.2 Introduction

Diabetes mellitus (DM) is the fast growing metabolic disorder and a leading cause of morbidity in developing countries. It is a heterogeneous group of metabolic disorders characterized by persistent hyperglycemia (Neelesh et al. 2010) and alteration in the metabolism of major cellular biomolecules as a result of defects in insulin secretion and/or insulin action (Maritim et al. 2003). Type 2 diabetes (T2D) is most prevalent among the major forms of DM. T2D is a heterogeneous disorder characterized by insulin resistance and pancreatic β-cell dysfunction leading to hyperglycemia (Hui et al. 2007). In chronic uncontrolled hyperglycemia, there is an increased production of reactive oxygen species (ROS) and reduced anti-oxidant defense system, which is referred as oxidative stress. This leads to initiation of the major processes that underlay the various diabetic associated complications, and in the absence of effective treatment result to death (ADA, 2015; Surampud et al. 2009; Maritim et al. 2003).

The current treatment option for T2D that is widely receiving much interest include the control of postprandial hyperglycemia by the use of inhibitors of carbohydrate digesting enzymes (Ademiluyi and Oboh, 2013). The inhibitors of carbohydrate digesting enzymes such as α-glucosidase and α-amylase can significantly delay the intestinal absorption of dietary glucose and therefore help in lowering postprandial hyperglycemia (Imam, 2015). However, the major available glucosidase inhibitors such as acarbose and miglitol, were reported to cause diarrhea and other gastrointestinal disturbances (Fujisawa et al. 2005). Therefore with increasing interest in alternatives for the control of T2D, search for anti-diabetic agent has been extended to plant-based remedies as less adverse effects are associated with plant-based therapy (Singh, 2001; Calixto, 2000).

Xylopia aethiopica (Dunal) A. Rich. (Annonaceae) commonly known as Ethiopian pepper is a spice widely used as flavoring and coloring agents. In addition, X. aethiopica is utilized in folk medicines for the treatment of diabetes in Africa (Soladoye et al. 2012; Diallo et al. 2012; Karou et al. 2011; Dièye et al. 2008). Recent studies have shown that the extracts from X. aethiopica fruit possesses anti-oxidative (Adefegha and Oboh, 2012a,c; 2011; Etoundi et al. 2010; Odukoya et al. 2005; George and Osiam, 2011), anti-microbial (Esekhiagbe et al. 2009), anti-cancer (Kuete et al. 2013; 2011; Choumessi et al. 2012) and anti-diabetic (Adefegha and Oboh, 2012a,c; 2011; Etoundi et al. 2010) activities in vitro. However, the majority of scientific investigations had only focused on the fruit only. No data is available on the validity or potential of extracts of the other parts of the plant. Therefore the present study was aimed to investigate and compare the anti-oxidative and anti-diabetic effects of various extracts from different parts (including fruit) of X. aethiopica using several in vitro models. Additionally, phytochemical analysis of the possible bioactive compounds present in most active extracts was also carried out using GC-MS analysis.
4.1.3 Materials and methods

For plant sample collection, identification and preparation of the extracts, including the detail experimental protocols, please refer to chapter 2, sub-sections 2.3 and 2.4.

4.1.4 Results

The yields recovered, total polyphenols and flavonoids contents from different solvent extracts of various parts of *X. aethiopica* are presented in Table 4.1. The ethanolic (EtOH) fruit extract showed significantly \((p < 0.05)\) higher yield, total polyphenols and flavonoids contents compared to other solvent extracts of the same or different parts of *X. aethiopica* (Table 4.1). The polyphenols and flavonoids contents of the leaf and stem EtOH extracts were also higher compared to the ethyl acetate (EtOAc) and aqueous extracts of these parts. In addition, the polyphenols and flavonoids contents of the roots were not detected.

Table 4.1: Percentage yield, total polyphenol and flavonoid contents in the various solvent extracts of *X. aethiopica* parts

<table>
<thead>
<tr>
<th>Samples</th>
<th>Yield (%)</th>
<th>Total polyphenols (mg/gGAE)</th>
<th>Total flavonoids (mg/gQE)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fruit</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>2.05</td>
<td>3.69 ± 0.09(^c)</td>
<td>1.86 ± 0.29(^c)</td>
</tr>
<tr>
<td>Ethanol</td>
<td>7.35</td>
<td>25.36 ± 0.32(^f)</td>
<td>8.06 ± 0.88(^e)</td>
</tr>
<tr>
<td>Aqueous</td>
<td>3.15</td>
<td>4.83 ± 0.15(^d)</td>
<td>0.17 ± 0.06(^a)</td>
</tr>
<tr>
<td><strong>Leaf</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>1.05</td>
<td>2.82 ± 0.26(^b)</td>
<td>0.69 ± 0.12(^b)</td>
</tr>
<tr>
<td>Ethanol</td>
<td>2.00</td>
<td>9.45 ± 0.34(^e)</td>
<td>3.50 ± 0.18(^d)</td>
</tr>
<tr>
<td>Aqueous</td>
<td>4.45</td>
<td>3.83 ± 0.34(^c)</td>
<td>0.36 ± 0.18(^a,b)</td>
</tr>
<tr>
<td><strong>Stem</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>0.78</td>
<td>0.88 ± 0.14(^a)</td>
<td>ND</td>
</tr>
<tr>
<td>Ethanol</td>
<td>2.95</td>
<td>5.13 ± 0.05(^d)</td>
<td>1.11 ± 0.18(^b,c)</td>
</tr>
<tr>
<td>Aqueous</td>
<td>1.05</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SD of triplicate determinations. \(^a\)-\(^g\): Values with different superscripted letters for a given parameter within a column are significantly different from each other extracts (Tukey’s-HSD multiple range post hoc test, \(p < 0.05\)). ND, Not determined.

The data of ferric (Fe\(^{3+}\)) reducing anti-oxidant power (expressed as gallic acid equivalents) of various extracts are presented in Figure 4.1. The fruit EtOH extract demonstrated significantly \((p < 0.05)\) higher Fe\(^{3+}\) to Fe\(^{2+}\) reducing ability compared to other solvent extracts of the same or different
part (Figure 4.1). The activity of leaf EtOH extract was also significantly ($p < 0.05$) higher than the other extracts from this part, when the action of stem EtOH extract was not significantly ($p < 0.05$) difference compared to the EtOAc extract (Figure 4.2).

**Figure 4.1.** Ferric reducing power (relative to gallic acid) of fruit (A), leaf (B) and stem (C) extracts of *X. aethiopica*. Data are presented as mean ± SD of triplicate determinations. *a-d* Different letters presented over the bars for a given concentration of each extract are significantly different from each other (Tukey’s-HSD multiple range post hoc test, $p < 0.05$).
The results of DPPH radical scavenging activities and the calculated IC$_{50}$ values of various extracts from different parts of *X. aethiopica* are presented in **Figure 4.2** and **Table 4.2**, respectively. According to the data, the fruit and leaf EtOH extracts had exhibited significantly ($p < 0.05$) lower IC$_{50}$ values (fruit: 0.04 ± 0.01 mg/mL; leaf: 0.08 ± 0.01 mg/mL) compared to other extracts that are comparable to the standards used in this study (ascorbic acid: 0.03±0.02 mg/mL; gallic acid: 0.05 ± 0.01 mg/mL). In all the parts investigated, EtOH extracts were the most active followed by EtOAc extracts while aqueous extracts were the least active (**Table 4.2**). Various extracts derived from the root part were inactive to scavenge the DPPH radical.

![Figure 4.2](image_url)

**Figure 4.2.** DPPH Radical scavenging activity (%) of fruit (A), leaf (B) and stem (C) extracts of *X. aethiopica*. Data are presented as mean ± SD of triplicate determinations. Different letters presented over the bars for a given concentration of each extract are significantly different from each other (Tukey’s HSD multiple range post hoc test, $p < 0.05$).
Table 4.2: IC$_{50}$ values of various solvent extracts of *X. aethiopica* parts in different anti-oxidative and anti-diabetic models.

<table>
<thead>
<tr>
<th>Samples</th>
<th>IC$_{50}$ (mg/ml)</th>
<th>DPPH scavenging activity</th>
<th>Inhibition of hemoglobin glycosylation</th>
<th>α-amylase inhibition</th>
<th>α-glucosidase inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Fruit</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>0.15 ± 0.01$^{b}$</td>
<td>0.66 ± 0.05$^{a,b}$</td>
<td>17.42 ± 1.77$^{c}$</td>
<td>0.78 ± 0.13$^{c}$</td>
<td></td>
</tr>
<tr>
<td>Ethanol</td>
<td>0.04 ± 0.01$^{a}$</td>
<td>0.16 ± 0.01$^{a}$</td>
<td>0.21 ± 0.01$^{a}$</td>
<td>0.08 ± 0.01$^{a}$</td>
<td></td>
</tr>
<tr>
<td>Aqueous</td>
<td>0.10 ± 0.01$^{a,b}$</td>
<td>8.04 ± 0.28$^{c}$</td>
<td>5.50 ± 0.66$^{d}$</td>
<td>2.20 ± 0.13$^{d}$</td>
<td></td>
</tr>
<tr>
<td><strong>Leaf</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>0.12 ± 0.02$^{a,b}$</td>
<td>0.51 ± 0.07$^{a,b}$</td>
<td>119.64 ± 23.05$^{g}$</td>
<td>65.85 ± 5.48$^{e}$</td>
<td></td>
</tr>
<tr>
<td>Ethanol</td>
<td>0.08 ± 0.01$^{a}$</td>
<td>0.27 ± 0.03$^{a}$</td>
<td>2.40 ± 0.11$^{c}$</td>
<td>0.28 ± 0.01$^{b}$</td>
<td></td>
</tr>
<tr>
<td>Aqueous</td>
<td>0.15 ± 0.01$^{b}$</td>
<td>0.73 ± 0.10$^{b}$</td>
<td>71.90 ± 6.63$^{f}$</td>
<td>0.95 ± 0.09$^{c}$</td>
<td></td>
</tr>
<tr>
<td><strong>Stem</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>1.01 ± 0.08$^{c}$</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Ethanol</td>
<td>0.26 ± 0.05$^{b}$</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Aqueous</td>
<td>5.98 ± 2.61$^{d}$</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>0.03 ± 0.02$^{a}$</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Gallic acid</td>
<td>0.05 ± 0.01$^{a}$</td>
<td>0.20 ± 0.01$^{a}$</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Acarbose</td>
<td>ND</td>
<td>ND</td>
<td>0.37 ± 0.03$^{b}$</td>
<td>0.21 ± 0.01$^{b}$</td>
<td></td>
</tr>
</tbody>
</table>

Data are presented as mean ± SD of triplicate determinations. $^{a-g}$ Values with different superscript letters presented within a column for a given parameter are significantly different from each other extract (Tukey’s-HSD multiple range post hoc test, $p < 0.05$). ND, Not determined.

The results of the inhibition of hemoglobin glycation by the extracts from different parts of *X. aethiopica* are presented in Figure 4.3 and Table 4.2 (IC$_{50}$ values). The EtOH and EtoAc extracts from the fruit and leaf parts had showed significantly ($p < 0.05$) lower calculated IC$_{50}$ values compared to other solvent extracts from the same or different parts. The calculated IC$_{50}$ value (0.16 ± 0.01 mg/mL) exhibited by the fruit EtOH extract was the least and comparable to the gallic acid (0.20 ± 0.01 mg/mL) used as a standard (Table 4.2). Moreover, all the extracts from the stem and root parts showed no inhibitory activity to hemoglobin glycosylation.
Figure 4.3. Inhibition of hemoglobin glycosylation (%) of fruit (A) and leaf (B) extracts of *X. aethiopica*. Data are presented as mean ± SD of triplicate determinations. **Different** letters presented over the bars for a given concentration of each extract are significantly different from each other (Tukey’s-HSD multiple range *post hoc* test, *p* < 0.05).

The data of inhibition of α-amylase action by various extracts from *X. aethiopica* are presented in **Figure 4.4** and **Table 4.2** (IC$_{50}$ values). It was observed from the results that, the calculated IC$_{50}$ value (0.21 ± 0.01 mg/mL) exhibited by the fruit EtOH extract was significantly (*p* < 0.05) lower compared to other solvent extracts of the same or different parts and the acarbose as well (**Table 4.2**). The leaf EtOH extract was also observed to show an IC$_{50}$ value (2.40 ± 0.11 mg/mL) that was significantly (*p* < 0.05) lower than the standard acarbose (0.37 ± 0.03 mg/mL).
The results of the α-glucosidase inhibitory action by various solvent extracts from X. aethiopica are presented in Figure 4.5 and Table 4.2. According to the results, fruit EtOH extract had demonstrated potent α-glucosidase inhibitory action with the calculated IC$_{50}$ value (0.08 ± 0.01 mg/mL) was significantly ($p < 0.05$) lower compared to the acarbose (0.21 ± 0.01 mg/mL). The inhibition of α-glucosidas action exhibited by leaf EtOH extract was not significantly ($p < 0.05$) compared to acarbose (Table 4.2). Furthermore, all the extracts from the stem and root parts showed no enzyme inhibitory action.
Figure 4.5. $\alpha$-Glucosidase inhibition (%) of fruit (A) and leaf (B) extracts of *X. aethiopica*. Data are presented as mean ± SD of triplicate determinations. * Different letters presented over the bars for a given concentration of each extract are significantly different from each other (Tukey’s-HSD multiple range post hoc test, $p < 0.05$).

The results of the phytochemical analysis of the most active extracts (EtOH fruit and leaf) from *X. aethiopica* are presented in Figures 4.6 (GC-MS) and Table 4.3 (list of compounds). Several peaks appeared in the chromatogram of the fruit EtOH extract and were identified and compared with standards from the NIST library (Figure 4.6A). The available constituents (1-16) in fruit EtOH extract include terpenes; $p$-menth-1-en-8-ol (1), camphene (4), caryophyllene (5), octahydro-1,4,9,9-tetramethyl-methanoazulene (6), cis-$\alpha$-copaene-8-ol (7), 3-ethynyldecahydro-pentamethyl naphthol (11), androst-5-en-4-one (15) and 4-methylcholest-3-en-ol (16), aromatic compounds; 2-ethyl-4,5-dimethyl-phenol (2), 3-hydroxy-4-pteridinone (3), 1-formylethyl-4-(1-buten-3-yl)-benzene (8) and 1,2-
benzenedicarboxylic acid, butyl 2-methylpropyl (9) and other long chain fatty acid derivatives; hexadecanoic acid, ethyl ester (10), ethyl oleate (12), linoleic acid ethyl ester (13) and octadecanoic acid, ethyl ester (14) (Figure 4.6A and Table 4.3). Furthermore peaks visible from the chromatogram of leaf EtOH extract (Figure 4.6B) lead to identification of terpenes; 3-methyl-6-(1-methylethyl)-2-cyclohexen-1-one (17), lilac alcohol formate C (18), camphene (4) and hexahydro-9-hydroxy trimethyl naphthol (25), a sugar 4-methyl-myo-inositol (20) and majorly long chain fatty acids and esters (19), 5-hydroxy-spiro[2,4]heptane-5-methanol (21), n-hexadecanoic acid (22), 9-octadecenoic acid, methyl ester (23) and 9-octadecenoic acid (24) (Table 4.3).

Figure 4.6. GC-MS Chromatograms of ethanolic extracts of fruit (A) and leaf (B) extracts of X. aethiopica
<table>
<thead>
<tr>
<th>Extracts</th>
<th>Compounds</th>
<th>Retention time (min)</th>
<th>Mass/amu</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fruit</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>p-Menth-1-en-8-ol</td>
<td>5.51</td>
<td>139 [M]+</td>
</tr>
<tr>
<td>2</td>
<td>2-Ethyl-4,5-dimethyl-phenol</td>
<td>6.26</td>
<td>150 [M]+</td>
</tr>
<tr>
<td>3</td>
<td>3-Hydroxy-4-pteridinone</td>
<td>6.68</td>
<td>164 [M]+</td>
</tr>
<tr>
<td>4</td>
<td>Camphene</td>
<td>7.23</td>
<td>136 [M]+</td>
</tr>
<tr>
<td>5</td>
<td>Caryophyllene</td>
<td>7.47</td>
<td>204 [M]+</td>
</tr>
<tr>
<td>6</td>
<td>Octahydro-1,4,9,9-tetramethyl-</td>
<td>8.26</td>
<td>191 [M]+</td>
</tr>
<tr>
<td></td>
<td>methanoazulene</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>cis-α-Copaene-8-ol</td>
<td>8.54</td>
<td>220 [M]+</td>
</tr>
<tr>
<td>8</td>
<td>1-Formylethyl-4-(1-butene-3-yl)-</td>
<td>8.95</td>
<td>188 [M]+</td>
</tr>
<tr>
<td></td>
<td>benzene</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>1,2-Benzenedicarboxylic acid, butyl</td>
<td>10.30</td>
<td>278 [M]+</td>
</tr>
<tr>
<td></td>
<td>2-methylpropyl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Hexadecanoic acid, ethyl ester</td>
<td>10.40</td>
<td>284 [M]+</td>
</tr>
<tr>
<td>11</td>
<td>3-Ethenyldodecahydro-pentamethyl</td>
<td>10.76</td>
<td>275 [M]+</td>
</tr>
<tr>
<td></td>
<td>naphthol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>Ethyl oleate</td>
<td>11.25</td>
<td>310 [M]+</td>
</tr>
<tr>
<td>13</td>
<td>Linoleic acid ethyl ester</td>
<td>11.27</td>
<td>308 [M]+</td>
</tr>
<tr>
<td>14</td>
<td>Octadecanoic acid, ethyl ester</td>
<td>11.36</td>
<td>312 [M]+</td>
</tr>
<tr>
<td>15</td>
<td>Androst-5-en-4-one</td>
<td>11.94</td>
<td>272 [M]+</td>
</tr>
<tr>
<td>16</td>
<td>4-Methylcholesten-3-ol</td>
<td>12.05</td>
<td>400 [M]+</td>
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<tr>
<td><strong>Leaf</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>3-Methyl-6-(1-methylethyl)-2-</td>
<td>5.99</td>
<td>152 [M]+</td>
</tr>
<tr>
<td></td>
<td>cyclohexen-1-one</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>Lilac alcohol formate C</td>
<td>6.61</td>
<td>198 [M]+</td>
</tr>
<tr>
<td>19</td>
<td>Hex-4-ynoic acid, methyl ester</td>
<td>7.21</td>
<td>125 [M]+</td>
</tr>
<tr>
<td>4</td>
<td>Camphene</td>
<td>7.35</td>
<td>136 [M]+</td>
</tr>
</tbody>
</table>
4.1.5 Discussion

In most of the previous studies, various extracts from *X. aethiopica* fruit were reported to be rich in polyphenols and flavonoids as well (Adefegha and Oboh, 2012a,c; 2011; George and Osioma, 2011; Etoundi et al. 2010; Essekhagbe et al. 2009; Odukoya et al. 2005). The highest amounts of polyphenols (4.2 ± 0.0 mg/g) and flavonoids (3.5 ± 0.6 mg/g) were that reported in *X. aethiopica* fruit aqueous extract (Adefegha and Oboh, 2012c). This is far lower compared to that of fruit EtOH extract (polyphenols: 25.36 ± 0.32 mg/g; flavonoids: 8.06 ± 0.88 mg/g) obtained in our present study (Table 4.1). Additionally, even the polyphenolic content of the fruit aqueous extract (4.83 ± 0.15 mg/g) obtained in our present study was higher than that reported earlier (Adefegha and Oboh, 2012c). These variations could be due to many factors, but not limited to the method and the solvent used for the extraction. It was also evident from our present results that polyphenolic and flavonoid contents vary from one part to another (Table 4.1). This again might be attributed to genetic and environmental factors (nature of the soil, high temperature and rain fall) in addition to growth or maturation stages for most of the parts of the plants (Mamphiswana et al. 2010; Pandey and Rizvi, 2009).

In a previous study, Odukoya et al. (2005) have reported that the *X. aethiopica* fruit aqueous extract showed excellent ferric (Fe$^{3+}$) reducing anti-oxidant ability. This is in line with the results of our present study, when the fruit EtOH extract was the most potent than other extracts (Figure 4.1). In addition although no correlation analysis was conducted in our present study, the reducing anti-oxidant ability of the various extracts correlates well with the polyphenolic and flavonoid contents (Table 4.1). It was also observed that extracts derived from the leaf part are good source for anti-oxidant molecules as they exhibited good anti-oxidant activity. The reducing anti-oxidant ability of various *X. aethiopica* extracts was further supported by the potent potent DPPH radical scavenging actions (Table 4.2).

The anti-radical potentials of *X. aethiopica* fruit aqueous extract (IC$_{50}$: 0.3 mg/mL) has been reported recently (Adefegha and Oboh, 2012a, c; 2011). However, according to our results, the calculated IC$_{50}$ value for the fruit aqueous extract (0.10 ± 0.01 mg/mL) was lower compared to that
reported by Adefegha and Oboh (2012c), when that of EtOH extract (0.04 ± 0.01 mg/mL) was by far lower compared to the aqueous extract (Table 4.2). This could be the least IC₅₀ value for DPPH scavenging of any extract from X. aethiopica so far reported. The higher activity of the fruit EtOH extract might be attributed to the higher polyphenol and flavonoid content mentioned above. This again could be the reason behind the higher anti-radical ability of the EtOH extracts from other parts of X. aethiopica than other solvent extracts (Table 4.2).

In order to further evaluate the anti-oxidant capacity of various extracts from different parts of X. aethiopica, the inhibitory effect of hemoglobin glycation by these extracts was investigated. Glycation is a non-enzymatic reaction that occurs when proteins such as hemoglobin and albumin are exposed to excess reducing sugars and contributes greatly to formation of advanced glycation end products that lead to oxidative stress (Yamagishi et al. 2008; Rahbar and Figarola, 2003; Brownlee et al. 1984). According to the results of previous studies, there is a positive correlation between polyphenolic contents and anti-glycation ability of several plant-derived extracts (Ramkissoon et al. 2013; Povichit et al. 2010; Rahbar and Figarola, 2003). Therefore, the higher anti-glycation of the EtOH extracts from fruit and leaf of X. aethiopica observed in our present study might be due to the greater amount of polyphenols in these parts (Table 4.1 and 4.2). The result was further supported by the inhibition of the activities of α-amylase and α-glucosidase by various extracts from X. aethiopica (Table 4.2).

It has been reported that X. aethiopica fruit aqueous extract exhibited moderate inhibition of α-amylase as well as α-glucosidase actions (Adefegha and Oboh, 2012a; Etoundi et al. 2010). The calculated IC₅₀ values were 2.81 ± 0.79 mg/mL and 2.57 ± 1.15 mg/mL for α-amylase and α-glucosidase actions, respectively (Adefegha and Oboh, 2012a). However, according to our present results, the IC₅₀ values (α-amylase: 0.21 ± 0.01 mg/mL α-glucosidase: 0.08 ± 0.01 mg/mL) demonstrated by the most potent extract (fruit EtOH) was lower than other extracts, indicating higher inhibitory actions than the aqueous extract reported previously (Adefegha and Oboh, 2012a; Etoundi et al. 2010). Therefore, it is suggested that the availability of bioactive inhibitors from X. aethiopica are higher in the fruit EtOH extract compared to other extracts of the same or different parts (Table 4.2). The inhibitors of α-amylase and α-glucosidase actions can significantly control the postprandial hyperglycemia by delaying the process of carbohydrate hydrolysis and absorption as well (Imam, 2015; Kwon et al. 2006).

Furthermore, the higher α-amylase and α-glucosidase actions as well as the anti-oxidant activities of fruit and leaf EtOH extracts indicated the presence of promising compounds and were therefore subjected to GC-MS analysis. Karioti et al. (2004) have reported that monoterpane hydrocarbons are the major compounds detected in the essential oils derived from the fruit, leaf stem and root barks. This is in line with our present data (Figure 4.6 and Table 4.3). Moreover, compounds such as p-menth-1-en-8-ol (1), camphene (4) and caryophyllene (5) detected in fruit EtOH extract were also reported previously (Karioti et al. 2004). Additionally, Karioti et al. (2004) have also attributed the anti-radical potentials of the essential oils derived from X. aethiopica to the presence of these
compounds. Therefore, the higher anti-oxidative and anti-diabetic actions of the fruit and leaf EtOH extracts might be due the presence of these compounds and constituents detected from the extracts.

4.1.6 Conclusions

In conclusion, various solvent extracts from fruit and leaf of X. aethiopica have demonstrated anti-oxidative as well as anti-diabetic effects in vitro when stem and root extracts showed very low or no significant activities in these regards. The fruit EtOH extract exhibited higher actions compared to other extracts of the same or different parts. Thus, it is selected for further study in order to fully investigate the in vivo anti-diabetic and anti-oxidative effects of this plant.
According to the in vitro anti-oxidative and anti-diabetic studies of the various solvent extracts derived from different parts of X. aethiopica stated above, fruit ethanolic extract demonstrated higher activities compared to other extracts. Therefore it was selected for further studies.

4.2 Anti-oxidative effect and inhibition of key enzymes linked to type 2 diabetes of various solvent fractions from fruit ethanolic extract of Xylopia aethiopica in vitro

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4.2.1 Abstract

Objectives: Xylopia aethiopica (Dun.) A. Rich (Annonaceae) has been widely used in food preparations and is locally used in treatment of various diseases. The present study was carried out to assess the anti-oxidative and anti-diabetic potentials of solvent-solvent fractions from crude ethanolic extract of X. aethiopica fruit using various in vitro models.

Methods: Crude ethanolic extract was fractionated using hexane, dichloromethane, ethyl acetate and acetone. They were subjected and investigated for 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging activity, ferric reducing anti-oxidant power (FRAP), inhibition of hemoglobin glycosylation, α-amylase and α-glucosidase activities as markers of in vitro anti-diabetic effects at various doses (30-240 µg/mL). Possible bioactive compounds were analyzed using Gas Chromatography-Mass Spectrometry (GC-MS) analysis.

Results: The results indicated that acetone fraction exhibited significantly (p < 0.05) higher total polyphenols and flavonoids contents and recorded lower IC_{50} values in all the models used (DPPH: 84.47 ± 0.71 µg/mL; inhibition of hemoglobin glycosylation: 148.15 ± 3.57 µg/mL; α-amylase: 155.41 ± 1.83 µg/mL; α-glucosidase: 86.23 ± 0.30 µg/mL) compared to other solvent fractions. Possible phytochemical present in acetone fraction were mostly phenolics and fatty acid derivatives. This finding clearly demonstrates that acetone fraction possesses anti-oxidative and anti-diabetic effects.

Keywords: Acetone fraction, Anti-oxidative, Ethanolic extract, Type 2 diabetes, Xylopia aethiopica,
4.2.2 Introduction

*Xylopia aethiopica* (Dun.) A. Rich (Annonaceae) also known as Ethiopian pepper is a plant, 20 m in height with short roots and smooth grey bark and usually scented when fresh (Iwu, 2014). Its popularity as a spice in many local dishes employed by various traditions in Africa (Kingsley, 2012). In addition, different parts of *X. aethiopica* are used locally either alone or in combination with other plant in the treatment of different disease conditions (Fall et al. 2003). In Nigeria, either *X. aethiopica* fruit decoction alone or in equal proportion with *Alstonia congensis* bark were reported to be used in the treatment of diabetes (Soladoye et al. 2012; Ogbonnia et al. 2008). The fruit is also used as pain reliever and in the treatment of stomach aches, dysentery, ulceration, skin infections and coughs (Ezekwesili et al. 2010). Previous studies have reported that *X. aethiopica* fruit extracts and/or its essential oils possess anti-cancer (Kuete et al. 2013; Choumessi et al. 2012), anti-anaphylactic and anti-inflammatory (Obiri and Osafo, 2013), hypolidemic (Etoundi et al. 2013) and anti-microbial (Esekhiagbe et al. 2009) effects. Furthermore, fruit aqueous extract from *X. aethiopica* was reported to exhibit anti-oxidative and carbohydrate digesting enzymes inhibitory effects *in vitro* (Adefegha and Oboh, 2012a; Etoundi et al. 2010; Odukoya et al. 2005; George and Osiama, 2011). In addition, our recently concluded study had showed that fruit ethanolic (EtOH) extract from *X. aethiopica* fruit demonstrated potent anti-oxidative and anti-diabetic actions *in vitro* (unpublished data). Therefore in our search for the plant-derived products with potent anti-oxidative and anti-diabetic effects, the fruit EtOH extract was further partitioned and fractionated using solvents of increasing polarity. The fractions obtained were subjected to anti-oxidative and anti-diabetic effects using various *in vitro* models. Phytochemical analysis of the fraction with higher activity was assessed using GC-MS analysis.

4.2.3 Materials and methods

For sample collection, identification and preparation of the fractions, including the detail experimental protocols, please refer to chapter 2, sub-sections 2.3 and 2.4.

4.2.4 Results

The results of percentage yield recovered, total polyphenols and flavonoids contents of all the fractions derived from *X. aethiopica* fruit are presented in Table 4.4. According to the data hexane fraction was observed to recover the highest yield while acetone fraction recovered the least amount. It was also observed that acetone fraction exhibited significantly (*p* < 0.05) higher total polyphenols and flavonoids contents compared to other solvent fractions (Table 4.4). In addition the flavonoids content of the hexane fraction was not detected.
Table 4.4: Percentage yield, total polyphenol and flavonoid contents of various fractions from ethanolic fruit extract of X. aethiopica

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Yield (%)</th>
<th>Total polyphenols (mg/gGAE)</th>
<th>Total flavonoids (mg/gQE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexane</td>
<td>17.80</td>
<td>0.63 ± 0.16&lt;sup&gt;a&lt;/sup&gt;</td>
<td>ND</td>
</tr>
<tr>
<td>Dichloromethane</td>
<td>10.54</td>
<td>1.14 ± 0.34&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.07 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>9.60</td>
<td>2.31 ± 0.05&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.23 ± 0.04&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Acetone</td>
<td>8.70</td>
<td>13.46 ± 0.05&lt;sup&gt;c&lt;/sup&gt;</td>
<td>5.13 ± 0.05&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SD values of triplicate determinations. <sup>a-c</sup> Different superscripted letters for a given value within a column are significantly different from each other fractions or standards (Tukey’s-HSD multiple range post hoc test, <i>p</i> < 0.05), ND, Not determined.

The results of ferric (Fe<sup>3+</sup>) reducing anti-oxidant power (FRAP) of the various fractions are presented in **Figure 4.7**. It was observed that acetone fraction showed a significantly (<i>p</i> < 0.05) higher and concentration dependent Fe<sup>3+</sup> to Fe<sup>2+</sup> reducing power compared to other solvent fractions, although the results were significantly (<i>p</i> < 0.05) lower than the ascorbic acid (**Figure 4.7**). The ethyl acetate (EtOAc) fraction exhibited moderate activity, whereas the hexane fraction was the least active.

**Figure 4.7.** Total reducing power (relative to gallic acid) of fruit (A) and leaf (B) extracts of X. aethiopica. Data are presented as mean ± SD of triplicate determinations. <sup>a-e</sup> Different letters presented over the bars for a given concentration of each extract are significantly different from each other fractions or standards (Tukey’s-HSD multiple range post hoc test, <i>p</i> < 0.05).

The data of the DPPH radical scavenging ability of various fractions are presented in **Table 4.5** and **Figure 4.8**. From the results, acetone fraction demonstrated significantly (<i>p</i> < 0.05) lower IC<sub>50</sub> value (84.47 ± 0.71 µg/mL) for the DPPH radical scavenging activity compared to other fractions and this was also comparable to the standards used (Ascorbate: 25.34 ± 6.19 µg/mL; Gallic acid: 20.01 ± 4.42 µg/mL). The IC<sub>50</sub> value depicted by the EtOAc fraction was significantly (<i>p</i> < 0.05) lower compared to that of the hexane and dichloromethane fractions as well (**Table 4.5**).
Figure 4.8. DPPH Radical scavenging activity (%) of various fractions from fruit ethanolic extract of *X. aethiopica*. Data are presented as mean ± SD of triplicate determinations. Values with different letters presented for a given concentration for each extract are significantly different from each other fractions or standards (Tukey’s-HSD multiple range post hoc test, $p < 0.05$).

The data of inhibition of hemoglobin glycation and the calculated IC$_{50}$ values of various fractions are presented in Figure 4.9 and Table 4.5 (IC$_{50}$ values). According to the results the acetone fraction demonstrated significantly ($p < 0.05$) lower IC$_{50}$ value (148.15 ± 3.57 µg/mL) compared to other solvent fractions and comparable to the gallic acid (199.09 ± 2.85 µg/mL). The IC$_{50}$ values exhibited by the dichloromethane and EtOAc fractions were not significantly ($p < 0.05$) different from each other (Table 4.5).

**Table 4.5:** IC$_{50}$ values of various solvent fractions from ethanolic fruit extract of *X. aethiopica* in different anti-oxidative and anti-diabetic models.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>DPPH scavenging activity</th>
<th>Anti-glycation of hemoglobin</th>
<th>$\alpha$-amylase inhibition</th>
<th>$\alpha$-glucosidase inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexane</td>
<td>3190.52 ± 147.53$^c$</td>
<td>5373.39 ± 90.74$^d$</td>
<td>2517.02 ± 194.33$^c$</td>
<td>4155.78 ± 188.35$^c$</td>
</tr>
<tr>
<td>Dichloromethane</td>
<td>1172.05 ± 5.81$^d$</td>
<td>4892.59 ± 53.78$^c$</td>
<td>6818.32 ± 804.96$^e$</td>
<td>1315.41 ± 122.27$^d$</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>317.74 ± 6.29$^c$</td>
<td>4912.24 ± 541.03$^c$</td>
<td>4086.76 ± 153.59$^d$</td>
<td>652.60 ± 58.47$^c$</td>
</tr>
<tr>
<td>Acetone</td>
<td>84.47 ± 0.71$^b$</td>
<td>148.15 ± 3.57$^a$</td>
<td>155.41 ± 1.83$^a$</td>
<td>86.23 ± 0.30$^a$</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>25.34 ± 6.19$^a$</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>20.01 ± 4.42$^a$</td>
<td>199.09 ± 2.85$^b$</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Acarbose</td>
<td>ND</td>
<td>ND</td>
<td>374.95 ± 26.81$^b$</td>
<td>211.59 ± 3.32$^b$</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SD values of triplicate determinations. Different superscripted letters for a given value within a column are significantly different from each other fractions or standards (Tukey’s-HSD multiple range post hoc test, $p < 0.05$). ND, Not determined.
Figure 4.9: Inhibition of hemoglobin glycation (%) of various fractions from fruit ethanolic extract of *X. aethiopica*. Data are presented as mean ± SD of triplicate determinations. *a-e* Values with different letters presented for a given concentration for each extract are significantly different from each other fractions or standards (Tukey’s-HSD multiple range post hoc test, *p* < 0.05).

The results of α-amylase inhibition by the various fractions and the calculated IC₅₀ values are presented in **Figure 4.10** and **Table 4.5** (IC₅₀ values). According to the data, acetone fraction showed a significantly (*p* < 0.05) lower calculated IC₅₀ value (155.41 ± 1.83 µg/mL) for α-amylase inhibition compared to other solvent fractions and acarbose as well (**Table 4.5**). The IC₅₀ values exhibited by the EtOAc and hexane fractions were not significantly (*p* < 0.05) difference compared to the standard acarbose, when that of dichloromethane fraction was the highest (**Table 4.5**).

Figure 4.10: α-Amylase inhibition (%) of various fractions from fruit ethanolic extract of *X. aethiopica*. Data are presented as mean±SD of triplicate determinations. *a-e* Values with different letters presented for a given concentration for each extract are significantly different from each other fractions or standards (Tukey’s-HSD multiple range post hoc test, *p* < 0.05).
The data of α-glucosidase inhibition by the various fractions and the calculated IC\textsubscript{50} values are presented in Figure 4.11 and Table 4.5 (IC\textsubscript{50} values). It was observed that the acetone fraction demonstrated significantly (\textit{p} < 0.05) lower IC\textsubscript{50} value (86.23 ± 0.30 µg/mL) for α-glucosidase inhibition compared to other solvent fractions as well as acarbose (211.59 ± 3.32 µg/mL) (Table 4.5). The IC\textsubscript{50} value depicted by the EtOAc fraction was not significantly different compared to the acarbose, when the other fractions showed the highest calculated IC\textsubscript{50} values (Table 4.5).

![Figure 4.11: α-glucosidase inhibition (%) of various fractions from fruit ethanolic extract of \textit{X. aethiopica}. Data are presented as mean ± SD of triplicate determinations. \textsuperscript{a-d} Values with different letters presented for a given concentration for each extract are significantly different from each other fractions or standards (Tukey’s-HSD multiple range post hoc test, \textit{p} < 0.05).](image)

The results of the kinetics of the mode of the α-glucosidase and amylase inhibition by the most active fraction (acetone) are presented in Figure 4.12. It was observed from the results that the acetone fraction showed a mixed inhibition type for α-amylase action. The K\textsubscript{M} value (control: 0.06%; with acetone: 0.09%) increased when the value of V\textsuperscript{max} (control: 35.67 µmol/min; with acetone: 21.58 µmol/min) was observed to decrease and the K\textsubscript{i} value for inhibiting α-amylase was 45.94 µg/mL. Figure 4.12A. Furthermore, the data revealed that the acetone fraction is a non-competitive inhibitor of α-glucosidase with The K\textsubscript{M} value remains unchanged (1.10 mmol/L), whereas the V\textsuperscript{max} was found to decreases from 471.56 µmol/min to 98.20 µmol/min and the equilibrium constant for inhibitor binding (K\textsubscript{i}) was calculated as 7.89 µg/mL. (Figure 4.12B).
The results of the phytochemical analysis of the most active fraction (acetone) are presented in Figure 4.13 and Table 4.6. Several peaks appeared in the chromatogram of the acetone fraction and were identified and compared with those in the NIST library (Figure 4.13). The major peaks correspond to 3-methyl phenol (1), 1,2-dimethoxy-4-(2-propenyl) benzene (2), 2-methoxy-4-(1-propenyl)-phenol (3), 4-methoxy-6-(2-propenyl)-1,3-benzodioxole (4), 1,2,3-trimethoxy-5-(2-propenyl) benzene (5), 2,6-dimethoxy-4-(2-propenyl)-phenol (6), isoelemicin (7), trans-Z-α-bisabolene epoxide (8), cumminic aldehyde (9) butyl isobutyl phthalate (10), oleic acid (11), kauran-16-ol (12), 4-[2,3-dihydro-7-methoxy-3-methyl-5-(1-propenyl)-2-benzofuranyl]-2-methoxy-phenol (13) and N-(4-methoxyphenyl)-2-hydroxyimino-acetamide (14) (Figure 4.14 and Table 4.6).
Table 4.6: Identified compounds from the acetone fraction from fruit ethanolic extract of *X. aethiopica* by GC-MS

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Compounds</th>
<th>Retention time (min)</th>
<th>Mass/amu</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone</td>
<td>1 3-Methyl phenol</td>
<td>4.45</td>
<td>108 [M]$^+$</td>
</tr>
<tr>
<td></td>
<td>2 1,2-Dimethoxy-4-(2-propenyl) benzene</td>
<td>6.96</td>
<td>178 [M]$^+$</td>
</tr>
<tr>
<td></td>
<td>3 2-Methoxy-4-(1-propenyl)-phenol</td>
<td>7.34</td>
<td>164 [M]$^+$</td>
</tr>
<tr>
<td></td>
<td>4 4-Methoxy-6-(2-propenyl)-1,3-benzodioxole</td>
<td>7.82</td>
<td>192 [M]$^+$</td>
</tr>
<tr>
<td></td>
<td>5 1,2,3-Trimethoxy-5-(2-propenyl) benzene</td>
<td>7.91</td>
<td>208 [M]$^+$</td>
</tr>
<tr>
<td></td>
<td>6 2,6-Dimethoxy-4-(2-propenyl)-phenol</td>
<td>8.25</td>
<td>194 [M]$^+$</td>
</tr>
<tr>
<td></td>
<td>7 Isoelemicin</td>
<td>8.54</td>
<td>208 [M]$^+$</td>
</tr>
<tr>
<td></td>
<td>8 <em>trans</em>-Z-α-Bisabolene epoxide</td>
<td>9.150</td>
<td>220 [M]$^+$</td>
</tr>
<tr>
<td></td>
<td>9 Cumminic aldehyde</td>
<td>10.22</td>
<td>256 [M]$^+$</td>
</tr>
<tr>
<td></td>
<td>10 Butyl isobutyl phthalate</td>
<td>10.26</td>
<td>278 [M]$^+$</td>
</tr>
<tr>
<td></td>
<td>11 Oleic acid</td>
<td>11.12</td>
<td>264 [M]$^+$</td>
</tr>
<tr>
<td></td>
<td>12 Kauran-16-ol</td>
<td>13.17</td>
<td>279 [M]$^+$</td>
</tr>
<tr>
<td></td>
<td>13 4-[2,3-dihydro-7-methoxy-3-methyl-5-(1-propenyl)-2-benzofuranyl]-2-methoxy-phenol</td>
<td>14.58</td>
<td>326 [M]$^+$</td>
</tr>
<tr>
<td></td>
<td>14 N-(4-Methoxyphenyl)-2-hydroxyimino-acetamide</td>
<td>15.40</td>
<td>194 [M]$^+$</td>
</tr>
</tbody>
</table>
Figure 4.14. Structures of compounds identified from the acetone fraction from fruit ethanolic extract of X. aethiopica.
4.2.5 Discussion

It has been reported that aqueous and methanolic extracts from *X. aethiopica* fruit contained high amount of polyphenols and flavonoids (Adefegha and Oboh, 2012a,c; 2011; George and Osiaoma, 2011; Etoundi et al. 2010; Esinkiiagbe et al. 2009; Odukoya et al. 2005). Likewise in our recent study, the fruit EtOH extract was observed to contain higher polyphenols and flavonoids compared to other extracts from the different parts of *X. aethiopica* (unpublished data). However, according to our present results the most polar solvent fraction (acetone) contained the highest amounts, indicating the role of polarity in the extraction of the polyphenols and flavonoids (Table 4.4). That is a positive correlation exists between the polyphenolic contents and polarity of the solvents. The polyphenols are the most available and diverse constituents in plant-derived extracts and impact crucial role in the prevention of the deleterious action of free radicals (Brewer, 2011; Cohen and Kennedy, 2010). The data were further supported by the higher anti-radical actions of the acetone fraction (Table 4.5).

The anti-radical scavenging abilities of the fruit aqueous extract have been reported in some recent studies (Adefegha and Oboh, 2012a,c). In addition, our recent data showed that the fruit EtOH extract exhibited the most active Fe$^{3+}$ to Fe$^{2+}$ and DPPH (IC$_{50}$: of 0.04 ± 0.01 mg/mL) reducing potentials (unpublished data). Moreover, based on our present results, fractionation of the crude EtOH extract yielded another potent anti-radical scavenger (acetone fraction), which was further supported by the higher Fe$^{3+}$ to Fe$^{2+}$ reducing potentials of this fraction (Figure 4.7 and Table 4.5). Therefore the higher electron quenching ability of the acetone fraction might be associated to the higher polyphenolic content, as previous studies have shown strong correlation between the polyphenolic content and the anti-oxidant activity of plant-derived products (Miladi and Demak, 2008; Pulido et al. 2000). Additionally, radical scavenging is among the underlying mechanisms by which plant-derived products prevent the deliterious oxidative damages in diabetes and aging-related complications (Perron and Brumaghim, 2009).

In some diseases such as diabetes, elevation of blood glucose levels and the increasing production of free radicals lead to glycation of the proteins such a as albumin and hemoglobin. This process contributes significantly to tissue and cellular damage in diabetics increase the production of advanced glycation endproducts (AGEs) as well (Lobo et al. 2010). However in a number of studies reported earlier, plant-derived extracts and/or fractions have showed potent anti-glycation that was attributed to their phenolic contents (Mudgal et al. 2010; Pandey and Rizvi, 2009; Rice-Evans et al. 1996). Therefore, the potent anti-glycation exhibited by the acetone fraction might be attributed to the higher phenolic contents of this fraction as well (Table 4.5). This result is partly supported by the higher inhibition of α-amylase and α-glucosidase actions by the acetone fraction (Table 4.5).

Inhibition of the activities of α-amylase and α-glucosidase is an important strategy for delaying carbohydrate digestion and subsequent intestinal absorption of the monosaccharides, and is thus crucial in reduction of postprandial hyperglycemia (Kawamura-Konishi et al. 2012). It has been reported that
X. aethiopica fruit aqueous exhibited good inhibition of α-amylase as well as α-glucosidase actions (Adefegha and Oboh, 2012a; Etoundi et al. 2010). This in addition to our recent study that showed potent action of fruit EtOH extract (IC$_{50}$ value: α-amylase: 0.21 ± 0.01 mg/mL; α-glucosidase: 0.08 ± 0.01 mg/mL) compared to other extracts of X. aethiopica (unpublished data). According to our present data, the inhibition of acetone fraction derived from the fruit EtOH extract was the highest (IC$_{50}$ value: α-amylase: 155.41 ± 1.83 µg/mL; α-glucosidase: 86.23 ± 0.30 µg/mL), indicating the greater availability of potent α-amylase and α-glucosidase inhibitors in this fraction (Table 4.5).

Furthermore, kinetic parameters calculated from the double reciprocal plot revealed that the mode of α-amylase inhibition exhibited by the acetone fraction is a mixed inhibition type (Figure 4.12A). In this type of inhibition, both the $K_m$ and $V_{max}$ are greatly altered in the presence or absence of the inhibitor. This causes some conformational modifications at the active site of the enzyme that hinder the binding of the substrate and thus decreases the enzyme activity. On the other hand, acetone fraction was observed to be a non-competitive inhibitor of the α-glucosidase action (Figure 4.12B). In this type, the km value remains unchanged in the presence or absence of the inhibitor. In the same way as in the mixed inhibition type, non-competitive inhibitors bound at separate site(s) (not the active site) of the enzyme (α-glucosidase) and caused conformational modification at the active site. This leads to the delay of effective binding of the substrate to the enzyme active site and consequently reducing the activity of the enzyme.

Based on the consistently higher anti-oxidant and anti-diabetic actions in vitro of the acetone fraction, it was subjected to GC-MS analysis to find possible phytochemicals present. In a previous study, Karioti et al. (2004) have reported that monoterpenic hydrocarbons are the major compounds detected in the essential oils derived from the fruit, leaf stem and root barks of X. aethiopica, which was further supported by our recent study (Unpublished data). However in our present study, fractionation of the crude EtOH extract yielded mostly terpenes and aromatic compounds (Table 4.6). The presence of compounds such as trans-Z-α-bisabolene epoxide, terpinen-4-ol, cumminic aldehyde and kauran-16-ol were previously linked to the potent anti-oxidative action of oil derived from the X. aethiopica fruit (Karioti et al., 2004). In another study, butyl isobutyl phthalate and cumminic aldehyde (cuminaldehyde) were reported demonstrated anti-diabetic action using various in vitro and in vivo models (Liu et al., 2011; Bu et al., 2010; Lee, 2005). Therefore, the promising anti-oxidative and anti-diabetic actions of acetone fraction observed in our present study might be attributed to the presence of these compounds and other aromatic molecules detected as well.

### 4.2.6 Conclusions

Conclusively, various solvent fractions from X. aethiopica fruit exhibited anti-oxidative as well as inhibition of α-amylase and α-glucosidase actions in vitro. The acetone fraction, which is the most
polar, has exhibited higher activities compared to other fractions. Therefore, it is recommended for *in vivo* anti-diabetic and anti-oxidative effects to fully establish the efficacy of this fraction.
According to the anti-oxidative and anti-diabetic study of the various solvent fractions derived from ethanolic extract of *X. aethiopica* fruit stated above, acetone fraction exhibited higher activities compared to other solvent fractions. Therefore it was selected for the *in vivo* studies.

### 4.3 Anti-diabetic effect of *Xylopia aethiopica* (Dunal) A. Rich. fruit acetone fraction in a type 2 diabetes model of rats

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²Department of Biochemistry, Faculty of Science, Ahmadu Bello University, Zaria-Nigeria

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#### 4.3.1 Abstract

**Objective:** In the traditional medicine from West Africa, fruit decoction of *Xylopia aethiopica* (Dunal) A. Rich. is widely used for the treatment of diabetes mellitus (DM) either alone or in combination with other plants. The present study is designed to investigate the anti-diabetic effects of *X. aethiopica* acetone fraction (XAAF) from fruit ethanolic extract in a type 2 diabetes (T2D) model of rats.

**Methods:** T2D was induced in rats by feeding a 10% fructose solution *ad libitum* for 2 weeks followed by a single intraperitoneal injection of streptozotocin (40 mg/kg body weight) and the animals were orally treated with 150 or 300 mg/kg body weight (bw) of the XAAF once daily for four weeks.

**Results:** After 4 weeks study period, diabetic untreated animals exhibited significantly higher serum glucose, serum fructosamine, LDH, CK-MB, serum lipids, liver glycogen, insulin resistance (HOMA-IR), AI, CRI and lower serum insulin, β-cell function (HOMA-β) and glucose tolerance ability compared to the normal animals. Histopathological examination of their pancreas revealed corresponding pathological changes in the islets and β-cells. These alterations were reverted to near-normal after the treatment of XAAF at 150 and 300 mg/kg bw when, the effects were more pronounced at 300 mg/kg bw compared to the 150 mg/kg bw.

**Conclusion:** The results of our study suggest that XAAF treatment showed excellent anti-diabetic effect in a T2D model of rats.

**Keywords:** Acetone fraction, Fruit ethanolic extract, Type 2 diabetes, Rats, *Xylopia aethiopica*. 
4.3.2 Introduction

The progressive increase of diabetes mellitus (DM) constitutes a global public health problem. The recent report by the International Diabetes Federation (IDF) estimate that DM affects more than 378 million people and this figure is likely to double by 2035, and type 2 diabetes (T2D) is the most prevalent one (IDF, 2014). T2D is a heterogeneous disorder characterized by insulin resistance and partially dysfunctional pancreatic β-cell to properly secrete insulin in response to hyperglycemia (Hui et al. 2007). Despite the currently available conventional drugs (sulfonylureas, biguanides, α-glucosidase inhibitors, thiazolidinediones and dipeptidyl peptidase-4 (DPP-4) inhibitors), the treatment of T2D has been a difficult task. This is due to the unwanted side effects associated with the use of the conventional drugs, which include hypoglycemia, weight gain, hypersensitivity, gastrointestinal discomfort, nausea, liver and heart failure, and diarrhoea (Hung et al. 2012). Therefore, in order to curve these problems, search for anti-diabetic agents has been extended to plant-derived products, since fewer side effects have been reported with the use of plants in the treatment of several diseases (Singh, 2001; Calixto, 2000).

*Xylopia aethiopica* (Dunal) A. Rich. (Annonaceae) also known as Ethiopian pepper is an indigenous spice widely distributed in almost all parts of Africa. It is an aromatic, evergreen plant, 20 m height, fruits in carpels, forming dense cluster, twisted bean-like pods, dark brown, cylindrical, 1.5-6 cm long and 4-7 mm thick (Iwu, 2014; Orwa et al. 2009). The fruit is popularly used as condiment in many local dishes by different traditions in Africa and Asia (Kingsley, 2012). In addition, *X. aethiopica* fruit is widely used locally in the treatment of various ailments and also as an excipient to many other medicines (Freiesleben et al. 2015). In Nigerian, Guinean, Togolese and Senegalese traditional medicines, the fruit decoction is widely used in the treatment of diabetes (Soladoye et al. 2012; Diallo et al. 2012; Karou et al. 2011; Dièye et al. 2008). Additionally, there are some anti-diabetic recipes such as Okudiabet, in Nigeria and Togo that are used for the treatment of diabetes containing *X. aethiopica* fruit as one of the ingredients of the poly-herbal drug (Soladoye et al. 2012; Karou et al. 2011; Ogbonnia et al. 2010; 2008). Moreover, previous studies have shown that various extracts from *X. aethiopica* fruit possessed anti-cancer (Kuete et al. 2013; 2011; Choumessi et al. 2012), anti-fertility (Nwangwa, 2012), anti-sickling (Uwakwe and Nwaoguikpe, 2008), anti-microbial (Esekhiagbe et al. 2009), anti-oxidative actions and inhibits the activities of carbohydrate hydrolysing enzymes *in vitro* as well (Adefegha and Oboh, 2012a,c; 2011; Etoundi et al. 2010; Odukoya et al. 2005; George and Osiaama, 2011).

Furthermore, the toxicological studies so far conducted have indicated that oral administration of *X. aethiopica* fruit aqueous extract for 2 weeks was found to cause less side effects in rats (Johnkennedy et al. 2011; Taiwo et al. 2009). Xylopic acid (15β-acetoxy-(−)-kaur-16-en-19-oic acid), kaurenoic, 15-oxo-kaurenoic acid, and kauran-16-α-ol are some of the compounds isolated from *X. aethiopica* fruit (Iwu, 2014; Ekong and Ogan, 1968). Moreover, *X. aethiopica* seed extract was reported
to exhibit hypolodemic and anti-oxidant actions in hypercholesterolemic rats (Nwozo et al. 2011). In a more recent study, Etoundi et al. (2013) have indicated that *X. aethiopica* fruit ethanolic extract improved glucose tolerance ability in high-sucrose containing diet-fed animals, when the detail anti-diabetic action of either extract or fraction from *X. aethiopica* fruit has not yet been conducted either in humans or in experimental animals.

Therefore, the present study was designed to investigate the *in vivo* anti-diabetic effects of the acetone fraction derived from the fruit ethanolic extract of *X. aethiopica* in a T2D model of rats.

### 4.3.3 Materials and methods

For sample collection, identification and preparation of the fraction, including the detail experimental protocols, please refer to chapter 2, sub-sections 2.3 and 2.5.1 to 2.5.8.

### 4.3.4 Results

The data of weekly body weight (bw) change of all the animals are presented in Figure 4.15. It was observed from the result that before the STZ injection, fructose feeding did not affect the bw of the animals. However, after one week of STZ injection, the bw of the diabetic animals were significantly (*p* < 0.05) decreased compared to non-diabetic animals. Treatment of *X. aethiopica* acetone fraction (XAAF) to diabetic animals for 4-weeks significantly (*p* < 0.05) increased the bw to near normal and comparable to metformin treated animals (Figure 4.15). The effect was more pronounced in DXAH group compared to DXAL group, when the increased in bw of NXAH group was observed not differ significantly (*p* < 0.05) compared to NC group (Figure 4.15).

**Figure 4.15.** Mean body weight change of all animal groups during the study period. Data are presented as the mean ± SD of 5-7 animals. *a-c* Values with different letter near the lines for a given week 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; DXAL, Diabetic *X. aethiopica* low dose; DXAH, Diabetic *X. aethiopica* high dose; DMF, Diabetic Metformin; NXAH, Normal *X. aethiopica* high dose.
The results of daily feed and fluid intake of all the animals are presented in Figure 4.16. According to the data, the feed and fluid intake of DBC group were significantly ($p < 0.05$) increased compared to the NC group. However, oral administration of the XAAF to diabetic animals significantly decreased the feed and fluid intake compared to the DBC group, when the reduction of fluid intake of the DXAH group was significantly ($p < 0.05$) lower compared to the DXAL group (Figure 4.16). Furthermore, the daily feed and fluid intake of NXAH group were not affected throughout the study period.

![Figure 4.16](image)

Figure 4.16. Food and fluid intake in different animal groups during the experimental period. Data are presented as the mean ± SD of 5-7 animals. *-d Values with different letters over the bars for a given parameter 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; Diabetic *X. aethiopica* low dose; DXAH, Diabetic *X. aethiopica* high dose; DMF, Diabetic Metformin; NXAH, Normal *X. aethiopica* high dose.

The weekly blood glucose levels (NFBG and FBG) of all the animals throughout the study period are presented in Figure 4.17. It was observed that, one week after the confirmation of diabetes (week 0), the blood glucose level of the diabetic animals was significantly ($p < 0.05$) elevated compared to the non-diabetic animals. But the treatment of XAAF for four weeks significantly ($p < 0.05$) reduced the elevated blood glucose compared to the DBC group and this reduction was comparable with the metformin-administered DMF group (Figure 4.17). Although not significant, better reduction was observed in the DXAH group compared to the DXAL group. It was also observed that, the NXAH group maintained the blood glucose level within the normal range throughout the study period.
Figure 4.17. Weekly NFBG in all animal groups during the intervention period. Data are presented as the mean SD of 5-7 animals. Values with different letter near the lines for a given week 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; Diabetic X. aethiopica low dose; DXAH, Diabetic X. aethiopica high dose; DMF, Diabetic Metformin; NXAH, Normal X. aethiopica high dose.

The results of oral glucose tolerance test (OGTT) and the calculated area under the curve (AUC) are presented in Figure 4.18 and Table 4.7, respectively. It was observed that, induction of the diabetes significantly ($p < 0.05$) affected the glucose tolerance of the DBC group compared to the NC group. On the other hand, significantly ($p < 0.05$) better glucose tolerance ability was observed in the DXAH and DXAL groups compared to the DBC group, when the effect was more pronounced in the DXAH group compared to the DXAL group, which is comparable to the DMF group (Figure 4.18). Similarly, the calculated AUC for DBC group was significantly ($p < 0.05$) higher compared to NC group when that of DXAL and DXAH groups were significantly ($p < 0.05$) lower compared to DBC groups and were comparable to the DMF group (Table 4.7).
Figure 4.18. Oral glucose tolerance test (OGTT) in all animal groups at the last week of the 4-week intervention period. Data are presented as the mean ± SD of 5-7 animals. *Values with different letter near the lines for a given time 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; Diabetic X. aethiopica low dose; DXAH, Diabetic X. aethiopica high dose; DMF, Diabetic Metformin; NXAH, Normal X. aethiopica high dose.

Table 4.7. Area under the curve (AUC) of different animal groups at the end of the experimental period

<table>
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<tr>
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<th>NC</th>
<th>DBC</th>
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<th>DMF</th>
<th>NXAH</th>
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<tbody>
<tr>
<td>AUC x10³</td>
<td>15.75 ± 0.97 ¹</td>
<td>60.15 ± 7.50 ²</td>
<td>33.89 ± 7.83 ³</td>
<td>30.65 ± 8.85 ³</td>
<td>28.68 ± 7.21 ³</td>
<td>15.78 ± 0.79 ³</td>
</tr>
</tbody>
</table>

Data are presented as the mean ± SD of 5-7 animals. **Values with different letter along a row for a given parameter 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; DXAL, Diabetic X. aethiopica low dose; DXAH, Diabetic X. aethiopica high dose; DMF, Diabetic Metformin; NXAH, Normal X. aethiopica high dose, AUC, Area under curve.

The results of serum insulin and fructosamine levels including the calculated HOMA-IR and HOMA-β scores are presented in Table 4.8. From the results, the serum insulin level and the calculated HOMA-β scores were decreased significantly (p < 0.05) whereas serum fructosamine as well as HOMA-IR scores were increased significantly (p < 0.05) in the DBC group compared to the NC group (Table 4.8). After 4-week treatment of XAAF, the serum insulin level was significantly (p < 0.05) increased and the serum fructosamine and HOMA-IR scores were significantly (p < 0.05) decreased in DXAH and DXAL groups compared to the DBC group (Table 4.8). The calculated HOMA-β scores of the diabetic treated animals was significantly (p < 0.05) in the DXAH and DMF group compared to the DBC group when no significant difference was observed between the DXAH and DMF groups (Table
4.8). The above-mentioned parameters were not affected in NXAH group throughout the test period (Table 4.8).
Table 4.8. Effect of XAAF on serum insulin and fructosamine levels and calculated HOMA-IR and HOMA-β scores in different animal groups at the end of the intervention period.

<table>
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<th>NC</th>
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<th>DXAL</th>
<th>DXAH</th>
<th>DMF</th>
<th>NXAH</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Insulin (pmol/L)</strong></td>
<td>76.50 ± 6.26c</td>
<td>47.05 ± 9.02a</td>
<td>54.42 ± 6.01a</td>
<td>67.07 ± 10.00b</td>
<td>75.88 ± 14.23c</td>
<td>73.03 ± 6.18b</td>
</tr>
<tr>
<td><strong>Fructosamine (μmol/l)</strong></td>
<td>239.20 ± 47.49a</td>
<td>365.43 ± 44.94c</td>
<td>301.00 ± 16.58b</td>
<td>288.00 ± 23.26ab</td>
<td>283.14 ± 32.46ab</td>
<td>235.00 ± 24.59a</td>
</tr>
<tr>
<td><strong>HOMA-IR</strong></td>
<td>2.50 ± 0.27a</td>
<td>6.30 ± 1.64b</td>
<td>3.43 ± 0.71a</td>
<td>3.17 ± 0.48a</td>
<td>3.82 ± 0.90a</td>
<td>2.33 ± 0.26a</td>
</tr>
<tr>
<td><strong>HOMA-β</strong></td>
<td>127.66 ± 43.71d</td>
<td>7.33 ± 1.26a</td>
<td>25.07 ± 7.13b</td>
<td>46.51 ± 12.09c</td>
<td>52.57 ± 25.76c</td>
<td>127.03 ± 22.24d</td>
</tr>
</tbody>
</table>

Data are presented as the mean ± SD of 5-7 animals. **Values with different letters along the rows for a given parameter 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; Diabetic *X. aethiopica* low dose; DXAH, Diabetic *X. aethiopica* high dose; DMF, Diabetic Metformin; NXAH, Normal *X. aethiopica* high dose. *HOMA-IR = [(Fasting serum insulin in U/l x Fasting blood glucose in mmol/L)/22.5]. **HOMA-β = (Fasting serum insulin in U/l x 20/Fasting blood glucose in mmol/L-3.5).

Figure 4.19. Histopathological examination of the pancreatic tissues of different animal groups at the end of the intervention period. The NC had a larger islet with high number of β-cells while the DBC had a smaller islet and morphologically deformed β-cells. The DXAL, DXAH and DMF groups had relatively larger islets with higher number of β-cells compared to the DBC group. NC, Normal Control; DBC, Diabetic Control; Diabetic *X. aethiopica* low dose; DXAH, Diabetic *X. aethiopica* high dose; DMF, Diabetic Metformin; NXAH, Normal *X. aethiopica* high dose.
The histopathological slides of the pancreas for all the groups are presented in Figure 4.19. The slides revealed that, there were fatty infiltrations, reduce number of pancreatic β-cells and smaller size of the pancreatic islet of the DBC group compared to the NC group. However, treatment with XAAF to diabetic animals reduced fatty infiltrations, improved the number of pancreatic β-cells and the pancreatic islet size in the DXAL and DXAH groups (Figure 4.19). Additionally, the fatty infiltrations in the DXAH group were less compared to that of DXAL group. Furthermore, the pancreatic morphology was not affected by the higher dose of XAAF in the NXAH group (Figure 4.19).

The results of absolute and relative liver weight including liver glycogen contents are presented in Table 4.9. According to the data, although the absolute liver weight was not affected by the induction of diabetes, the significantly ($p < 0.05$) increased relative liver weight and glycogen content was observed in the DBC group compared to the NC group. Oral administration of the XAAF significantly ($p < 0.05$) restored the relative liver weight and glycogen content in the DXAH and DXAL groups which is comparable to the NC and DMF groups (Table 4.9). The liver weights and glycogen content of the NXAH group were not affected by the administration of XAAF throughout the experimental period.

In Table 4.10, the serum lipid profile and calculated atherogenic index (AI) and coronary risk index (CRI) are presented. The increased serum total cholesterol (TC), triglycerides (TG) and low density lipoprotein (LDL) cholesterol as well as calculated AI and CRI along with the reduction of serum high density lipoprotein (HDL) cholesterol were observed in the DBC group compared to the NC group. After 4-week of oral intervention with XAAF, the serum TC, TG, LDL-cholesterol, calculated AI and CRI were significantly ($p < 0.05$) decreased in the DXAL and DXAH groups compared to the DBC group in a dose-dependent manner (Table 4.10) when, the serum HDL-cholesterol level of diabetic treated groups was not significantly ($p < 0.05$) difference compared to the DBC group. Administration of the high dose of the fraction to non-diabetic animals did not affect these parameters in the NXAH group compared to the NC group (Table 4.10).

The results of serum ALT, AST, ALP, urea, uric acid, creatinine, LDH and CK-MB are presented in Table 4.11. It was observed from the data that, the serum ALT, ALP, urea, creatinine, LDH and CK-MB levels were elevated in the DBC group compared to the NC group, when the serum ALP and uric acid levels were not affected by the induction of diabetes (Table 4.11). Treatment with XAAF to diabetic animals significantly ($p < 0.05$) reduced serum ALT, ALP, urea, creatinine, LDH and CK-MB levels compared to the DBC group (Table 4.11). Moreover, treatment to non-diabetic animals with high dose of XAAF did not alter the serum levels of these parameters compared to the NC group.
Table 4.9. Effect of XAAF on liver weights and liver glycogen concentrations in T2D rats

<table>
<thead>
<tr>
<th></th>
<th>NC</th>
<th>DBC</th>
<th>DXAL</th>
<th>DXAH</th>
<th>DMF</th>
<th>NXAH</th>
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<tbody>
<tr>
<td>Liver weight (g)</td>
<td>9.25 ± 1.04</td>
<td>10.34 ± 0.31</td>
<td>9.62 ± 0.84</td>
<td>10.05 ± 1.03</td>
<td>9.10 ± 1.33</td>
<td>9.42 ± 0.35</td>
</tr>
<tr>
<td>Relative liver weight (%)*</td>
<td>2.89 ± 0.29²</td>
<td>4.55 ± 0.34¹b</td>
<td>3.27 ± 0.35¹a</td>
<td>3.40 ± 0.22¹a</td>
<td>3.10 ± 0.45¹a</td>
<td>2.93 ± 0.18¹a</td>
</tr>
<tr>
<td>Liver glycogen (mg/g tissue)</td>
<td>6.76 ± 0.81¹a</td>
<td>8.25 ± 0.83¹b</td>
<td>7.17 ± 0.46¹a</td>
<td>6.93 ± 0.45¹a</td>
<td>6.92 ± 0.56¹a</td>
<td>6.58 ± 0.62¹a</td>
</tr>
</tbody>
</table>

Data are presented as the mean ± SD of 5-7 animals. *Values with different letter along the rows for a given parameter 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; Diabetic *X. aethiopica* low dose; DXAH, Diabetic *X. aethiopica* high dose; DMF, Diabetic Metformin; NXAH, Normal *X. aethiopica* high dose.

*Relative liver weight (%) = [weight of the liver (g)/body weight (g)] x 100
Table 4.10. Serum lipid profiles and other biochemical parameters in different animal groups at the end of the intervention period

<table>
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<th>NC</th>
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<th>DXAH</th>
<th>DMF</th>
<th>NXAH</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC (mg/dL)</td>
<td>76.20 ± 7.40&lt;sup&gt;a&lt;/sup&gt;</td>
<td>116.00 ± 12.92&lt;sup&gt;c&lt;/sup&gt;</td>
<td>76.29 ± 8.36&lt;sup&gt;a&lt;/sup&gt;</td>
<td>78.86 ± 5.21&lt;sup&gt;a&lt;/sup&gt;</td>
<td>95.71 ± 7.74&lt;sup&gt;b&lt;/sup&gt;</td>
<td>73.20 ± 8.23&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>TG (mg/dL)</td>
<td>70.20 ± 18.10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>150.86 ± 24.57&lt;sup&gt;b&lt;/sup&gt;</td>
<td>86.57 ± 26.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>91.86 ± 8.15&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>100.71 ± 32.47&lt;sup&gt;b&lt;/sup&gt;</td>
<td>69.80 ± 3.83&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>LDL-Cholesterol (mg/dL)*</td>
<td>12.96 ± 4.16&lt;sup&gt;a&lt;/sup&gt;</td>
<td>57.54 ± 14.45&lt;sup&gt;c&lt;/sup&gt;</td>
<td>32.26 ± 11.55&lt;sup&gt;b&lt;/sup&gt;</td>
<td>31.63 ± 5.69&lt;sup&gt;b&lt;/sup&gt;</td>
<td>42.86 ± 8.93&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>26.64 ± 7.96&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>HDL-Cholesterol (mg/dL)</td>
<td>49.20 ± 8.35&lt;sup&gt;b&lt;/sup&gt;</td>
<td>28.29 ± 6.25&lt;sup&gt;a&lt;/sup&gt;</td>
<td>26.71 ± 7.30&lt;sup&gt;a&lt;/sup&gt;</td>
<td>28.86 ± 5.87&lt;sup&gt;a&lt;/sup&gt;</td>
<td>32.71 ± 7.43&lt;sup&gt;a&lt;/sup&gt;</td>
<td>32.60 ± 4.51&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>AI</td>
<td>0.57 ± 0.13&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.30 ± 1.08&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.04 ± 0.90&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.81 ± 0.48&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>2.06 ± 0.69&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.27 ± 0.32&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>CRI</td>
<td>1.57 ± 0.13&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.30 ± 1.08&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.04 ± 0.90&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.81 ± 0.48&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>3.06 ± 0.69&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.27 ± 0.32&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data are presented as the mean SD of 5-7 animals. **Values with different letters along the rows for a given parameter 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; Diabetic X. aethiopica low dose; DXAH, Diabetic X. aethiopica high dose; DMF, Diabetic Metformin; NXAH, Normal X. aethiopica high dose; TC, Total cholesterol; TG, Triglyceride; LDL-cholesterol, Low density lipoprotein-cholesterol; HDL-cholesterol; High density lipoprotein-cholesterol; AI, Atherogenic index; CRI, Coronary risk index

*LDL-Cholesterol (mg/dL)= [TC- HDL- (TG/5)]
Table 4.11. Serum biochemical parameters in different animal groups at the end of the intervention period

<table>
<thead>
<tr>
<th></th>
<th>NC</th>
<th>DBC</th>
<th>DXAL</th>
<th>DXAH</th>
<th>DMF</th>
<th>NXAH</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT (U/L)</td>
<td>77.40 ± 20.38a</td>
<td>116.14 ± 37.37c</td>
<td>81.29 ± 8.46a</td>
<td>87.57 ± 9.86ab</td>
<td>93.57 ± 6.86b</td>
<td>73.80 ± 14.1a</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>88.40 ± 11.41</td>
<td>83.29 ± 15.57</td>
<td>80.29 ± 13.09</td>
<td>83.43 ± 9.45</td>
<td>94.71 ± 9.39</td>
<td>82.60 ± 16.33</td>
</tr>
<tr>
<td>ALP (U/l)</td>
<td>149.40 ± 11.33a</td>
<td>451.14 ± 35.10d</td>
<td>317.71 ± 33.60c</td>
<td>234.86 ± 25.79b</td>
<td>208.86 ± 27.58b</td>
<td>146.40 ± 19.32a</td>
</tr>
<tr>
<td>Creatinine (mg/dL)</td>
<td>2.52 ± 0.78b</td>
<td>3.12 ± 0.45b</td>
<td>2.27 ± 0.34b</td>
<td>2.13 ± 0.40b</td>
<td>1.68 ± 0.27a</td>
<td>1.35 ± 0.34a</td>
</tr>
<tr>
<td>Urea (mg/dL)</td>
<td>22.00 ± 4.69a</td>
<td>60.71 ± 15.71c</td>
<td>29.43 ± 6.43b</td>
<td>30.00 ± 8.91b</td>
<td>26.00 ± 5.00b</td>
<td>17.80 ± 4.66a</td>
</tr>
<tr>
<td>Uric acid (mg/dL)</td>
<td>2.06 ± 0.67</td>
<td>2.85 ± 1.18</td>
<td>2.05 ± 1.07</td>
<td>2.40 ± 1.20</td>
<td>2.56 ± 1.36</td>
<td>1.48 ± 0.40</td>
</tr>
<tr>
<td>LDH (U/L)</td>
<td>284.00 ± 62.56a</td>
<td>458.86 ± 68.05c</td>
<td>339.86 ± 64.41b</td>
<td>301.71 ± 36.94b</td>
<td>361.57 ± 80.66ab</td>
<td>295.60 ± 37.96a</td>
</tr>
<tr>
<td>CK-MB (U/L)</td>
<td>374.54 ± 82.56a</td>
<td>1064.36 ± 537.18b</td>
<td>582.53 ± 86.00a</td>
<td>492.01 ± 51.28a</td>
<td>743.97 ± 75.29ab</td>
<td>371.52 ± 9.06a</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SD of 5-7 animals. *-d Values with different superscript letters along a row for a given parameter are significantly different from each other group of animals (Tukey's-HSD multiple range *post hoc* test, *p* < 0.05). ALT, Alanine transaminase; AST, Alanine transaminase; ALP, Alkaline phosphate; LDH, Lactate dehydrogenase; CK-MB, Creatine kinase.
4.3.5 Discussion

In the traditional medicine from West Africa, *X. aethiopica* fruit is used locally for anti-diabetic remedy and other related metabolic disorders (Soladoye et al. 2012; Diallo et al. 2012; Karou et al. 2011; Dièye et al. 2008). In addition, the acetone fraction derived from the fruit ethanolic extract has demonstrated higher inhibitory effect of α-glucosidase and α-amylase actions *in vitro* (unpublished data) compared to other fractions and therefore was selected for the present study. This is the first comprehensive study that reports the anti-diabetic potential of *X. aethiopica* fruit, despite the popularity of the plant in the treatment of diabetes in West Africa. The result of our present study is quite promising as the both dosages of the fraction (150 and 300 mg/kg bw) used have demonstrated strong anti-diabetic activity in the diabetic animals.

Etoundi et al. (2013) have reported that the daily administration of *X. aethiopica* fruit ethanolic extract (400 mg/kg bw) for 30 days reverted the gained body weight in high-sucrose fed rats. In another study, non-diabetic animals maintained in diets with 2% and 4% of *X. aethiopica* fruit for 2 weeks showed no effect on the bw of the treated animals (Adefegha and Oboh, 2012a). However, the effect *X. aethiopica* fruit ethanolic extracts or fruit itself may be different from the *X. aethiopica* acetone fraction of fruit ethanolic extract. Additionally, the effects of *X. aethiopica* fruit extracts or fraction may not be similar in non-diabetic and diabetic animals. Hence, the effects XAAF in our study are not exactly consistent with the above-mentioned studies. However, despite of lower feed and fluid intake (Figure 4.16) the significantly improved body weight in the XAAF administered groups (DXAL and DXAH) compared to the DBC group (Figure 4.15) revealed its potent anti-diabetic effects, when no significant alteration was observed in the NXAH group (Figure 4.15 and 4.16). The result was further supported by the significantly reduced blood glucose level in the DXAL and DXAH groups compared to the DBC group (Figure 4.17).

In our present study, the persistent hyperglycemia was observed in the DBC group throughout the experimental period (Figure 4.17), which is an indication of successful induction of diabetes. At the same time, significantly higher HOMA-IR scores and significantly lower HOMA-β scores in the DBC group compared to the NC group (Table 4.7) confirmed the induction of insulin resistance and partial pancreatic beta-cell dysfunction. However, a dose-dependent reduction of blood glucose level was observed in the diabetic treated animals (Figure 4.17), which was further supported by the OGTT data (Figure 4.18). In a previous study, Etoundi et al. (2013) have reported that, high-sucrose fed rats treated with 400 mg/kg bw of fruit ethanolic extract improved the blood glucose tolerance ability, which is in line with our present data. This could be due to the potent inhibitory effects of *X. aethiopica* fruit on the activities of carbohydrate hydrolyzing enzymes as reported previously (Adefegha and Oboh, 2012a; Etoundi et al. 2010). The inhibitors of these enzymes cause a decrease in the digestion of carbohydrates and intestinal absorption of glucose and therefore reduced the hyperglycemia in T2D (Ademiluyi and Oboh, 2013). In addition, even though, the dosages used in our present study are lower
compared to the earlier study (Etoundi et al. 2013), the significantly lower blood glucose levels and better glucose tolerance ability of the DXAL and DXAH groups compared to DBC the group (Figures 4.17 and 4.18) revealed the potential anti-diabetic effects XAAF, at least in the case of type 2 diabetes.

It has been reported that chronic uncontrolled hyperglycemia in T2D causes an impairment or decline in insulin secretion (Nattrass and Bailey, 1999), with subsequent distortion of pancreatic architecture, β-cell integrity and function as well (Bonner-Weir and O'Brien, 2008). These effects were fully manifested in the DBC group in our present study (Table 4.8 and Figure 4.19). On the other hand, the oral treatment of XAAF dose dependently improved serum insulin levels, decreased insulin resistance and restored the pancreatic morphology to near normal in diabetic animals. In one of our previous studies, the XAAF was also found to possess high in vitro anti-oxidative activity (data not shown) in addition to other studies conducted on the crude extracts of *X. aethiopica* fruit (Adefegha and Oboh, 2012a; 2011; Etoundi et al. 2010; Odukoya et al. 2005; George and Osiama, 2011). Therefore, we may propose that the protection of the pancreatic islets morphology could be mediated through the anti-oxidant potentials because oxidative stress is the major causal factor for the pancreatic β-cell damage inT2D (Huang et al. 2011). Interestingly, the above hypothesis was further supported by the significant attenuation of the serum fructosamine level in diabetic treated animals (Table 4.8). The reduction of serum fructosamine level by XAAF is an indication of decrease in protein glycosylation, a major contributor to the production of advanced glycation ends products in diabetic condition.

In consisten with the earlier reports, the absolute liver weight was not affected but the relative liver weight and liver glycogen content were significantly increased by the induction of diabetes (Clore et al. 1992; Oliveira et al. 2008). The elevation of hepatic glycogen content could possibly be linked to the fructose feeding that triggers hepatic glycogenesis via stimulation of glucose-6-phosphatase activity and cause higher accumulation of glycogen in the liver despite low circulating insulin (Ciudad et al. 1988). However, these effects were completely attenuated to near normal in DXAH and DXAL groups and were comparable to DMF group (Table 4.9). This suggests that, XAAF may modulate glycogen biosynthesis by suppressing the activity of glucose-6-phosphatase through a reduction in glucose-6-phosphate/ATP ratio and therefore, decreases the hepatic glycogen synthesis and storage as well. Though the diabetic treated animals have shown insulinotropic ability, which invariably mobilize hepatic glucose input for glycogenesis, the hepatic glycogen level was maintained within the normal range. This has demonstrated that treatment of XAAF to diabetic animals restored the normal metabolic processes altered by the induction of diabetes, which was further supported by the anti-hyperlipidemic effect of XAAF (Table 4.10).

Considerable evidence has been accumulated to correlate insulin resistance in the etiology of dyslipidemia in T2D. It has been postulated that the insulin resistance stimulate lipolysis from the fats depots, which promote the activity of HMG-CoA reductase and decrease lipoprotein lipase, which lead to the elevation of TG and TC levels (De Silva and Frayling, 2010; Hotamisligil, 2000). In a previous study, the oral treatment of *X. aethiopica* fruit aqueous extract (200 mg/kg bw) to non-diabetic animals
for 2 weeks exhibited hypolipidemic action (Johnkennedy et al. 2011). Moreover, Adefegha and Oboh (2012a) also reported similar findings when *X. aethiopica* fruit (2% and 4%) was included in the diet of the same animal model and for the same period of time. In another study, administration of *X. aethiopica* aqueous extract (250 mg/kg bw) for 8 weeks demonstrated potent anti-hyperlipidemic effect in hypercholesterolemic rats (Nwozo et al. 2011). Conversely, in a more recent study, Etoundi et al. (2013) have shown that ethanolic extract (400 mg/kg bw) did not show any significant anti-hyperlipidemic action after 2-day post-treatment period in hyperlipidemic rats. However, in our study, the *X. aethiopica* fruit acetone fraction showed potent anti-hyperlipidemic ability with no alterations in serum lipid profile of NXAH group (Table 4.10). Therefore, the lipid-lowering effect of *X. aethiopica* fruit can only be achieved after prolong treatment (sub-chronic or chronic) against the acute treatment as recently reported by Etoundi et al. (2013). In addition, the anti-hyperlipidemic action of XAAF observed in our present study could be attributed to the reduction of insulin resistance in the diabetic treated animals.

On the other hand, Etoundi et al. (2013) have also reported the hepato-protective ability of *X. aethiopica* fruit (400 mg/kg bw) in hyperlipidemic rats via reduction of serum ALT and AST levels in the treated animals. Additionally, no significant alterations of serum ALT, AST and ALP were observed in non-diabetic animals treated with *X. aethiopica* fruit (3% and 4%) in diet for 2 weeks (Adefegha and Oboh, 2012a). This was apparently in line with our present data (Table 4.11). Treatment of the fraction protects the integrity of the liver as well as heart via reductions of serum ALT, ALP, urea, creatinine, LDH and CK-MB in the treated diabetic animals. The serum AST was found not to be affected by the diabetes throughout the study period. In comparison with ALT, AST is less specific for the detection of hepatic injury due to its shorter half-life compared to ALT, particularly in experimental animals (Center, 2007).

**4.3.6 Conclusion**

In conclusion, oral intervention of *X. aethiopica* fruit acetone fraction (150 and 300 mg/kg bw) have demonstrated anti-diabetic actions via improving bw gain, reducing food and fluid intake and hyperglycemia, improving glucose tolerance ability, insulin sensitivity, amelioration of pancreatic β-cell histology and β-cell functions and improving dyslipidemia in T2D model of rats. Hence, our findings suggest that acetone fraction derived from *X. aethiopica* fruit may provide an excellent anti-diabetic remedy with no considerable side effects. Further clinical study is required to confirm the effects in human subjects. The isolation and partial characterization of the active principle from XAAF is currently underway to find the compound(s) responsible for this activity.
4.4 *In vivo* anti-oxidant potential of *Xylopia aethiopica* fruit acetone fraction in a type 2 diabetes model of rats

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4.4.1 Abstract

**Objectives:** The present study investigated the *in vivo* anti-oxidative actions of *Xylopia aethiopica* acetone fraction (XAAF) from fruit ethanolic extract in a type 2 diabetes (T2D) model of rats.

**Methods:** T2D was induced in rats by feeding a 10% fructose solution *ad libitum* for two weeks followed by a single intraperitoneal injection of streptozotocin (40 mg/kg body weight(bw)) and the animals were orally treated with 150 or 300 mg/kg bw of the XAAF once daily for four weeks.

**Results:** After four weeks of intervention, diabetic untreated animals showed significantly higher levels of thiobarbituric acid reactive substances (TBARS) and reductions of reduced glutathione (GSH) in the serum and organs (liver, kidney, heart and pancreas) compared to the normal animals. The activities of endogenous anti-oxidant enzymes (superoxide dismutase, catalase, glutathione peroxidase and reductase) were greatly impaired in in the serum and organs of diabetic untreated animals compared to the normal animals. These alterations were reverted to near-normal after the treatment of XAAF, especially at the dose of 300 mg/kg bw.

**Conclusion:** The results of our study suggest that XAAF treatment at 300 mg/kg bw showed potent anti-oxidative action in a T2D model of rats.

**Keywords:** *Xylopia aethiopica*, Anti-oxidant, acetone fraction, Fruit ethanolic extract, Type 2 diabetes, Rats.

4.4.2 Introduction

Recently, tremendous evidences are available, correlating the cellular injury arising from ROS in the etiology of some diseases such as Alzheimer disease, inflammation, viral infections and T2D (Kroner, 2009; Atawodi, 2005; Rahimi et al. 2005). In T2D, the mechanisms involve in hyperglycemia-induced cellular and tissue damages have been previously described (Sabu and Kuttan, 2002; Boynes, 1991; Collier et al. 1990). This condition causes a depletion of *in vivo* anti-oxidant defense system and increase the ROS production, a term referred as oxidative stress. Therefore, due to decrease in the
overall in vivo defense system, the increased intake of anti-oxidants cannot be underestimated. Interestingly, within the last two decades, there has been an upsurge of interest in the utilization of natural anti-oxidants present in plants such as Xylopia aethiopica (Dunal) A. Rich. (Annonaceae) in attenuating the deleterious ROS-induced tissue damage (Sangeetha et al. 2011).

X. aethiopica also known as Ethiopian pepper is an indigenous spice widely distributed in almost all parts of Africa (Iwu, 2014). In some previous studies, extracts derived from the X. aethiopica fruit have showed potent anti-oxidative activity using various in vitro models (Adefegha and Oboh, 2012a,c; 2011; Etoundi et al. 2010; Odukoya et al. 2005; George and Osia, 2011). In another study, the seed aqueous extract was reported to exhibit hypolidemic and anti-oxidant actions in hypercholesterolemic rats (Nwozo et al. 2011). In addition, our recent investigation have shown that X. aethiopica acetone fraction (XAAF) from fruit EtOH extract demonstrated anti-oxidative potential in vitro, ameliorated β-cell dysfunction and decreased blood glucose levels in a T2D model of rats (Mohammed and Islam, 2015; Unpublished work). Therefore in order to fully validate the anti-diabetic action of XAAF, our present study investigate the effect of XAAF in attenuating hyperglycemia-induced alterations on the in vivo anti-oxidant defense system in type 2 diabetic animals.

4.4.3 Materials and methods

For sample collection, identification and preparation of the fraction, including the detail experimental protocols, please refer to chapter 2, sub-sections 2.3 and 2.5.1 to 2.5.8.

4.4.4 Results

The data of the lipid peroxidation, expressed as malondialdehyde (MDA) concentration of the serum and organs (liver, kidney, heart and pancreas) of all the groups are presented in Figure 4.20. According to the results, the MDA concentration of diabetic control (DBC) group has significantly \( p < 0.05 \) increased in the serum, liver, heart and pancreas compared to the NC group. Additionally, the elevation of MDA level in the kidney of the the DBC group was not significantly \( p < 0.05 \) affected by the diabetes (Figure 4.20). However, oral administration of XAAF has significantly \( p < 0.05 \) reduced the MDA levels in the serum, liver and pancreas of the DXAL and DXAH groups, comparable to the DMF group. The reduction of MDA in the kidney and heart of the diabetic treated groups was observed not to differ significantly \( p < 0.05 \) compared to DBC group (Figure 4.20).
Figure 4.20. Serum and organs thiobarbituric acid reactive substances of all animal groups at the end of the intervention period. Data are presented as the mean ± SD of 5-7 animals. "Values with different letter over 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; DXAL, Diabetic *X. aethiopica* low dose; DXAH, Diabetic *X. aethiopica* high dose; DMF, Diabetic Metformin; NXAH, Normal *X. aethiopica* high dose; MDA, Malondialdehyde.

The results of the reduced glutathione (GSH) concentrations of the serum and organs of all the groups are presented in Figure 4.21. The concentration of GSH in the serum, liver and pancreas was significantly \( (p < 0.05) \) decreased in the DBC group compared to the NC group, when the reduction in kidney and heart was not statistically difference \( (p < 0.05) \) compared to the NC group (Figure 4.21). Oral treatment of the fraction in the DXAH group significantly \( (p < 0.05) \) increased the levels of GSH in the serum, liver and pancreas compared to the DBC group (Figure 4.21). Furthermore, the increase of GSH was not greatly affected in in the kidney and heart of the diabetic treated groups compared to the NC group. Additionally, the GSH level of NXAH group was not altered throughout the study period.
Figure 4.21. Serum and organs reduced glutathione (GSH) content in all animal groups at the end of the study period. Data are presented as the mean ± SD of 5-7 animals. *Values with different letter over 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; DXAL, Diabetic X. aethiopica low dose; DXAH, Diabetic X. aethiopica high dose; DMF, Diabetic Metformin; NXAH, Normal X. aethiopica high dose; GSH, Reduced glutathione.

The results of the activity of superoxide dismutase (SOD) of serum and organs of all the groups are presented in Figure 4.22. It was observed from the results that the activity of SOD in serum and organs of the DBC group was significantly \( (p < 0.05) \) decreased. After oral intervention of XAAF for 4 weeks, the activity of SOD significant \( (p < 0.05) \) increased in the serum and organs of the DXAH group compared to the DBC group (Figure 4.22). In addition, the activity of SOD in the liver of the NXAH group was significantly \( (p < 0.05) \) increased compared to the NC group (Figure 4.22).

Figure 4.22. Superoxide dismutase (SOD) activities in the serum and organs of different animal groups at the end of the study period. Data are presented as the mean ± SD of 5-7 animals. *Values with different letter over 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; DXAL, Diabetic X. aethiopica low dose; DXAH, Diabetic X. aethiopica high dose; DMF, Diabetic Metformin; NXAH, Normal X. aethiopica high dose.
The results of catalase activity in serum and organs of all the groups are presented in Figure 4.23. According to the data, the activity of the catalase in the serum and organs was significantly \( (p < 0.05) \) decreased in the DBC group compared to the NC group. However, administration of XAAF to diabetic animals significantly \( (p < 0.05) \) increased the activity of catalase in the organs, when the increased in serum was did not differ significant \( (p < 0.05) \) compared to DBC group (Figure 4.23).

![Figure 4.23. Catalase activities in the serum and different organs of different animal groups at the end of the study period. Data are presented as the mean ± SD of 5-7 animals. **Values with different letter over 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; DXAL, Diabetic *X. aethiopica* low dose; DXAH, Diabetic *X. aethiopica* high dose; DMF, Diabetic Metformin; NXAH, Normal *X. aethiopica* high dose.]

The results of the activity of glutathione peroxidase (GPx) in serum and organs of all the groups are presented in Figure 4.24. The activity of the GPx in the serum and organs was significantly \( (p < 0.05) \) decreased in the DBC group compared to the NC group. Treatment of XAAF especially in the DXAH group significantly \( (p < 0.05) \) increased the GPx activity comparable to the DMF group (Figure 4.24). The activity of GPx in the serum and organs of the NXAH group was altered within the study period (Figure 4.24).
Figure 4.25. Glutathione peroxidase activities in the serum and organs of different animal groups at the end of the study period. Data are presented as the mean ± SD of 5-7 animals. Values with different letters over 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; DXAL, Diabetic *X. aethiopica* low dose; DXAH, Diabetic *X. aethiopica* high dose; DMF, Diabetic Metformin; NXAH, Normal *X. aethiopica* high dose.

The data of glutathione reductase (GR) activity in the serum and organs of all the groups are presented in Figure 4.25. The activity of this enzyme was not affected by the diabetes in the kidney and heart, whereas significantly (p < 0.05) decreased in the serum, liver and pancreas of the DBC group compared to NC group (Figure 4.25). However, oral administration of the XAAF especially in the DXAH significantly (p < 0.05) and dose-dependently increased in activity of GR in serum, liver, kidney and pancreas, when in the eart the effect was not statistically different from the DBC group (Figure 4.25). In addition, treatment of XAAF had significantly (p < 0.05) increased the activity of GR in the liver, kidney and heart of NAMH compared to the NC group (Figure 4.25).
4.4.5 Discussion

In a previous study, Nwozo et al. (2011) have reported that administration of *X. aethiopica* aqueous extract (250 mg/kg bw) for 8 weeks decrease MDA level in the serum, liver and kidney of hypercholesterolemic rats. This is in line with the result of our present study and was similarly observed in the heart and pancreas of the diabetic treated animals (Figure 4.20). Additionally, the result is promising even at the lower dose (150 mg/kg bw) of XAAF used in our present study. Lipid peroxidation manifested due to impair lipid metabolism plays a crucial role in the pathogenesis of cellular damage in T2D (Fenercioğlu et al. 2010; Memısogullari et al. 2003). Therefore, the potent reduction of MDA levels after treatment of XAAF indicated the ability of the XAAF in modulating diabetes-induced cellular injury and oxidative stress as well. The data was further supported by the increase in reduced glutathione (GSH) levels of the diabetic treated animals (Figure 4.21).

GSH is an important cellular anti-oxidant biomolecule that prevents the oxidative damage by alleviating the ROS mediated lipid peroxidation (Rahimi et al. 2005). Previously, treatment of *X. aethiopica* fruit aqueous extract at 250 mg/kg bw for 8 weeks to hypercholesterolemic rats was reported to elevate serum GSH level to near normal (Nwozo et al. 2011). This again accord with the result of our present study, when the effect was more pronounced in the DXAH group than DXAL group (Figure 4.21). The increase of GSH concentrations in the serum and organs of diabetic treated animals might be due to the reduction of lipid peroxidation highlighted above. This also shows that the ratio of GSH/GSSG greatly improved in diabetic treated groups than in the DBC group, which was further supported by improved activity of superoxide dismutase (Figure 4.22).

The termination of the deleterious effect of superoxide radical is achieved in a reaction catalyzed by superoxide dismutase (SOD) to less harmful hydrogen peroxide (Sati et al. 2010). Moreover in a study by Nwozo et al. (2011), the SOD activity was observed to greatly improve after 8 weeks treatment of *X. aethiopica* fruit aqueous extract (250 mg/kg bw) in hypercholesterolemic rats. However in our present study, when the dose was increased to 300 mg/kg bw and treated for 4 weeks, the activity of SOD in the DXAH group was even comparable to the NC group (Figure 4.22). This indicates that the higher dose of XAAF can shown better activity in a shorter period of time, when no alteration in the SOD activity was observed in the NXAH group.

Furthermore, in the same study by Nwozo et al. (2011), daily treatment of *X. aethiopica* fruit aqueous extract (250 mg/kg bw) to hypercholesterolemic rats increased the catalase activity in the serum, liver and kidney. This is also in line with our present data, when the catalase activity in the organs of the DXAL group was even comparable to the NC group. Interestingly, the catalase activity in the serum and organs correlate well with the activity of SOD, as these enzymes mostly work closely to attenuate the oxidative damage mediated by lipid peroxidation (Figure 4.23).

On the other hand, the decrease in the activities of glutathione dependent enzymes; GPx and GR in the serum and organs in the DBC group could be attributed to the reduction of intracellular GSH
concentration (Memisogullari et al. 2003). Treatment of XAAF revealed positive response and attenuated the reduction of GPx and GR activities of the diabetic treated groups to near normal (Figure 4.24 and Figure 4.25). This reflects the potency of the XAAF in ameliorating oxidative stress associated with T2D.

4.4.6 Conclusion

Oral administration of XAAF especially at higher dose showed excellent anti-oxidant activity via reduction of lipid peroxidation, increasing glutathione levels and endogenous anti-oxidants enzymes in serum and organs mostly affected by diabetic-complications. Therefore, XAAF is a good anti-oxidant agent with no considerable side effects. Further clinical study is required to confirm its efficacy in human subjects.
CHAPTER 5

5.0 *In vitro* and *in vivo* anti-diabetic and anti-oxidative effects of various extracts and fractions from the different parts of *Capsicum annuum*

5.1 Anti-oxidative and anti-diabetic effects of various parts of *Capsicum annuum* L. (*Solanaceae*) *in vitro*

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5.1.1 Abstract

**Objective:** *Capsicum annuum* L. is widely consumed as spice worldwide and is endowed with abundant medicinal properties. The present study examined the anti-oxidative as well as anti-diabetic effects of various solvent extracts from fruit, leaf and stem of *Capsicum annuum* L. using various *in vitro* models.

**Methods:** The plant samples were sequentially extracted using solvents of increasing polarity and investigated for 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging activities, total reducing power, inhibition of hemoglobin glycosylation, α-amylase and α-glucosidase activities as markers of *in vitro* anti-diabetic effects.

**Results:** Our results showed that fruit and leaf ethanolic (EtOH) extracts exhibited significantly (*p < 0.05*) higher total polyphenols and flavonoids contents with greater anti-oxidative and anti-diabetic actions, when that of fruit EtOH extract showed higher activity compared to the leaf EtOH extract. Furthermore, various solvent extracts from the stem part showed no ant-diabetic effect. The GC-MS analysis of the most active extracts (fruit and leaf EtOH) revealed the presence of phenolic compounds such as phenol-4-methyl, gingerol, Phenol, 2-methoxy-4-(2-propenyl)-, and eugenol acetate.

**Conclusion:** The EtOH fruit extract exhibited strong anti-oxidative and anti-diabetic effects compared to other solvent extracts and thus recommended for further studies to confirm these effects *in-vivo*

**Key words:** *Capsicum annuum*, Anti-oxidative, Anti-diabetic, *In vitro*
5.1.2 Introduction

Oxidative modifications are crucial for the survival of cells. A side effect of this process is the increased reactive oxygen species (ROS) production (Winrow et al. 1993). ROS are normal products of biochemical reactions, capable of oxidizing cellular and tissue molecules (Cui et al. 2004). Increase production of ROS and depletion of endogenous defense system lead to oxidative stress, and this has been observed in several metabolic disorders, such as obesity, atherosclerosis and type 2 diabetes (T2D) (Atawodi, 2005). In T2D, oxidative stress is a major contributor to the pancreatic β-cell failure and the overproduction of ROS usually occurs via but not limited to; protein kinase C dependent activation of NADH/NADPH oxidase (Inoguchi et al. 2003), increased flux of glucose through the polyol pathway (Brownlee, 2001), increased formation of advanced glycation end products (AGEs) and their receptors (Giocco and Brownlee, 2010) and over activity of the hexosamine pathways (Ceriello and Testa, 2009). Therefore, in the context of T2D, oxidative stress can also be regarded as a trigger of postprandial hyperglycemia which is a prime target for the control of T2D (Ademiluyi and Oboh, 2013; Brownlee, 2005).

There are several approaches employed to control the postprandial hyperglycemia in T2D, among such strategy is by delaying the activities of α-amylase and α-glucosidase, which are key enzymes involved in the digestion of carbohydrates and absorption of glucose in the small intestinal tract (Tucci et al. 2010; Ortiz-Andrade et al. 2007). Therefore, inhibition of these enzymes will limit the absorption of glucose to bloodstream and hence, achieved in reduction of hyperglycemia in T2D. However, the major drawback to this is the unwanted side effects associated with the use of the conventional α-glucosidase inhibitors, such as acarbose and miglitol (Fujisawa et al. 2005). This cause a shift to look for option from natural products that pose limited adverse consequences. Furthermore, convincing evidences are now available that strongly correlate inhibitory effects of α-amylase and α-glucosidase by the plant-derived products (Xiao et al. 2013a,b; Rastija et al. 2012; Hsieh et al. 2010; Hanhineva et al. 2010), which again have reported to possess high anti-oxidant actions as well (Sati et al. 2010; Rahimi et al. 2005; Chanwittheesuk et al. 2005).

In view of this, there is a growing interest in T2D therapy and control by natural anti-oxidants from plants source capable of decreasing the production of ROS as well as delaying glucose absorption at intestinal tract. Among such species endowed with tremendous potentials is Capsicum annuum L. It belongs to Solanaceae family and is readily available in almost all parts of the world. The fruit is widely consumed in vast quantities as flavoring agent in various traditional foods (Khan et al. 2014), and also used as a blood tonic, stimulant and in the treatment of nerve weakness due to diabetes in some parts of Africa and Asia (Iwu, 2014; Jayakumar et al., 2010).

Previous studies have demonstrated that C. annuum fruit possessed high polyphenolic contents and strong anti-oxidative activities (Deepa et al. 2007; Ghasemnezhad et al. 2011; Chen et al. 2012; Silva et al. 2013). Some other studies reported the α-amylase and α-glucosidase inhibitory and anti-
oxidative effects of *C. annuum* fruit *in vitro* (Tundis et al. 2013; Tundis et al. 2011; Kwon et al. 2007). Furthermore, Kim et al. (2011) reported the anti-radical scavenging effect of fruit and leaf, when the detail anti-oxidant and anti-diabetic actions of leaf and stem of *C. annuum* have not yet been conducted. Additionally, to our knowledge no study is available that investigated and compared the anti-oxidative and anti-diabetic effects of *C. annuum* fruit, leaf and stem in a single study either *in vitro* or *in vivo*. Therefore, this study is aimed to investigate and compare the anti-oxidative and anti-diabetic effects of the various solvent extracts from fruit, leaf and stem of *C. annuum*. In addition, possible phytochemicals content in the most active extract (s) were also analyzed by using GC-MS.

### 5.1.3 Materials and methods

For sample collection, identification and preparation of the extracts, including the detail experimental protocols, please refer to chapter 2, sub-sections 2.3 and 2.4.

### 5.1.4 Results

The results on the yield recovered, polyphenols and flavonoids contents of various solvent extracts from different parts of *C. annuum* are presented in Table 5.1. According to the data, aqueous extracts from different parts of *C. annuum* have recovered greater yield compared to other solvent extracts. It was also observed that ethanolic (EtOH) extracts showed significantly (*p < 0.05*) higher total polyphenols and flavonoids contents compared to other solvent extracts with fruit EtOH extract containing the highest amount (Table 5.1).

**Table 5.1:** Percentage yield, total polyphenol and flavonoid contents of various solvent extracts of *C. annuum* parts

<table>
<thead>
<tr>
<th>Samples</th>
<th>Yield (%)</th>
<th>Total polyphenols content (mg/gGAE)</th>
<th>Total flavonoids content (mg/gQE)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fruit</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>0.23</td>
<td>5.34 ± 0.29&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2.15 ± 0.31&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ethanol</td>
<td>1.73</td>
<td>19.75 ± 0.59&lt;sup&gt;f&lt;/sup&gt;</td>
<td>9.94 ± 0.53&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Aqueous</td>
<td>4.70</td>
<td>1.40 ± 0.11&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>0.15 ± 0.03&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Leaf</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>1.60</td>
<td>1.91 ± 0.01&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.58 ± 0.11&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ethanol</td>
<td>2.25</td>
<td>14.11 ± 0.07&lt;sup&gt;e&lt;/sup&gt;</td>
<td>5.33 ± 0.40&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Aqueous</td>
<td>3.00</td>
<td>1.78 ± 0.08&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.11 ± 0.08&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Stem</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>0.15</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Ethanol</td>
<td>0.30</td>
<td>0.98 ± 0.05&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.15 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Aqueous</td>
<td>3.75</td>
<td>0.14 ± 0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.13 ± 0.03&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SD of triplicate determinations. */* Different superscripted letters for a given value within a column are significantly different from each other (Tukey’s-HSD multiple range *post hoc* test, *p < 0.05*), ND, Not determined.
The data of the ferric (Fe³⁺) ion reducing anti-oxidant potential of various extracts from *C. annuum* are presented in Figure 5.1. It was observed from the results that fruit and leaf EtOH extracts have demonstrated significantly (*p* < 0.05) higher reducing potentials compared to other solvent extracts from the same or different parts of *C. annuum*. Again the reducing ability of the fruit EtOH extract was higher compared to the leaf EtOH extract, when the EtOAc extracts from the fruits and leaf showed reducing ability which was significantly (*p* < 0.05) higher compared to the aqueous extracts (Figure 5.1). Additionally, various solvent extracts from stem part exhibited no reducing potential at different concentrations used in this study (Figure 5.1).

**Figure 5.1.** Ferric reducing power (relative to gallic acid) of fruit (A) and leaf (B) extracts of *C. annuum*. Data are presented as mean ± SD of triplicate determinations. *abcd* Different letters presented over the bars for a given concentration of each extract are significantly different from each other (Tukey’s-HSD multiple range *post hoc* test, *p* < 0.05).
The data of DPPH radical scavenging activities of various parts of *C. annuum* are presented in Figure 5.2 and Table 5.2. Various extracts showed variable DPPH radical scavenging activities. Fruit and leaf EtOH extracts significantly (*p < 0.05*) exhibited lower IC$_{50}$ values compared to other extracts, when that of the fruit had the least IC$_{50}$ value (0.03 ± 0.01 mg/mL). The activities of the fruit and leaf EtOH extracts were comparable to that of the standards (Ascorbic acid: 0.03 ± 0.02 mg/mL; Gallic acid: 0.05 ± 0.01 mg/mL) used in this study (Table 5.2). It was also observed that stem ethyl acetate (EtOAc) showed lower IC$_{50}$ value compared to other extracts from this part (Table 5.2).

Table 5.2: IC$_{50}$ values of various extracts of *C. annuum* parts in different anti-oxidative and anti-diabetic models.

<table>
<thead>
<tr>
<th>Samples</th>
<th>DPPH scavenging activity</th>
<th>Non-enzymatic glycation of hemoglobin</th>
<th>α-amylase inhibition</th>
<th>α-glucosidase inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fruit</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>0.59 ± 0.05$^b$</td>
<td>10.04 ± 1.48$^c$</td>
<td>1.57 ± 0.04$^c$</td>
<td>17.52 ± 2.83$^c$</td>
</tr>
<tr>
<td>Ethanol</td>
<td>0.03 ± 0.01$^a$</td>
<td>0.22 ± 0.02$^a$</td>
<td>0.15 ± 0.01$^a$</td>
<td>0.05 ± 0.02$^a$</td>
</tr>
<tr>
<td>Aqueous</td>
<td>1.31 ± 0.20$^c$</td>
<td>9620.74 ± 50.69$^c$</td>
<td>0.94 ± 0.07$^c$</td>
<td>32386.4 ± 228.79$^f$</td>
</tr>
<tr>
<td><strong>Leaf</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>0.32 ± 0.01$^b$</td>
<td>4.21 ± 0.04$^b$</td>
<td>300.13 ± 101.53$^d$</td>
<td>4.16 ± 0.25$^d$</td>
</tr>
<tr>
<td>Ethanol</td>
<td>0.09 ± 0.01$^a$</td>
<td>0.30 ± 0.01$^a$</td>
<td>0.29 ± 0.01$^{a,b}$</td>
<td>2.06 ± 0.04$^c$</td>
</tr>
<tr>
<td>Aqueous</td>
<td>2.35 ± 0.27$^d$</td>
<td>49.92 ± 28.86$^d$</td>
<td>27100.5 ± 1450.35$^e$</td>
<td>16.22 ± 0.16$^c$</td>
</tr>
<tr>
<td><strong>Stem</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>5.98 ± 0.62$^e$</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Ethanol</td>
<td>222.62 ± 36.28$^g$</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Aqueous</td>
<td>111.23 ± 32.36$^f$</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>0.03 ± 0.02$^a$</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>0.05 ± 0.01$^a$</td>
<td>0.20 ± 0.01$^a$</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Acarbose</td>
<td>ND</td>
<td>0.37 ± 0.03$^b$</td>
<td>0.21 ± 0.01$^b$</td>
<td>ND</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SD values of triplicate determinations. $^a-g$ Different superscript letters presented within a column for a given parameter are significantly different from each other (Tukey’s-HSD multiple range post hoc test, *p < 0.05*). ND, Not determined.
Figure 5.2. DPPH Radical scavenging activity (%) of fruit (A), leaf (B) and stem (C) extracts of *C. annuum*. Data are presented as mean ± SD of triplicate determinations. Different letters presented over the bars for a given concentration of each extract are significantly different from each other (Tukey’s HSD multiple range post hoc test, $p < 0.05$).
The results of inhibition of hemoglobin glycosylation of various solvent extracts from *C. annuum* are presented in Figure 5.3 and Table 5.2. In a similar pattern with the previous anti-oxidative parameters, fruit and leaf EtOH extracts demonstrated significantly (*p* < 0.05) the least IC$_{50}$ values (fruit: 0.22 ± 0.02 mg/mL; leaf: 0.30 ± 0.01 mg/mL) compared to other solvent extracts that were not significantly (*p* < 0.05) different from that of gallic acid (0.20 ± 0.01 mg/mL). The degree of inhibitory effects of the fruit and leaf extracts were in the order of EtOH > EtOAc > aqueous (Table 5.2).

![Figure 5.3](image)

*Figure 5.3.* Inhibition of hemoglobin glycosylation (%) of fruit (A) and leaf (B) extracts of *C. annuum*. Data are presented as mean ± SD of triplicate determinations. *a-d* Different letters presented over the bars for a given concentration of each extract are significantly different from each other (Tukey’s HSD multiple range post hoc test, *p* < 0.05).
The results of the inhibitory effect of α-amylase action of various solvent extracts from \textit{C. annuum} are presented in Figure 5.4 and Table 5.2. According to the data, EtOH extract derived from the fruit have exhibited lower IC$_{50}$ value ($0.15 \pm 0.01$ mg/mL) compared to other extracts of the same or different parts as well as acarbose. Various extracts from the stem part have shown no inhibitory action towards α-amylase action (Table 5.2).

**Figure 5.4.** The α-amylase inhibition (%) of fruit (A) and leaf (B) extracts of \textit{C. annuum}. Data are presented as mean ± SD of triplicate determinations. a–d Different letters presented over the bars for a given concentration of each extract are significantly different from each other (Tukey’s HSD multiple range post hoc test, $p < 0.05$).
The data of the inhibitory effect towards α-glucosidase action of various solvent extracts from *C. annuum* are presented in Figure 5.5 and Table 5.2. Interestingly, fruit EtOH extract had demonstrated significantly (*p* < 0.05) lower IC₅₀ value (0.05 ± 0.02 mg/mL) compared to other extracts from the same or different parts and acarbose (0.21 ± 0.01 mg/mL) as well (Table 5.2). The pattern of inhibition of the fruit and leaf extracts were in the decreasing order of EtOH> EtAOc> aqueous. Additionally, various solvent extracts from stem part exhibited no inhibitory effect towards α-glucoside action at different concentrations used in this study (Table 5.2).

**Figure 5.5.** The α-Glucosidase inhibition (%) of fruit (A), leaf (B) and stem (C) extracts of *C. annuum*. Data are presented as mean ± SD of triplicate determinations. *a-d* Different letters presented over the bars for a given concentration of each extract are significantly different from each other (Tukey’s HSD multiple range post hoc test, *p* < 0.05).
The results of the GC-MS analyses of the most active extracts (fruit and leaf EtOH extracts) are presented in Table 5.3, Figures 5.6 and 5.7. The major peaks detected from the chromatogram of the fruit EtOH extract correspond to phenol 4-methyl (1), eugenol (2), caryophyllene (3), eugenol acetate (4), 4-(4-hydroxy-3-methoxyphenyl)-butanone (5), 1, 2-benzenedicarboxylic acid, butyl 2-methylpropyl ester (6), 9-octadecenoic acid (7), 1-(4-hydroxy-3-methoxyphenyl)-3-decanone (8), gingerol (9) and capsaicin (10) (Table 5.3, Figures 5.6A and 5.7). On the other hand, the based on the chromatogram of leaf EtOH extract, the major peaks that correspond to the NIST library include; eugenol (2), tetradecanoic acid (11), dibutyl phthalate (12) and 1,2-benzene dicarboxylic acid, mono(2-ethylhexyl) ester (13) (Table 5.3, Figures 5.6B and 5.7).

Figure 5.6. GC-MS Chromatograms of ethanolic extracts of fruit (A) and leaf (B) of C. annuum
Table 5.3: Identified compounds from the EtOH fruit, leaf and stem extracts of *C. annuum* by GC-MS

<table>
<thead>
<tr>
<th>Extracts</th>
<th>Compounds</th>
<th>Retention time (min)</th>
<th>Mass/amu</th>
</tr>
</thead>
<tbody>
<tr>
<td>EtOH fruit</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Phenol, 4-methyl</td>
<td>4.44</td>
<td>107 [M]+</td>
</tr>
<tr>
<td>2</td>
<td>Eugenol</td>
<td>6.67</td>
<td>164 [M]+</td>
</tr>
<tr>
<td>3</td>
<td>Caryophyllene</td>
<td>7.22</td>
<td>204 [M]+</td>
</tr>
<tr>
<td>4</td>
<td>Phenol, 2-methoxy-4-(2-propenyl)-acetate</td>
<td>7.72</td>
<td>206 [M]+</td>
</tr>
<tr>
<td>5</td>
<td>4-(4-Hydroxy-3-methoxyphenyl)-butanone</td>
<td>8.57</td>
<td>194 [M]+</td>
</tr>
<tr>
<td>6</td>
<td>1,2-Benzenedicarboxylic acid, butyl 2-methylpropyl ester</td>
<td>10.26</td>
<td>214 [M]+</td>
</tr>
<tr>
<td>7</td>
<td>9-Octadecenoic acid</td>
<td>11.11</td>
<td>282 [M]+</td>
</tr>
<tr>
<td>8</td>
<td>1-(4-Hydroxy-3-methoxyphenyl)-3-decanone</td>
<td>11.63</td>
<td>278 [M]+</td>
</tr>
<tr>
<td>9</td>
<td>Gingerol</td>
<td>12.12</td>
<td>294 [M]+</td>
</tr>
<tr>
<td>10</td>
<td>Capsaicin</td>
<td>13.08</td>
<td>308 [M]+</td>
</tr>
<tr>
<td>EtOH leaf</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Eugenol</td>
<td>6.67</td>
<td>164 [M]+</td>
</tr>
<tr>
<td>11</td>
<td>Tetradecanoic acid</td>
<td>9.14</td>
<td>228 [M]+</td>
</tr>
<tr>
<td>12</td>
<td>Dibutyl phthalate</td>
<td>10.26</td>
<td>278 [M]+</td>
</tr>
<tr>
<td>13</td>
<td>1,2-Benzene dicarboxylic acid, mono(2-ethylhexyl) ester</td>
<td>13.16</td>
<td>279 [M]+</td>
</tr>
</tbody>
</table>
Figure 5.7. Structures of identified compounds from ethanolic extracts of fruit and leaf of *C. annuum*
5.1.5 Discussion

In our present study, we have investigated and compared the anti-oxidative and anti-diabetic effects of various solvent extracts derived from different parts of *C. annuum*. This is the first study that systematically compared the activities of different parts (fruit, leaf and stem) of *C. annuum* in a single study, despite several studies that reported the anti-oxidative and anti-diabetic effects of *C. annuum* *in vitro*.

The polyphenols and flavonoids contents of *C. annuum* fruit and leaf parts have been previously studies in a number of studies. Kim et al. (2011) highlighted that the polyphenolic and flavonoid contents of the EtOH extracts from the leaf were higher than the fruit part of *C. annuum* cultivated in Korea, which contradict our present data. This could partly be due to some genetic and environmental (nature of the soil, high temperature and rain fall) factors in addition to growth and maturation stages of the *C. annuum* parts (Mamphiswana et al. 2010; Pandey and Rizvi, 2009). To support our present data, several studies have reported the fruit EtOH extract to contain greater amount of polyphenols and flavonoids (Tundis et al. 2013; Deepa et al. 2007; Perucka and Materska, 2007; Materska and Perucka, 2005). Furthermore, our present results have further support the fact that EtOH is basically the best solvent for proper extraction of polyphenols and other related compounds, which has been observed across all the parts (fruit, leaf and stem) used in our current study (Table 5.1). It was also observed that *C. annuum* leaf could be a potential source of polyphenols and flavonoids, which in most of the time is considered as wasted vegetable.

In assessing the anti-oxidant status of plants species, several *in vitro* models were developed. In our present study, the *in vitro* methods used include the ferric (Fe$^{3+}$) reducing power, DPPH radical scavenging ability as well as the inhibition of hemoglobin glycosylation. Deepa et al. (2007) have reported that fruit EtOH extracts of *C. annuum* obtained at different stages of maturation, have demonstrated variable Fe$^{3+}$ to Fe$^{2+}$ reducing potentials. This was similarly observed in our present data (Figure 5.2). Therefore, although no correlation analysis was carried out in our present study, it might be suggested that the Fe$^{3+}$ to Fe$^{2+}$ reducing potential is correlated to the polyphenols and flavonoids contents, which is in line with a previous published data (Pulido et al. 2000). The result was further supported by the DPPH radical scavenging activity by the various extracts from *C. annuum* (Table 5.2).

The DPPH radical scavenging activity of various solvent extracts from the fruit of *C. annuum* has been extensively reported (Azeez et al. 2012; Chen et al. 2012; Tundis et al. 2011; Perucka and Materska, 2007; Conforti et al. 2007; Kwon et. 2007). In some of these studies, fruit methanolic extract had exhibited IC$_{50}$ values of 1.15 mg/mL and 0.13 mg/mL for DPPH scavenging activity (Azeez et al. 2012; Conforti et al. 2007). Similarly, Tundis et al. (2011) and Kim et al. (2011) have also reported an IC$_{50}$ value of 0.15 mg/mL for fruit EtOH extract, when that of leaf EtOH extract was 0.46 mg/mL. However, in our present results, the IC$_{50}$ values recorded for the DPPH scavenging was lower for the fruit (IC$_{50}$: 0.03 ± 0.01 mg/mL) and leaf (IC$_{50}$: 0.09 ± 0.01 mg/mL) extracts compared to previously
reported data (Table 5.2). The higher activity of fruit over the leaf and stem parts observed in our present study indicated the greater amounts of active ingredients that could scavenge and terminate the oxidative damages of ROS (Miladi and Demak, 2008; Manach et al. 2004).

Furthermore, increased protein glycation contributes significantly to the progression and manifestation of diabetic complications (Calisti and Tognetti, 2005). The glycation process involves a cascade of reactions and rearrangements between reducing sugars and proteins which ultimately forms AGEs (Rahbar and Figarola, 2002). Hence, inhibitors of this process could play a crucial role in the prevention of T2D associated complications. In our present study, fruit EtOH extract maintained higher anti-oxidative nature by exhibiting the lower IC$_{50}$ value (0.22 ± 0.02 mg/mL) toward hemoglobin glycation, indicating higher activity compared to other solvent extracts. Interestingly, leaf EtOH extract also showed similar inhibition of glycosylation with fruit EtOH extract (Table 5.2). This could possibly be utilized and will reduce the over usage of fruit for nutritional and medicinal purposes.

On the other side, inhibitors of carbohydrate hydrolyzing enzymes are recently being utilized as therapeutic targets in prevention and control of T2D and its associated complications (Tucci et al. 2010; Ortiz-Andrade et al., 2007). However, the major glucosidase inhibitors, acarbose and miglitol, were reported to cause abdominal distension, flatulence, bowel necrosis and diarrhea (Fujisawa et al. 2005). This paves way for search of inhibitors from plants origin. Furthermore, the α-amylase and α-glucosidase inhibitory effects of various extracts from the fruit of C. annuum have been reported (Tundis et al. 2013; 2012; Kwon et al. 2007). Tundis et al. (2012) have reported that fruit EtOH extract is a potential inhibitor of α-amylase (IC$_{50}$: 0.26 mg/mL) and α-glucosidase (IC$_{50}$: 0.36 mg/mL) actions. However, when compared to our data, the calculated IC$_{50}$ values for α-amylase and α-glucosidase inhibitory effects are lower, which could be the least reported for fruit of C. annuum (Table 5.2). Additionally, the observed higher α-glucosidase inhibitory action of the fruit EtOH extract over α-amylase action is of enormous importance. This is because some of the unwanted effects attributed with the currently utilized drugs are linked to strong inhibition of α-amylase effect (Ademiluyi and Oboh, 2013). Moreover, the better inhibitory effect of the fruit extracts than leaf extracts is an indication of the greater availability of the inhibitors in fruit compared to leaf part.

Based on the higher anti-oxidative and anti-diabetic effects of fruit and leaf EtOH extracts, the GC-MS analysis was carried out to identify major possible phytochemicals present. It was observed that most of the compounds detected were phenolics in nature with some long chain fatty derivatives. Phenolics and other related compounds are known to directly or indirectly contribute to the anti-oxidative and enzyme inhibitory actions of various plant-derived products (Mai et al. 2007). Therefore, without the exclusion of the possible contribution of other compounds present in the two extracts, the consistently higher anti-oxidative and anti-diabetic effects could be associated to the presence of these phenolics compounds.
5.1.6 Conclusions

In conclusion, various solvent extracts from fruit and leaf possessed potent anti-oxidative and anti-diabetic effects *in vitro*, when the fruit EtOH extracts exhibited the highest. Therefore, bioassay guided fractionation and investigation of anti-oxidative and anti-diabetic effects *in vivo* of most active extract has been conducted and presented in the following part of this chapter.
According to the in vitro anti-oxidative and anti-diabetic studies of the various solvent extracts derived from different parts of C. annuum stated above, fruit ethanolic extract demonstrated higher activities compared to other extracts. Therefore it was selected for further studies.

5.2 Acetone fraction from Capsicum annuum fruit possesses anti-oxidative effects and inhibits the activities of carbohydrate digesting enzymes in vitro

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5.2.1 Abstract

Objectives: The present study was carried out to investigate the anti-oxidative and inhibition of the activities of carbohydrate digesting enzymes by various solvent fractions from crude ethanolic extract of C. annuum using various in vitro models.

Methods: Crude ethanolic extract of fruit was fractionated using hexane, dichloromethane, ethyl acetate and acetone. They were subjected and investigated for 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging activity, ferric reducing anti-oxidant power (FRAP), inhibition of hemoglobin glycosylation, α-amylase and α-glucosidase activities as markers of in vitro anti-diabetic effects at various doses (30-240 µg/ml). Possible bioactive compounds were analyzed using Gas Chromatography-Mass Spectrometry (GC-MS) analysis.

Results: The results indicated that acetone fraction showed significantly (p < 0.05) higher total polyphenols and flavonoids contents and recorded lower IC₅₀ values in all the models used (DPPH: 111.97 ± 2.06 µg/mL; inhibition of hemoglobin glycosylation: 150.64 ± 6.10 µg/ml; α-amylase: 803.34 ± 22.18 µg/mL; α-glucosidase: 65.11 ± 6.78 µg/mL) compared to other solvent fractions, and also demonstrated excellent reducing Fe³⁺ to Fe²⁺ power. The phytochemical studies identified six possible compounds (tridecanoic acid, 9, 12-octadecadienoic acid, oleic acid, kauran-16-ol, capsaicin and 1, 2-benzenedicarboxylic acid, mono (2-ethylhexyl) ester) from the acetone fraction. This results clearly demonstrates that acetone fraction from C. annuum possesses potent anti-oxidative and inhibition of the activities of carbohydrate digesting enzymes, with potential bioactive compounds.
Keywords: Anti-oxidative, Capsicum annuum, Inhibitory effect, Type 2 diabetes mellitus

5.2.2 Introduction

In recent time, natural products such as plants are regarded as good source of potential therapeutic agents. This is in addition to the fact that a number of widely used modern drugs were initially derived from plant source. For instance, the discoveries of metformin (anti-diabetic drug) and artemisinin (anti-malarial drug) from Galega officinalis L. and Artemisia annua L., respectively just to mention few (Gurib-Fakim, 2006). Similarly, plant-derived formulations or compounds were reported to cause limited or no adverse side effects compared to the conventional synthetic drugs (Harvey, 2014). In view of this, there is a widespread search for newer chemical lead from plants to treat various diseases which may offer better protection with lesser side effects.

On the other hand, the prevalence of diseases such as type 2 diabetes (T2D) is quite alarming globally; reaching epidemic proportion. T2D is characterized by chronic insulin resistance and loss of pancreatic β-cell function (Hui et al. 2007). This leads to hyperglycemia, which has been associated with increase formation of reactive oxygen species (ROS), and eventually causes oxidative stress (Devasagayam et al. 2007). Oxidative stress is a condition of imbalance that exists between pro-oxidants and in-vivo anti-oxidant levels in such a way that pro-oxidant production overwhelms anti-oxidant defenses (Powers and Jackson, 2008). Moreover, oxidative stress plays a crucial role in the pathogenesis of diabetes associated complications such as neuropathy, nephropathy and retinopathy (Azenabor et al. 2011). Therefore, control of oxidative stress could be a potential strategy to control T2D in addition to the inhibition of the activities of carbohydrate digesting enzymes, such as α-glucosidase and α-amylase that are currently employed to treat T2D. This process involves retarding the digestion of carbohydrates via inhibition of key enzymes; α-glucosidase and α-amylase, which may limit the hydrolysis and absorption of glucose in the into the bloodstream (Kawamura-Konishi et al. 2012). Hence, the search for natural inhibitors of α-glucosidase and α-amylase activities and ROS scavengers have gradually increased to achieve in the proper control of T2D.

The fruit from Capsicum annuum L. has been a major component of various food preparations worldwide. In addition to this, C. annuum fruit has been reported to be an excellent source of vitamins, phenolics and other potentially active phytochemicals which are anti-oxidants that may prevent the risk of T2D. More importantly, there is sufficient available data that support the anti-oxidative as well as anti-diabetic effects of crude extracts of C. annuum in vitro (Tundis et al. 2013; Tundis et al. 2011; Kwon et al. 2007). Although not much comparative studies have been conducted using various solvent extracts, in most of the reported studies (including ours), fruit ethanolic extract showed promising and better activity compared to other solvent extracts (Tundis et al. 2013; 2011; Kim et al. 2011; Deepa et al. 2007). In view of this and our continued efforts to explore potential leads with anti-oxidative and
anti-diabetic effects from *C. annuum*, we further partition and fractionate the crude ethanolic extract using solvents of increasing polarity in order to find the most potent fraction with higher activities.

Thus, this study was aimed to investigate the anti-oxidative and inhibition of the activities of carbohydrate digesting enzymes *in vitro* by various solvent fractions from the crude ethanolic extract.

## 5.2.3 Materials and methods

For sample collection, identification and preparation of the fractions, including the detail experimental protocols, please refer to chapter 2, sub-sections 2.3 and 2.4.

## 5.2.4 Results

The results on yield recovered, total polyphenols and flavonoids contents from the various fractions are presented in Table 5.4. It was observed that acetone fraction recovered higher yield compared to other solvent fractions while ethyl acetate (EtOAc) fraction was the least recovered. Furthermore, the total polyphenols and flavonoids contents of the most polar fractions (EtOAc and acetone) were significantly (*p* < 0.05) higher compared to the less polar fractions (Table 5.4). The contents of the acetone fraction were significantly (*p* < 0.05) higher compared to the EtOAc fraction. In addition, the polyphenols and flavonoids contents of dichloromethane fractions and hexane fraction were observed not to differ significantly.

### Table 5.4: Percentage yield, total polyphenol and flavonoid contents of various fractions from ethanolic fruit extract of *C. annuum*

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Yield (%)</th>
<th>Total polyphenols content (mg/gGAE)</th>
<th>Total flavonoids content (mg/gQE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexane</td>
<td>11.0</td>
<td>1.44 ± 0.06&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.22 ± 0.06&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Dichloromethane</td>
<td>6.65</td>
<td>1.14 ± 0.41&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.55 ± 0.16&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>2.87</td>
<td>6.38 ± 0.06&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.73 ± 0.06&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Acetone</td>
<td>14.5</td>
<td>12.66 ± 0.05&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4.77 ± 0.12&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SD values of triplicate determinations. Different superscripted letters for a given value within a column are significantly different from each other (Tukey’s-HSD multiple range *post hoc* test, *p* < 0.05).

The data of ferric reducing anti-oxidant power (FRAP) assay is presented in Figure 5.8. From the result, a significantly (*p*<0.05) higher reducing ability was demonstrated by acetone fraction in concentration dependent response compared to other fractions, although lower than the standard ascorbic acid. It was also observed that the reducing potentials of the remaining solvent fractions were comparatively similar to each other (Figure 5.8).
The data of DPPH scavenging ability of various fractions are presented in Figure 5.9 while the extent of inhibition is expressed base on the calculated IC$_{50}$ values is shown in Table 5.5. Acetone fraction demonstrated a significantly ($p < 0.05$) lower IC$_{50}$ value (111.97 ± 2.06 µg/mL) for DPPH scavenging activity compared to other fractions. The IC$_{50}$ values for dichloromethane and ethyl acetate fractions did not differ significantly (Table 5.5). Similarly, the pattern of inhibition was in the order of acetone > dichloromethane > ethyl acetate > hexane, with acetone been the highest.

The results of the abilities of the fractions to inhibit hemoglobin glycation are presented in Figure 5.10 and Table 5.5. It was observed that acetone fraction exhibited a significantly ($p < 0.05$) lower IC$_{50}$ value (150.64 ± 6.10 µg/mL) compared to other fractions, which is comparable to the gallic
acid (199.09 ± 2.85 µg/mL). The extent of inhibitory effects of hexane and EtOAc fractions were found not to differ significantly, when dichloromethane fraction was observed to exhibit the highest IC\textsubscript{50} value (Table 5.5).

![Figure 5.10: Inhibition of hemoglobin glycation (%) of various fractions from fruit ethanolic extract of C. annuum. Data are presented as mean ± SD of triplicate determinations. a-d Values with different letters presented for a given concentration for each extract are significantly different from each other. (Tukey’s-HSD multiple range post hoc test, p < 0.05).]

Table 5.5: IC\textsubscript{50} values of various solvent fractions from ethanolic fruit extract of C. annuum in different anti-oxidative and anti-diabetic models.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>DPPH scavenging activity</th>
<th>Non-enzymatic glycation of hemoglobin</th>
<th>α-amylase inhibition</th>
<th>α-glucosidase inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexane</td>
<td>1862.88 ± 720.3\textsuperscript{d}</td>
<td>844.84 ± 32.85\textsuperscript{c}</td>
<td>8403.23 ± 806.97\textsuperscript{c}</td>
<td>724.89 ± 95.61\textsuperscript{d}</td>
</tr>
<tr>
<td>Dichloromethane</td>
<td>462.30 ± 14.32\textsuperscript{c}</td>
<td>2127.97 ± 987.21\textsuperscript{d}</td>
<td>2798.53 ± 143.65\textsuperscript{d}</td>
<td>454.01 ± 63.34\textsuperscript{c}</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>503.73 ± 7.74\textsuperscript{c}</td>
<td>503.73 ± 7.70\textsuperscript{b}</td>
<td>2143.46 ± 213.67\textsuperscript{c}</td>
<td>380.31 ± 20.35\textsuperscript{b,c}</td>
</tr>
<tr>
<td>Acetone</td>
<td>111.97 ± 2.06\textsuperscript{b}</td>
<td>150.64 ± 6.10\textsuperscript{a}</td>
<td>164.11 ± 5.28\textsuperscript{a}</td>
<td>65.11 ± 6.78\textsuperscript{a}</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>25.34 ± 6.19\textsuperscript{a}</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>20.01 ± 4.42\textsuperscript{a}</td>
<td>199.09 ± 2.85\textsuperscript{a}</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Acarbose</td>
<td>ND</td>
<td>ND</td>
<td>374.95 ± 26.81\textsuperscript{b}</td>
<td>211.59 ± 3.39\textsuperscript{b}</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SD values of triplicate determinations. \textsuperscript{a-e} Different superscripted letters for a given value within a column are significantly different from each other (Tukey’s-HSD multiple range post hoc test, p < 0.05). ND, Not determined

The results of α-amylase inhibition of various solvent fractions from the fruit EtOH extract are presented in Figure 5.11 and Table 5.5. Based on the data, the IC\textsubscript{50} value demonstrated by the acetone fraction (164.11 ± 5.28 µg/mL) for α-amylase inhibition was significantly (p<0.05) lower compared to other fractions and the acarbose as well. The IC\textsubscript{50} value of EtOAc fraction was significantly (p < 0.05)
lower compared to dichloromethane fraction, when that of hexane fraction had observed to be the highest (Table 5.5).

Moreover, the data of α-glucosidase inhibition of various solvent fractions are presented in Figure 5.12 and Table 5.5. It was observed that all the fraction have exhibited considerable inhibitory effect of α-glucosidase action. The IC₅₀ value demonstrated by the acetone fraction (65.11 ± 6.78 µg/mL) was significantly (p<0.05) lower compared to other fractions and the acarbose as well. Similarly, IC₅₀ values of dichloromethane and EtOAc fractions were found not to differ significantly (p<0.05) compared to the acarbose, when that of hexane was the highest (Table 5.5).
The results of the kinetics on the inhibitory mode of the α-glucosidase and α-amylase by the most active (acetone fraction) are presented in Figure 5.13. According to the results, acetone fraction has exhibited a non-competitive inhibition towards α-amylase and α-glucosidase actions. The $K_M$ values remain unchanged (α-amylase: 0.05 mmol/L; α-glucosidase: 1.0 mmol/L), whereas the values of the $V_{max}$ were found to decrease (α-amylase: 35.67 µmol/min to 11.18 µmol/min; α-glucosidase: 471.56 µmol/min to 166 µmol/min). The equilibrium constants for inhibitor binding ($K_i$) were calculated as 13.51 µg/mL and 16.30 µg/mL for α-amylase and α-glucosidase actions, respectively (Figure 5.13).

![Figure 5.13: Lineweaver-Burke’s plot of (A) α-amylase and (B) α-glucosidase catalyzed reactions in the presence and in the absence of the acetone fraction derived from the C. annuum fruit ethanolic extract.](image)

Based on the results of the above in vitro studies, acetone fraction exhibited the higher activities and thus, was subjected to GC-MS analysis to find possible phytochemicals present. The data of phytochemical analysis of the acetone fraction are presented in Figure 5.14 and Table 5.6. Six peaks were prominent from the chromatogram of the GC-MS analysis of the acetone fraction, which were
compared with those present in the NIST library (Figure 5.14A). The compounds were identified as tridecanoic acid (1), 9, 12-octadecadienoic acid (2), oleic acid (3), kauran-16-ol (4) and two phenolics identified as capsaicin (5) and 1, 2-benzenedicarboxylic acid, mono (2-ethylhexyl) ester (6) (Figure 5.14B and Table 5.6).

Table 5.6: Identified compounds from the acetone fraction from fruit ethanolic extract of *C. annuum* by GC-MS

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Compounds</th>
<th>Retention time (min)</th>
<th>Mass/amu</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone</td>
<td>1 Tridecanoic acid</td>
<td>10.24</td>
<td>214 [M]^+</td>
</tr>
<tr>
<td></td>
<td>2 9,12-Octadecadienoic acid</td>
<td>10.26</td>
<td>280 [M]^+</td>
</tr>
<tr>
<td></td>
<td>3 Oleic acid</td>
<td>11.11</td>
<td>282 [M]^+</td>
</tr>
<tr>
<td></td>
<td>4 Kauran-16-ol</td>
<td>11.39</td>
<td>290 [M]^+</td>
</tr>
<tr>
<td></td>
<td>5 Capsaicin</td>
<td>12.49</td>
<td>239 [M]^+</td>
</tr>
<tr>
<td></td>
<td>6 1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester</td>
<td>13.15</td>
<td>279 [M]^+</td>
</tr>
</tbody>
</table>
Figure 5.14. GC-MS chromatogram (A) and structures of identified compounds (B) from acetone fraction from fruit of *C. annuum*
5.2.5 Discussion

Natural products such as plants are now considered as an integral component of health care system, particularly in developing countries where most people have limited resources and access to modern treatments. In addition, the unwanted adverse effect of most of the modern synthetic drugs for treatment of diseases such as T2D is also an issue of concern (Marles and Farnsworth, 1994). However, the number of plants with potential anti-diabetic actions is very limited and many of their anti-diabetic effects have not yet been scientifically validated. Therefore, our present study is designed to investigate the anti-oxidative potentials and inhibition of the activities of α-glucosidase and α-amylase actions by various fractions derived from crude EtOH extract of *C. annuum* fruit *in vitro*.

Plant-derived bioactive compounds such as polyphenols and flavonoids are reported to scavenge reactive oxygen species (ROS) and also modulate the activities of α-amylase and α-glucosidase that are crucial in treatment of T2D (Ani and Akhilender, 2008; Pulok et al. 2006; Atawodi, 2005). The polyphenols and flavonoids contents of different extracts of *C. annuum* were extensively documented (Tundis et al. 2013; Kim et al. 2011; Deepa et al. 2007; Perucka and Materska, 2007; Materska and Perucka, 2005). In most of the earlier studies, crude ethanolic extract was reported to contain high amount of polyphenols and flavonoids. However, according to our present data, most of these compounds were observed to be available in the acetone fraction, which is the most polar fraction (*Table 5.4*). This is due to the greater polarity of acetone compared to other solvents and therefore retains most of the polyphenols from the crude extract.

In complex systems such as living organisms, oxidative stress may arise from different ways to cause deleterious effects, which contribute to high levels of ROS (Hinneburg et al. 2006). The ferric reducing anti-oxidant power (FRAP) is a method used to assess the anti-oxidant potentials of the plant extracts, fractions and/or compounds. The assay depends on the reduction of the Fe$^{3+}$ to Fe$^{2+}$ monitored by via the formation of Perl’s Prussian blue at 700 nm (Chung et al. 2002). According to our present results, acetone fraction showed higher anti-oxidant potential by demonstrating excellent reducing power in concentration dependent manner partly correlated to the rich-phenolic its contents (*Figure 5.8*).

The DPPH radical scavenging activity was used to further support the anti-radical action of the fractions. It has been highlighted previously that extracts derived from *C. annuum* fruit have demonstrated potent DPPH radical scavenging ability (Azeez et al. 2012; Chen et al. 2012; Tundis et al. 2011; Perucka and Materska, 2007; Conforti et al. 2007; Kwon et. 2007). This is in line with the results of our present study (*Table 5.5*). The lower IC$_{50}$ value for DPPH scavenging ability exhibited by the acetone fraction might be attributed to the higher polyphenols and flavonoids contents which are known to exert anti-oxidative effects (Sen et al. 2010; Atawodi, 2005).

Hemoglobin glycation induced by hyperglycemia, is a non-enzymatic reaction that contributes to increase in the formation of advanced glycated end products (AGEs), which lead to most of the
complications associated with diabetes (Calisti and Tognetti, 2005). Therefore, inhibitors of AGEs formation may prevent and attenuate the progression and development of complications due to hyperglycemia. It is evident from our present data that acetone fraction is a potential inhibitor of hyperglycemia-induced glycation (Table 5.5). The results accord with the DPPH and FRAP assays as described above, which correlate well with the total polyphenols and flavonoids contents of acetone fraction that again supported by the α-glucosidase and α-amylase inhibitory actions (Table 5.5).

The two important carbohydrate-digesting enzymes, α-glucosidase and α-amylase are the key enzymes involved in the in the breakdown of dietary carbohydrates. The α-amylase converts starch into disaccharides and oligosaccharides, after which α-glucosidase catalyzes the breakdown of disaccharides to monosaccharide (glucose) which is subsequently absorbed into bloodstream via small intestinal mucos (Tucci et al. 2010; Ortiz-Andrade et al. 2007). Therefore, inhibitors of these enzymes can slowdown carbohydrate digestion and increase the overall transit time. This will reduce the rate of glucose absorption, which may apparently abrupt the postprandial elevation of blood glucose levels, and therefore provide effective approaches in controlling T2D. Interestingly, the α-amylase and α-glucosidase inhibitory effects of aqueous and ethanolic extracts from the fruit of C. annuum have been reported (Tundis et al. 2013; 2012; Kwon et al. 2007). However, when the crude ethanolic extract was fractionated, acetone fraction has exerted the highest inhibitions (least IC \(_{50}\) values), which may be due to the rich-polyphenolic content of the fraction (Table 5.5).

The kinetic results have demonstrated that the nature of α-glucosidase and α-amylase inhibitions were of non-competitive type (Figure 5.13). This is the first study that report on the mode of inhibition of these enzymes by any extracts, fractions or compounds derived from C. annuum. Therefore acetone fraction exerts the inhibitory effect toward α-glucosidase and α-amylase actions by binding to the enzymes at regions other than active sites and may not be affected by higher amount of substrate. This induces some conformational changes in the three-dimensional structure of the enzymes that slowdown the activity of the enzymes.

Based on the higher anti-oxidative and anti-diabetic actions exhibited by the acetone fraction, it was subjected to GC-MS analysis to detect possible bioactive compounds present in this fraction. The six bioactive compounds detected in the acetone fraction were previously reported to be present in the C. annuum fruit (Tundis et al. 2013; Conforti et al. 2007). These compounds may act individually or in synergy to confer the strong activities demonstrated by the acetone fraction observed in our present study.

5.2.6 Conclusion

Conclusively, various solvent fractions from C. annuum fruit exhibited anti-oxidative as well as inhibitory effect towards carbohydrates digesting enzymes activities \textit{in vitro}. The acetone fraction, which is the most polar, has exhibited higher activities compared to other fractions. Therefore, it is
recommended for *in vivo* anti-diabetic and anti-oxidative effects and to isolate the active principle from this fraction which is responsible for these actions.
Based on the results of the study above, acetone fraction demonstrated higher anti-oxidative action and inhibits the activities of carbohydrate digesting enzymes \textit{in vitro} compared to other solvent fractions. It was therefore selected for the \textit{in vivo} studies.

5.3 Anti-diabetic effects of \textit{Capsicum annuum} L. fruit acetone fraction in a type 2 diabetes model of rats

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5.3.1 Abstract

Objective: \textit{Capsicum annuum} L. (Solanaceae) is a spice that is readily available in different parts of the world. The fruit is locally used as a blood tonic, stimulant and in the treatment of nerve weakness due to diabetes in some parts of Africa and Asia. The present study investigated the anti-diabetic effect of \textit{C. annuum} acetone fraction (CAAF) derived from fruit ethanolic extract in type 2 diabetes (T2D) model of rats.

Methods: T2D was induced in rats by feeding a 10\% fructose solution \textit{ad libitum} for two weeks followed by a single intraperitoneal injection of streptozotocin (40 mg/kg body weight (bw)). The animals were orally treated with 150 or 300 mg/kg bw of the CAAF once daily for 4 weeks.

Results: Oral treatment of CAAF for 4 weeks especially at 300 mg/kg bw to diabetic animals demonstrated anti-hyperglycemic and anti-hyperlipidemic effects, improved glucose intolerance and insulin resistance and restored pancreatic morphology to near normal. Additionally, the alterations of other organ related serum markers were ameliorated in the treated diabetic animals compared to diabetic control group.

Conclusion: The results of our study suggest that CAAF possessed potent anti-diabetic effect in a T2D model of rats when no toxicological effects were observed for the above-mentioned parameters. Hence, CAAF can not only be used as a potential anti-diabetic natural medicine but also can be used to find novel compounds to develop anti-diabetic medicine.

Keywords: \textit{Capsicum annuum}, Fruit ethanolic extract, Acetone fraction, Type 2 diabetes, Rats.
5.3.2 Introduction

Type 2 diabetes (T2D) is a metabolic disorder characterized by chronic insulin resistance and loss of functional β-cells due to relative (rather than absolute) deficiency of insulin (ADA, 2015). Inadequate secretion of insulin by pancreatic β-cell in type 2 diabetic individuals disrupts the regulation of hepatic gluconeogenesis, muscles glucose uptake and lipolysis in adipose tissue. The consequence is postprandial hyperglycemia which subsequently causes T2D (Gastaldelli, 2011). Furthermore, the control of postprandial hyperglycemia is crucial for the prevention of the progression of diabetic complications (Rahati et al. 2014). One therapeutic strategy to reduce postprandial hyperglycemia in T2D is to retard absorption of glucose via inhibition of carbohydrate-digesting enzymes, such as α-glucosidase and α-amylase located at the intestinal brush border (Holman et al. 1999). Acarbose and miglitol are the major available glucosidase inhibitors currently used in treatment of T2D. However, lack of effective treatment and the resulting adverse effects with the existing drugs motivated further search for better anti-diabetic agents with fewer side effects as well (Hung et al. 2012). Therefore, with the increasing interest in alternatives the use of food based approaches is emerging and receiving much attention. This is in addition to the fact that consumers prefer natural functional ingredients than the synthetic chemicals (Taveira et al. 2010). Vegetables such as spices are important food adjuncts that have been used as flavoring or coloring agents in various food preparations. Additionally, considerable evidence have shown that spices can enhance the overall health status of diabetic patients by blood glucose lowering ability, amelioration of dyslipidemia, improving β-cell function, body weight and preventing cellular oxidative damage (Asif, 2011; Srinivasan, 2005a, b; Khan and Safdar, 2003; Broadhurst et al. 2000).

*Capsicum annuum* L. (Solanaceae) also known as red pepper is a spice that is readily available in almost all parts of the world. The fruit is widely consumed in vast quantities as flavoring agent in various traditional foods (Khan et al. 2014). The fruit is locally used as a blood tonic, stimulant and in the treatment of nerve weakness or pain due to diabetes in some parts of Africa and Asia (Iwu 2014; Jayakumar et al. 2010). In addition, *C. annuum* is endowed with variety of medicinal values and a good source of important phytochemicals such as dihydrocapsaincin, carotenoids, vitamin C, feruloyl O-glucosides, kaempferol O-pentosylhexosides, dihydroxyflavone O-hexoses, quercetin-3-O-L-rhamnoside and capsaicin (Hernández-Ortega et al. 2012; Park et al. 2012; Luthria and Mukhopadhyay 2006; Materska and Perucka, 2005). These ingredients have beneficial effects on human health and could play a role in the prevention of disease such as diabetes, cardiovascular and other age-related disorders (Materska and Perucka, 2005; Sun et al. 2007). The beneficial influence of *C. annuum* fruit and its pungent principle capsaicin as anti-oxidative agents have been well documented (Tundis et al. 2011; 2013; Kwon et al. 2007; Materska and Perucka, 2005; Prasad et al. 2004; Kogure et al. 2002; Reddy and Lokesh, 1992). In addition, consumption of the whole fruit extract or capsaicin was reported
to attenuate dyslipidemia, improve satiety and impact thermogenic effect in hyperlipidemic animals (Kempaiah and Srinivasan 2006; Kawada et al. 1986; Sambaiah et al. 1978).

On the other hand, the anti-diabetic action of *C. annuum* is still poorly understood due to inconsistency of the anti-diabetic action of the pure capsaicin, one of the active ingredients of *C. annuum* fruit. It has been highlighted that various extracts from the whole fruit and pure capsaicin were reported to inhibit the activities of α-glucosidase and α-amylase *in vitro*, which are potential target for the treatment of T2D (Tundis et al. 2011, 2013; Kwon et al. 2007). Tsui et al. (2007) have reported that ‘substance P’ a neuropeptide released by capsaicin, was able to reduce insulin resistance and hyperglycemia in diabetic mice. Conversely, dietary inclusion of pure capsaicin to diabetic animals did not show any beneficial anti-hyperglycemic and hypolipidemic effects in diabetic animals (Babu and Srinivasan, 1997), when consumption of the aqueous extract of the whole fruit improved glycemic control in non-diabetic animals after 1 h post-treatment period (Monsereenusorn, 1980). Therefore, based on the above-mentioned studies and diverse array of compounds present in plant-based food, we may hypothesize that consumption of either extract or fraction from the whole fruit of *C. annuum* may have anti-diabetic action. Additionally, to the best of our knowledge no study is available that comprehensively reported the anti-diabetic effect of the extract or fraction from *C. annuum* whole fruit either in humans or in experimental animals. Therefore, the present study was design to investigate the *in vivo* anti-diabetic effects of the *C. annuum* acetone fraction (CAAF) derived from the fruit ethanolic extract in a T2D model of rats.

5.3.3 Materials and methods

For sample collection, identification and preparation of the fraction, including the detail experimental protocols, please refer to chapter 2, sub-sections 2.3 and 2.5.1 to 2.5.8.

5.3.4 Results

The results of body weight (bw) change of all animals are presented in Figure 5.15. According to the data, before the STZ-injection, fructose feeding did not affect the mean bw of the treated animals compared to the untreated animals. But one week after the STZ-injection, the mean bw of the diabetic animals significantly decreases compared to non-diabetic groups. However, at 2nd, 3rd and 4th week of CAAF treatment the mean bw of diabetic treated groups were significantly (*p* < 0.05) increased compared to the DBC group (Figure 5.15) which is comparable to the DMF group. Furthermore, administration of the fraction to non-diabetic animals did not affect the mean bw change of the treated animals (Figure 5.15).
Figure 5.15. Mean body weight change of all animal groups during the study period. Data are presented as the mean ± SD of 5-7 animals. *c Values with different letter near the lines for a given week 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; DCAL, Diabetic C. annuum low dose; DCAH, Diabetic C. annuum high dose; DMF, Diabetic Metformin; NCAH, Normal C. annuum high dose.

The data of feed and fluid intake are presented in Figure 5.16. It was observed that feed and fluid intake of the DBC group significantly (p < 0.05) increased compared to the NC group. Oral intervention of CAAF and metformin for 4 weeks to diabetic animals reduced feed and fluid intake, when the effect is more pronounced in the DCAH group compared to the DCAL group (Figure 5.16). However, treatment of CAAF at high dose did not affect the eating behavior of the non-diabetic treated animals.

Figure 5.16. Food and fluid intake in different animal groups during the experimental period. Data are presented as the mean ± SD of 5-7 animals. *c Values with different letters over the bars for a given parameter 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; DCAL, Diabetic C. annuum low dose; DCAH, Diabetic C. annuum high dose; DMF, Diabetic Metformin; NCAH, Normal C. annuum high dose.
The results of weekly NFBG and FBG (last week only) of all the animal groups are presented in Figure 5.17. Induction of T2D significantly \((p < 0.05)\) elevated the blood glucose levels of DBC group compared to the NC group. However, oral treatment of CAAF for 4 weeks significantly \((p < 0.05)\) decreased the blood glucose levels in the DCAL and DCAH groups, when the effect was more prominent in DCAH group which is comparable to DMF group. Additionally, treatment of CAAF to NCAH group did not affect NFBG and FBG throughout the study period (Figure 5.17).

![Figure 5.17](image)

**Figure 5.17.** Weekly NFBG in all animal groups during the intervention period. Data are presented as the mean SD of 5-7 animals. \(^{a-d}\)Values with different letter near the lines for a given week 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; DCAL, Diabetic *C. annuum* low dose; DCAH, Diabetic *C. annuum* high dose; DMF, Diabetic Metformin; NCAH, Normal *C. annuum* high dose; NFBG, Non-fasting blood glucose; FBG, Fasting blood glucose.

The data of oral glucose tolerance test (OGTT) and the calculated area under the curve (AUC) are presented in Figure 5.18 and Table 5.7, respectively. The oral glucose tolerance ability of DBC group was significantly impaired compared to the NC group. However, significantly \((p < 0.05)\) better glucose tolerance ability was observed in the DCAL and DCAH groups compared to the DBC group throughout the test period. Moreover, better glucose tolerance ability was observed in the DCAH group compared to the DCAL group when at 60 min the blood glucose of DCAH was significantly \((p < 0.05)\) better compared to the DMF group. The glucose tolerance ability of NCAH group is not affected during the entire period of the test (Figure 5.18). The calculated AUC for DBC group was observed to be significantly \((p < 0.05)\) higher compared to NC group when that of DCAL and DCAH groups were significantly \((p < 0.05)\) lower compared to DBC groups and were comparable to the DMF group (Table 5.7).
Figure 5.18. OGTT of all animal groups in the last week of the 4-week intervention period. Data are presented as the mean ± SD of 5-7 animals. *-**Values with different letter near the lines for a given time 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; DCAL, Diabetic *C. annuum* low dose; DCAH, Diabetic *C. annuum* high dose; DMF, Diabetic Metformin; NCAH, Normal *C. annuum* high dose.

Table 5.7. Area under the curve (AUC) of different animal groups at the end of the experimental period

<table>
<thead>
<tr>
<th></th>
<th>NC</th>
<th>DBC</th>
<th>DCAL</th>
<th>DCAH</th>
<th>DMF</th>
<th>NCAH</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC*10^3</td>
<td>15.75 ± 0.97^a</td>
<td>60.15 ± 7.50^c</td>
<td>35.97 ± 9.01^b</td>
<td>23.36 ± 2.97^b</td>
<td>28.68 ± 7.21^b</td>
<td>15.47 ± 0.47^a</td>
</tr>
</tbody>
</table>

Data are presented as the mean ± SD of 5-7 animals. *-**Values with different letter along a row for a given parameter 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; DCAL, Diabetic *C. annuum* low dose; DCAH, Diabetic *C. annuum* high dose; DMF, Diabetic Metformin; NCAH, Normal *C. annuum* high dose, AUC, Area under curve.

The results of serum insulin and fructosamine levels including the calculated HOMA-IR and HOMA-β scores are presented in Table 5.8. According to the data, the serum insulin level and the calculated HOMA-β scores were decreased significantly (p < 0.05) whereas serum fructosamine as well as HOMA-IR were increased significantly (p < 0.05) in the DBC group compared to the NC group (Table 5.8). Treatment of the fraction to diabetic animals caused a significant (p < 0.05) and dose-dependent increase of serum insulin levels and the calculated HOMA-β scores with a concomitant reduction of serum fructosamine levels and HOMA-IR scores. Similarly, the reduction of serum fructosamine levels and HOMA-IR scores did not differ significantly (p < 0.05) within the diabetic treated groups (DCAL and DCAH) although more pronounced results were observed in the DCAH group which is also comparable to the DMF group. These parameters were not altered in the non-diabetic treated group (Table 5.8).
Table 5.8. Effect of CAAF on serum insulin and fructosamine levels and calculated HOMA-IR and HOMA-β scores in different animal groups at the end of the intervention period.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>NC</th>
<th>DBC</th>
<th>DCAL</th>
<th>DCAH</th>
<th>DMF</th>
<th>NCAH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin (pmol/L)</td>
<td>76.50 ± 6.26&lt;sup&gt;b&lt;/sup&gt;</td>
<td>47.05 ± 9.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>57.15 ± 16.32&lt;sup&gt;a&lt;/sup&gt;</td>
<td>78.74 ± 6.32&lt;sup&gt;a&lt;/sup&gt;</td>
<td>75.88 ± 14.23&lt;sup&gt;b&lt;/sup&gt;</td>
<td>77.64 ± 4.39&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Fructosamine (μmol/L)</td>
<td>239.20 ± 47.49&lt;sup&gt;a&lt;/sup&gt;</td>
<td>365.43 ± 44.94&lt;sup&gt;b&lt;/sup&gt;</td>
<td>288.60 ± 28.43&lt;sup&gt;a&lt;/sup&gt;</td>
<td>289.57 ± 20.94&lt;sup&gt;a&lt;/sup&gt;</td>
<td>283.14 ± 32.46&lt;sup&gt;a&lt;/sup&gt;</td>
<td>267.60 ± 10.29&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>HOMA-IR&lt;sup&gt;٭&lt;/sup&gt;</td>
<td>2.50 ± 0.27&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.30 ± 1.64&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.94 ± 1.30&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.72 ± 0.54&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.82 ± 0.90&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.49 ± 0.22&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>HOMA-β&lt;sup&gt;٭٭&lt;/sup&gt;</td>
<td>127.66 ± 43.71&lt;sup&gt;d&lt;/sup&gt;</td>
<td>7.33 ± 1.26&lt;sup&gt;a&lt;/sup&gt;</td>
<td>21.20 ± 5.28&lt;sup&gt;b&lt;/sup&gt;</td>
<td>57.43 ± 17.54&lt;sup&gt;c&lt;/sup&gt;</td>
<td>52.57 ± 25.76&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>132.17 ± 16.81&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data are presented as the mean ± SD of 5-7 animals. <sup>a–d</sup>Values with different letters along the rows for a given parameter are significantly different from each other group of animals (Tukey's-HSD multiple range post hoc test, <i>p</i> < 0.05). NC, Normal Control; DBC, Diabetic Control; DCAL, Diabetic <i>C. annuum</i> low dose; DCAH, Diabetic <i>C. annuum</i> high dose; DMF, Diabetic Metformin; NCAH, Normal <i>C. annuum</i> high dose. <sup>٭</sup>HOMA-IR = [(Fasting serum insulin in U/l x Fasting blood glucose in mmol/L)/22.5]. <sup>٭٭</sup>HOMA-β = (Fasting serum insulin in U/l x 20/Fasting blood glucose in mmol/L-3.5).

Figure 5.19: Histopathological examination of the pancreatic islets of different animal groups at the end of the intervention period. The NC had a larger islet with high number of β-cells while the DBC had a smaller islet and morphologically deformed β-cells. The DCAL, DCAH and DMF groups had relatively larger islets with higher number of β-cells compared to the DBC group. NC, Normal Control; DBC, Diabetic Control; DCAL, Diabetic <i>C. annuum</i> low dose; DCAH, Diabetic <i>C. annuum</i> high dose; DMF, Diabetic Metformin; NCAH, Normal <i>C. annuum</i> high dose.
The histopathological slides of the pancreas for all the groups are presented in Figure 5.19. It was observed from the slides that the pancreas of the DBC group shows increased fatty infiltrations of the islets and reduced number of β-cells compared to the NC group (Figure 5.19). On the other hand, the pancreatic morphology of DCAL, DCAH and DMF groups were significantly improved with larger islets and higher number of β-cells compared to the DBC group. No significant difference was observed between the pancreatic islets integrity of NCAH and NC group.

The data of absolute and relative liver weight including liver glycogen contents are presented in Table 5.9. Although absolute liver weight was not affected by the induction of diabetes, the relative liver weight and hepatic glycogen content of DBC group were significantly \( (p < 0.05) \) increased compared to the NC group (Table 5.9). Oral intervention of CAAF to diabetic animals did not significantly \( (p < 0.05) \) affect the relative liver weight and hepatic glycogen content compared to the DBC group (Table 5.9). Similarly, the glycogen content of NCAH group was not altered at the end of the intervention period.

The data for serum lipid profile and calculated atherogenic index (AI) and coronary risk index (CRI) are presented in Table 5.10. The serum total cholesterol (TC), triglycerides (TG) and low density lipoprotein (LDL) cholesterol as well as calculated AI and CRI with subsequent reduction on serum high density lipoprotein (HDL) cholesterol were observed in the DBC group compared to the NC group (Table 5.10). Treatment of CAAF to the diabetic animals significantly \( (p < 0.05) \) decreased serum TC, TG and LDL-cholesterol levels, calculated AI and CRI indices compared to the DBC group. Moreover, the increased on serum HDL-cholesterol level in CAAF diabetic treated groups did not differ significantly \( (p < 0.05) \) compared to the DBC group. Additionally, the levels of the above-mention parameters were not affected in NCAH group at the end of the intervention period (Table 5.10).

The results of serum ALT, AST, ALP, urea, uric acid, creatinine, LDH and CK-MB are presented in Table 5.11. According to the data, the serum ALT, ALP, urea, creatinine, LDH and CK-MB levels were elevated in DBC group compared to the NC group, when the serum creatinine, ALP and uric acid levels were not affected by the induction of diabetes (Table 5.11). However, oral intervention of CAAF for 4 weeks to diabetic animals significantly reduced serum ALT, ALP, urea, creatinine, LDH and CK-MB levels compared to the DBC group. Although these reductions were not significantly \( (p < 0.05) \) different between DCAL and DCAH groups, they were comparable with the DMF group. Furthermore, treatment of CAAF did not cause any alterations in the serum levels of the above-stated parameters in NCAH group (Table 5.11).
Table 5.9. Effect of CAAF on liver weights and liver glycogen concentrations in T2D rats

<table>
<thead>
<tr>
<th></th>
<th>NC</th>
<th>DBC</th>
<th>DCAL</th>
<th>DCAH</th>
<th>DMF</th>
<th>NCAH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver weight(g)</td>
<td>9.25 ± 1.04</td>
<td>10.34 ± 0.31</td>
<td>9.52 ± 0.35</td>
<td>9.62 ± 0.85</td>
<td>9.10 ± 1.33</td>
<td>9.65 ± 0.41</td>
</tr>
<tr>
<td>Relative liver weight (%)</td>
<td>2.89 ± 0.29a</td>
<td>4.55 ± 0.34b</td>
<td>4.31 ± 0.10b</td>
<td>4.27 ± 0.20b</td>
<td>3.10 ± 0.45ab</td>
<td>2.85 ± 0.13a</td>
</tr>
<tr>
<td>Liver glycogen (mg/g tissue)</td>
<td>6.76 ± 0.81a</td>
<td>8.25 ± 0.83b</td>
<td>8.12 ± 0.32b</td>
<td>8.08 ± 0.49b</td>
<td>6.92 ± 0.56a</td>
<td>6.72 ± 0.27a</td>
</tr>
</tbody>
</table>

Data are presented as the mean ± SD of 5-7 animals. Values with different letters along the rows for a given parameter 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; DCAL, Diabetic C. annuum low dose; DCAH, Diabetic C. annuum high dose; DMF, Diabetic Metformin; NCAH, Normal C. annuum high dose.

*Relative liver weight (%) = [weight of the liver (g)/body weight (g)] x100
Table 5.10. Serum lipid profiles and other biochemical parameters in different animal groups at the end of the intervention period

<table>
<thead>
<tr>
<th>Parameter</th>
<th>NC</th>
<th>DBC</th>
<th>DCAL</th>
<th>DCAH</th>
<th>DMF</th>
<th>NCAH</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC (mg/dl)</td>
<td>76.20 ± 7.40a</td>
<td>116.00 ± 12.92c</td>
<td>83.14 ± 13.69ab</td>
<td>79.43 ± 7.91ab</td>
<td>95.71 ± 7.74b</td>
<td>75.80 ± 9.39a</td>
</tr>
<tr>
<td>HDL-Cholesterol (mg/dl)</td>
<td>49.20 ± 8.35b</td>
<td>28.29 ± 6.25a</td>
<td>32.57 ± 7.30a</td>
<td>30.57 ± 4.47a</td>
<td>32.71 ± 7.43a</td>
<td>30.40 ± 3.21a</td>
</tr>
<tr>
<td>LDL-Cholesterol (mg/dl)*</td>
<td>12.96 ± 4.16a</td>
<td>57.54 ± 14.45c</td>
<td>33.26 ± 9.10b</td>
<td>34.31 ± 9.97b</td>
<td>42.86 ± 8.93bc</td>
<td>32.84 ± 12.74ab</td>
</tr>
<tr>
<td>TG (mg/dl)</td>
<td>70.20 ± 18.10a</td>
<td>150.86 ± 24.57b</td>
<td>86.57 ± 29.40a</td>
<td>72.71 ± 20.89a</td>
<td>100.71 ± 32.47a</td>
<td>62.80 ± 6.06a</td>
</tr>
<tr>
<td>AI</td>
<td>0.57 ± 0.13a</td>
<td>3.30 ± 1.08c</td>
<td>1.60 ± 0.37ab</td>
<td>1.63 ± 0.38ab</td>
<td>2.06 ± 0.69b</td>
<td>1.53 ± 0.38ab</td>
</tr>
<tr>
<td>CRI</td>
<td>1.57 ± 0.13a</td>
<td>4.30 ± 1.08c</td>
<td>2.60 ± 0.37ab</td>
<td>2.63 ± 0.38ab</td>
<td>3.06 ± 0.69b</td>
<td>2.93 ± 0.38ab</td>
</tr>
</tbody>
</table>

Data are presented as the mean ± SD of 5-7 animals. * Values with different letters along the rows for a given parameter 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; DCAL, Diabetic *Capsicum annuum* low dose; DCAH, Diabetic *Capsicum annuum* high dose; DMF, Diabetic Metformin; NCAH, Normal *Capsicum annuum* high dose; TC, Total cholesterol; TG, Triglyceride; LDL-cholesterol, Low density lipoprotein-cholesterol; HDL-cholesterol, High density lipoprotein-cholesterol; AI, Atherogenic index; CRI, Coronary risk index.

*LDL-Cholesterol (mg/dl)= [TC- HDL- (TG/5)]
Table 5.11. Serum biochemical parameters in different animal groups at the end of the intervention period

<table>
<thead>
<tr>
<th></th>
<th>NC</th>
<th>DBC</th>
<th>DCAL</th>
<th>DCAH</th>
<th>DMF</th>
<th>NCAH</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT (U/l)</td>
<td>77.40 ± 20.38&lt;sup&gt;a&lt;/sup&gt;</td>
<td>116.14 ± 37.37&lt;sup&gt;b&lt;/sup&gt;</td>
<td>78.43 ± 6.63&lt;sup&gt;a&lt;/sup&gt;</td>
<td>71.43 ± 15.87&lt;sup&gt;a&lt;/sup&gt;</td>
<td>93.57 ± 6.86&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>73.80 ± 8.93&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>AST (U/l)</td>
<td>88.40 ± 11.41</td>
<td>83.29 ± 15.57</td>
<td>87.86 ± 1158</td>
<td>81.86 ± 17.07</td>
<td>94.71 ± 9.39</td>
<td>85.80 ± 10.33</td>
</tr>
<tr>
<td>ALP (U/l)</td>
<td>149.40 ± 11.33&lt;sup&gt;a&lt;/sup&gt;</td>
<td>451.14 ± 35.10&lt;sup&gt;c&lt;/sup&gt;</td>
<td>205.43 ± 46.62&lt;sup&gt;b&lt;/sup&gt;</td>
<td>207.57 ± 16.44&lt;sup&gt;b&lt;/sup&gt;</td>
<td>208.86 ± 27.58&lt;sup&gt;b&lt;/sup&gt;</td>
<td>154.20 ± 29.88&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>Creatinine (mg/dL)</td>
<td>2.52 ± 0.78&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.12 ± 0.45&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.66 ± 0.31&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.00 ± 0.10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.68 ± 0.27&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.86 ± 0.19&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Urea (mg/dL)</td>
<td>22.00 ± 4.69&lt;sup&gt;a&lt;/sup&gt;</td>
<td>60.71 ± 15.71&lt;sup&gt;b&lt;/sup&gt;</td>
<td>25.57 ± 10.10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>26.00 ± 9.31&lt;sup&gt;a&lt;/sup&gt;</td>
<td>26.00 ± 5.00&lt;sup&gt;a&lt;/sup&gt;</td>
<td>26.20 ± 6.02&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Uric acid (mg/dL)</td>
<td>2.06 ± 0.67</td>
<td>2.85 ± 1.18</td>
<td>2.32 ± 0.78</td>
<td>2.40 ± 1.20</td>
<td>2.56 ± 1.36</td>
<td>2.14 ± 0.84</td>
</tr>
<tr>
<td>LDH (U/l)</td>
<td>284.00 ± 62.56&lt;sup&gt;a&lt;/sup&gt;</td>
<td>458.86 ± 68.05&lt;sup&gt;b&lt;/sup&gt;</td>
<td>424.43 ± 107.14&lt;sup&gt;b&lt;/sup&gt;</td>
<td>372.71 ± 72.63&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>361.57 ± 80.66&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>287.60 ± 34.50&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>CK-MB (U/l)</td>
<td>374.54 ± 82.56&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1064.36 ± 537.18&lt;sup&gt;b&lt;/sup&gt;</td>
<td>714.33 ± 69.02&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>579.44 ± 71.72&lt;sup&gt;a&lt;/sup&gt;</td>
<td>743.97 ± 75.29&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>399.02 ± 58.82&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SD of 5-7 animals. *Values with different superscript letters along a row for a given parameter are significantly different from each other group of animals (Tukey’s-HSD multiple range post hoc test, p < 0.05). ALT, Alanine transaminase; AST, Alanine transaminase; ALP, Alkaline phosphate; LDH, Lactate dehydrogenase; CK-MB, Creatine kinase.
5.3.5 Discussion

Apart from dietary and life style modifications, the current treatment options of T2D involve in the use of conventional oral hypoglycemic drugs. However, the use of oral hypoglycemic drugs was associated with a number of short- and long-time consequences. For instance α-glucosidase inhibitors are reported to cause diarrhea, bloating, flatulence, cramping and abdominal pain (Fujisawa et al. 2005). Therefore, this has triggered researchers to scientifically investigate the anti-diabetic actions of a number of plants including plant-based spices as possible alternative therapies for diabetes. This is partly because plant-derived preparations are important component of health care delivery system, particularly in developing countries and were reported to cause minimum side effects (Cragg and Newman, 2013; Balunas and Kinghorn, 2005). Spices are important food supplements which have been used as flavoring agents and their ethnomedical usage in the treatment of diabetes has been documented for a long time (Srinivasan, 2005). In the present study, we have evaluated the effect of the 4-week oral administration of a low (150 mg/kg bw) and a high (300 mg/kg bw) dose of *C. annum* acetone fraction (CAAF) of fruit ethanolic extract in a T2D model of rats. Although a number of recent and previous studies reported the anti-diabetic effects of *C. annum* fruit extract *in vitro* (Tundis et al. 2013; Tundis et al. 2011; Kwon et al. 2007), as per our knowledge, this is the first *in vivo* study that comprehensively conducted the anti-diabetic effect of *C. annum* fruit.

According to the American Diabetes Association (ADA), diabetes causes polyuria, increase catabolic processes and weight loss, in addition to increase of feed and fluid intake (ADA, 2015). On the other hand, capsaicin is the major bioactive compound of *C. annum* and it has been reported that dietary inclusion of capsaicin (0.015%) for 8 weeks to diabetic animals did not show any effect on the bw change (Babu and Srinivasan, 1997). In another study, consumption dietary *C. annum* (0.9 g four times daily) increased energy expenditure, decreased appetite and food intake in human subjects within 2 days of study period (Westerterp-Plantenga et al. 2005). However, in our study, oral treatment of CAAF to diabetic animals for 4 weeks caused a significant increase in bw despite of lower feed and fluid intake compared to DBC group (*Figure 5.15* and *5.16*). In the above-mentioned studies, either pure capsaicin or whole dietary *C. annum* was used when acetone fraction of the fruit ethanolic extract was used in our study which chemical compositions might be different from the above. Hence, the other compounds from the *C. annum* might be contributed to this activities compared to the major active principle, capsaicin, or whole *C. annum*. Furthermore, the effects were more pronounced in DCAH group compared to the DCAL and were comparable to the DMF group. This indicates possible recovery from the diabetes-induced alterations in eating behavior of the treated animals, which was further supported by the significant decrease in blood glucose levels in CAAF treated groups (*Figure 5.17*).
Successful control of either fasting or postprandial blood glucose levels remains a prime target in preventing and attenuating diabetes and its associated complications (Bavarva and Narasimhacharya, 2008). Babu and Srinivasan (1997) have reported that diabetic animals maintained on capsaicin (0.015%) containing diet, for 8 weeks have shown no significant reduction on blood glucose levels. Likewise, in another study, "substance P" (a neuropeptide released by capsaicin) was reported to cause fluctuations in blood sugar levels in human subjects (Brown and Vale, 2011). Converesely, Monsereenusorn, (1980) has reported that intraperitoneal injection of fruit extract (500-700 mg/kg bw) showed a significant hypoglycemic action after 30 minutes post-treatment period. In our study, oral intervention of the CAAF to diabetic animals for 4 weeks significantly lowered blood glucose levels, when it was more pronounced in DCAH group compared to DCAL group (Figure 5.17). This has shown a dose-dependent anti-hyperglycemic response in the diabetic treated groups, which was further supported by the OGTT data (Figure 5.18). In the OGTT result, better glucose utilizing capacities was observed in CAAF diabetic treated animals compared to DBC group, when it was more prominent in the DCAH group compared to the DCAL group and was comparable to DMF group. This again supports the possible contribution of other compounds in CAAF apart from the major capsaicin in the observed anti-hyperglycemic action of CAAF. On the other hand, the CAAF did not show any effect on the blood glucose level of NCAH group throughout the study period, when in another study higher dosages (500-700 mg/kg bw) of the extract have shown hypoglycemic effects in non-diabetic animals (Monsereenusorn, 1980). This could be due to the lower dosages (300 mg/kg bw) of the fraction used in our study. The anti-hyperglycemic action of CAAF could be attributed to the higher α-glucosidase and α-amylase inhibitory actions which apparently could delay the intestinal absorption of glucose (Imam, 2015) in addition to the pancreatic β-cell ameliorating as well as insulin secreting effects of CAAF in our study (Figure 5.19 and Table 5.8).

It has been reported that chronic hyperglycemia in T2D causes an impairment or decline in insulin secretion (Nattrass and Bailey, 1999), with subsequent distortion of pancreatic architecture (Bonner-Weir and O'Brien, 2008). Based on the current available data, this is the first study that investigates the effect of CAAF of fruit on serum insulin levels and pancreatic β-cell integrity in diabetic state. According to our results, treatment of CAAF to diabetic animals especially at the higher dose (300 mg/kg bw) have shown a significant insulinotrophic effect, lowered peripheral insulin resistance, stimulated pancreatic β-cell function and restored the pancreatic morphology to near normal compared to the DBC group (Figure 5.19 and Table 5.8). Therefore, the anti-hyperglycemic effect of CAAF could partly be mediated via insulinotrophic actions and decreasing insulin resistance. This was further supported by the significantly lower serum fructosamine levels in the DCAL and DCAH groups compared to the DBC group which did not differ significantly with the NC and DMF groups (Table 5.8). This could be due to the strong anti-oxidative nature of C. annuum.
fruit (extracts or fractions) reported in previous studies (Tundis et al. 2013; Tundis et al. 2011; Kwon et al. 2007; Oboh et al. 2007).

On the other side, previous studies have shown that supplementation of 0.015% capsaicin containing diet to high-fat diet-fed diabetic animals for 8 weeks have shown no significant effect on the relative liver weight (Kempaiah and Srinivasan, 2006; Babu and Srinivasan, 1997), which was similarly observed in our present data (Table 5.9). Additionally, oral treatment of CAAF did not show any significantly effect on the hepatic glycogen content of the treated diabetic animals (Table 5.9). Hence, our data are in line with the previous capsaicin-based studies.

On the other side, *C. annuum* and its pungent principle capsaicin have been reported to play beneficial role on lipid metabolism in a number of previous studies. Capsaicin (0.015%) included in diet for 8 weeks was reported to decrease serum TC, TG and LDL-cholesterol levels in diabetic and high-fat diet animals (Kempaiah and Srinivasan, 2006; Babu and Srinivasan, 1997). Similarly, in a chronic study, oral treatment of capsaicin (50 mg/kg bw) or whole fruit extract (500 mg/kg bw) for 2 months demonstrated hypolipemic actions (Monsereenusorn, 1983). Our results are also in line with the above-mentioned studies. Additionally, a moderate increase of serum HDL-cholesterol levels was also observed in DCAL and DCAH groups which did not differ significantly from DBC groups (Table 5.10). These indicate the anti-hyperlipidemic effect of CAAF in diabetic animals. Moreover significantly lower calculated AI and CRI indices in DCAL and DCAH groups compared to the DBC in our study again support the anti-hyperlipidemic effect of CAAF in diabetic condition (Table 5.10).

In some disease conditions such as T2D, an increase of serum ALT, ALP, creatinine, urea, LDH and CK-MB into blood circulation induces hepatic injuries, myocardial infarction and functional disturbance of hepatic and renal tissues (Abolfathi et al. 2012; Jaramillo-Juarez et al. 2008). Therefore, based on the results of the above-mentioned parameters, treatment of CAAF has demonstrated hepatoprotective, cardio-protective as well as reno-protective effects in diabetic condition, when serum AST level was not affected anyway (Table 5.11). In comparison with ALT, AST is less specific for the detection of hepatic injury due to its shorter half-life compared to ALT, particularly in experimental animal (Center, 2007). Therefore, in a condition of acute or sub-chronic liver injury, there will be an elevation in both serum ALT and AST levels. However, the serum AST level may likely return to normal more rapidly compared to the level of serum ALT, which make ALT more sensitive in assessing hepatic damage in disease conditions (Ramaiah, 2007; Meyer and Harvey, 2004). Therefore, the unaffected serum AST level in our present result could be link to the above-mentioned factor.
5.3.6 Conclusion

Oral intervention of CAAF from whole fruit especially at the higher dose (300 mg/kg bw) have demonstrated anti-diabetic actions via improving bw gain, reducing food and fluid intake and hyperglycemia, improving glucose tolerance ability, insulin sensitivity, amelioration of pancreatic β-cell histology and β-cell functions and improving dyslipidemia in T2D. Hence, our findings suggest that acetone fraction of the whole *C. annuum* fruit ethanolic extract may provide an excellent anti-diabetic remedy with no considerable side effects. Further clinical study is required to confirm the effects in human subjects. The isolation and partial characterization of the active principle from CAAF is currently underway to find the compound(s) responsible for this activity.
5.4 Anti-oxidant action of *Capsicum annuum* fruit acetone fraction in in a type 2 diabetes model of rats

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5.4.1 Abstract

**Objectives:** The present study evaluated the *in vivo* anti-oxidative actions of *Capsicum annuum* acetone fraction (CAAF) from fruit ethanolic extract in a type 2 diabetes (T2D) model of rats.

**Methods:** T2D was induced in rats by feeding a 10% fructose solution *ad libitum* for two weeks followed by a single intraperitoneal injection of streptozotocin (40 mg/kg body weight (bw)) and the animals were orally treated with 150 or 300 mg/kg bw of the CAAF once daily for 4 weeks.

**Results:** After four weeks of intervention, diabetic untreated animals showed significantly higher levels of thiobarbituric acid reactive substances (TBARS) and reductions of reduced glutathione (GSH) in the serum and organs (liver, kidney, heart and pancreas) compared to the normal animals. The activities of endogenous anti-oxidant enzymes (superoxide dismutase, catalase, glutathione peroxidase and reductase) were greatly impaired in the serum and organs of diabetic untreated animals compared to the normal animals. These alterations were attenuated after the treatment of CAAF, especially at the dose of 300 mg/kg bw.

**Conclusion:** The results of our study suggest that CAAF treatment at 300 mg/kg bw showed potent anti-oxidative potential in a T2D model of rats.

**Keywords:** Acetone, Anti-oxidant, *Capsicum annuum*, Fruit ethanolic extract, Type 2 diabetes, Rats.

5.4.2 Introduction

Diabetes mellitus (DM) is a major threat to global public health that is rapidly escalating, and the highest impact on adults in most of the developing countries. The recent data indicate that more than 387 million people have DM and this figure is likely to be doubled by 2035, with type 2 diabetes (T2D) been the most prevalent one, accounting for more than 90% of all diabetic cases (IDF, 2014; Guariguata et al.
T2D is a heterogeneous disorder characterized by insulin resistance and partially dysfunctional pancreatic β-cells which cannot properly secrete insulin in response to hyperglycemia (Hui et al. 2007).

T2D is associated with severe chronic complications that are partly or solely depend on uncontrolled hyperglycemia (Monnier et al. 2006). The hyperglycemia in T2D induces generation of reactive oxygen species (ROS), which are responsible for oxidative stress induced pancreatic β-cell destruction (Ceriello and Testa, 2009). Oxidative stress is a condition due to the presence of ROS that usually arise either from an excess production of ROS or from the inactivation of the in vivo anti-oxidants defense system, thus shifting the ROS/anti-oxidant equilibrium towards stress (Ibrahim et al. 2012).

In aerobic system, oxygen is required for energy production (in the form of ATP) via the metabolism of biomolecules such as fatty acids, glucose, lactate, ketones, and amino acids. During this complex process, an unstable ROS, superoxide anion radical is produced, which is regarded as primary ROS and later generate the secondary ROS either directly or indirectly by a variety of enzyme or metal catalyzed processes (Sati et al. 2010). An enzyme, superoxide dismutase (SOD) catalyzes the conversion of superoxide to hydrogen peroxide, which subsequently cross the membranes and causes enzyme inactivation through oxidations of their thiol groups (Rajasekaran et al. 2005). The hydrogen peroxide is later converted to hydroxyl radicals in the presence of transition metals. This initiates a sequence for the generation of other ROS such as singlet oxygen and peroxyl (ROO•) radicals.

Therefore, due to the shift in the ROS/anti-oxidant equilibrium balance in living organisms, the increased intake of anti-oxidants is necessary to prevent the oxidative damages due to oxidative stress. However, the use of synthetic anti-oxidants such as butylated hydroxy anisole (BHA), butylated hydroxy toluene (BHT), tertiary butylated hydroquinon and gallic acid esters in food protection and disease control have been associated with unwanted consequences. This has motivated researchers to look for options from plant-derived anti-oxidants.

The fruit from Capsicum annuum L. has been a major component of various food preparations worldwide. In addition to this, C. annuum fruit has been reported to be an excellent source of important anti-oxidant biomolecules such as vitamins, polyphenols and other potentially active phytochemicals that may prevent the risk of T2D (Rahimi et al. 2005). More importantly, there is sufficient available data that support the anti-oxidative crude extracts of C. annuum in vitro (Azeez et al. 2012; Chen et al. 2012; Tundis et al. 2011; Perucka and Materska, 2007; Conforti et al. 2007; Kwon et. 2007), when no available study on the in vivo anti-oxidant action of any extracts, fractions or isolated compounds from C. annuum either in normal or diseased conditions.

Therefore, the present study was designed to comprehensively investigate the in vivo anti-oxidant effects of the acetone fraction derived from the fruit ethanolic extract of C. annuum in a T2D model of rats.
5.4.3 Materials and methods

For sample collection, identification and preparation of the fraction, including the detail experimental protocols, please refer to chapter 2, sub-sections 2.3 and 2.5.1 to 2.5.8.

5.4.4 Results

The results of thiobarbituric acid reactive substances (TBARS) expressed as malondialdehyde (MDA) concentration of the serum and organs (liver, kidney, heart and pancreas) of all the groups are presented in Figure 5.20. The TBARS concentrations were significantly \( p < 0.05 \) elevated in the serum, liver, heart and pancreas of the DBC group compared to the NC group. The increase in kidney of the DBC group was not significantly \( p < 0.05 \) different compared to NC group. However, treatment of CAAF to diabetic animals (DCAL and DCAH) significantly \( p < 0.05 \) decreased the serum and pancreatic TBARS concentrations comparable to the DMF group (Figure 5.20). Moreover, the reductions of TBARS levels in the liver, kidney and heart of the DCAL and DCAH groups were not significantly \( p < 0.05 \) different compared to the DBC group.

Figure 5.20: Serum and organs thiobarbituric acid reactive substances of all animal groups at the end of the intervention period. Data are presented as the mean ± SD of 5-7 animals. a-d Values with different letter over 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; DCAL, Diabetic *C. annuum* low dose; DCAH, Diabetic *C. annuum* high dose; DMF, Diabetic Metformin; NCAH, Normal *C. annuum* high dose; MDA, Malondialdehyde.
The data of reduced glutathione (GSH) levels of serum and organs of all the groups are presented in Figure 5.21. According to the results, the concentration of GSH in the serum, liver and pancreas were significantly \( (p < 0.05) \) decreased in the DBC group compared to the NC group. Similarly, the decrease of GSH levels in the kidney and heart of the DBC group were not significantly \( (p < 0.05) \) different compared to the NC group. Oral administration of CAAF to diabetic animals significantly \( (p < 0.05) \) increased the levels of GSH in the liver, when that of the serum, kidney, heart and pancreas was not significantly \( (p < 0.05) \) different compared to the DBC group (Figure 5.21). In addition, the effect was more pronounced in the DCAH compared to DCAL group. Furthermore, the GSH level in the NCAH was comparatively within normal range throughout the study period (Figure 5.21).

![Figure 5.21: Serum and organs reduced glutathione (GSH) content in all animal groups at the end of the study period. Data are presented as the mean ± SD of 5-7 animals. *Values with different letter over 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; DCAL, Diabetic C. annuum low dose; DCAH, Diabetic C. annuum high dose; DMF, Diabetic Metformin; NCAH, Normal C. annuum high dose; GSH, Reduced glutathione.](image)

The results of the activity of superoxide dismutase (SOD) of serum and organs of all the groups are presented in Figure 5.22. It was observed from the data that induction of diabetes significantly \( (p < 0.05) \) decrease the activity of SOD in serum, liver, heart and pancreas of DBC group, whereas the reduction in kidney did not differ significantly \( (p < 0.05) \) compared to NC group. However, treatment of the fraction to diabetic animals, especially the DCAH group showed a significant \( (p < 0.05) \) increased in the SOD activity of the serum and organs, comparable to DMF group (Figure 5.22). Additionally, the SOD activity in the serum and organs of NCAH group was not affected throughout the study period.
Figure 5.22: Superoxide dismutase (SOD) activities in the serum and organs of different animal groups at the end of the study period. Data are presented as the mean ± SD of 5-7 animals. *-Values with different letter over 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; DCAL, Diabetic C. annuum low dose; DCAH, Diabetic C. annuum high dose; DMF, Diabetic Metformin; NCAH, Normal C. annuum high dose.

The data of catalase activities in serum and organs of all the groups are presented in Figure 5.23. According the results, the activity of the catalase in the serum and organs was significantly (p < 0.05) decreased in the DBC group compared to the NC group. Treatment of CAAF to the diabetic animals increases the catalase activity, when the effect was more pronounced in the DCAH group than the DCAL group (Figure 5.23). Additionally, treatment of the fraction to the non-diabetic animals did not affect the serum or organ catalase activity throughout the study period (Figure 5.23).

Figure 5.23: Catalase activities in the serum and different organs of different animal groups at the end of the study period. Data are presented as the mean ± SD of 5-7 animals. *-Values with different letter over 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; DCAL, Diabetic C. annuum low dose; DCAH, Diabetic C. annuum high dose; DMF, Diabetic Metformin; NCAH, Normal C. annuum high dose.
The activity of glutathione peroxidase (GPx) in serum and organs of all the groups are presented in **Figure 5.24**. The GPx activity of the serum and organs was significantly \((p < 0.05)\) decreased in the DBC group compared to the NC group. Oral intervention of the CAAF, especially at the higher dose, significantly \((p < 0.05)\) increased the GPx activity, which is comparable to the DMF group (**Figure 5.24**).

![Glutathione peroxidase activities in the serum and organs of different animal groups at the end of the study period. Data are presented as the mean ± SD of 5-7 animals. Values with different letter over 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; DCAL, Diabetic C. annuum low dose; DCAH, Diabetic C. annuum high dose; DMF, Diabetic Metformin; NCAH, Normal C. annuum high dose.](image)

The data of glutathione reductase (GR) activity in serum and organs of all the groups are presented in **Figure 5.25**. The activity of GR was not affected by the diabetes in kidney and heart, whereas greatly decreased in serum, liver and the pancreas of DBC group compared to the NC group. Treatment of the CAAF to diabetic animals improved the activity of the enzyme, when in DCAH group the activity was significantly \((p < 0.05)\) increased in pancreas compared to the DBC group (**Figure 5.25**). In addition, the activity of GR in NCAH group was not affected throughout the study period (**Figure 5.25**).
Figure 5.25: Glutathione reductase activities in the serum and organs of different animal groups at the end of the study period. Data are presented as the mean ± SD of 5-7 animals. *Values with different letter over 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; DCAL, Diabetic *C. annuum* low dose; DCAH, Diabetic *C. annuum* high dose; DMF, Diabetic Metformin; NCAH, Normal *C. annuum* high dose.

5.4.5 Discussion

Natural products such as plants are currently utilized as alternative in the treatment and control of several disorders including T2D. The prevention of diabetes has been associated with consumption of plants rich in natural anti-oxidants. This is because oxidative stress has been the major causal factor for most of the complications associated with diabetes (Ceriello, 2006). In our present study, we intent to comprehensively investigate the *in vivo* anti-oxidant effects of the acetone fraction derived from the fruit ethanolic extract of *C. annuum* in a T2D model of rats. This is the first study that explore the *in vivo* anti-oxidant potentials of any parts, extracts or fractions derived from *C. annuum* despite several studies on the *in vitro* anti-oxidant activity (Azeez et al. 2012; Chen et al. 2012; Tundis et al. 2011; Perucka and Materska, 2007; Conforti et al. 2007; Kwon et. 2007).

Diabetes has been associated with marked elevation of lipid peroxidation levels (thiobarbituric acid reactive substances (TBARS), which is an indication of increased ROS production (Maritim et al. 2003). This was clearly manifested in the DBC group, a direct evidence of increased cellular oxidative stress and depletion of anti-oxidant defense system in diabetic condition. However, oral treatment of CAAF attenuates
this alteration, suggesting the protective role of CAAF in terminating the deleterious effect of ROS and oxidative stress as well (Figure 5.20).

In living system, there are some low molecular weight non-enzymatic molecules such as α-tocopherol, ascorbic acid, bilirubin and glutathione (GSH) that support the anti-oxidant defense system and counteract the effects of oxidative stress (Rahimi et al. 2005). Moreover, diabetes has been associated with reduction of GSH levels, due to the depletion of in vivo anti-oxidant system in diabetic condition, which was observed in the DBC group in our present data (Figure 5.21). Treatment of the fraction to diabetic animals was found to increase the serum and organ GSH levels, which may be liked to either increased GSH biosynthesis or attenuated the oxidative processes leading to GSH reduction.

Superoxide anion radical has been considered as a primary ROS formed when molecular oxygen accept electron in the metabolism of complex macromolecules (Sati et al. 2010). The deleterious effect of this radical is prevented by an enzyme superoxide dismutase (SOD), which converts the superoxide radical to less toxic hydrogen peroxide. The activity of this enzyme was reported to be impaired in diabetic condition, due to increased ROS production that cannot be quenched by the enzyme action (Niki, 2010; Valko et al. 2007). In our present study, this was clearly observed in the DBC group compared to the NC group (Figure 5.22). However, the increased SOD activity observed in diabetic treated animals suggests that the fraction contributes to in vivo anti-oxidant system via increasing the SOD activity, which was further supported by the increased catalase activity (Figure 5.23).

Furthermore, the hydrogen peroxide released by the SOD is immediately converted to non-toxic molecules, water and oxygen by catalase (McCrod et al. 1976). In most of degenerative disorders, the activity of catalase is greatly altered; leading to increase in the conversion of hydrogen peroxide to hydroxyl radicals, which invariably increased the oxidative stress. Therefore, the decreased catalase activity in the DBC group observed in our present study may be attributed to increase conversion of hydrogen peroxide to hydroxyl radicals that overpowered and reduced the catalase activity. Interestingly, treatment of CAAF was found to improve the activity of catalase in the serum and organs, when is more pronounced in the DCAH than DCAL (Figure 5.23). This was again supported by the increased activities of glutathione peroxidase (GPx) and glutathione reductase (GR) (Figure 5.24 and 5.24).

According to our present data, the reduction of the activities of two glutathione dependent enzymes; GPx and GR in the serum and organs of the DBC group could be due to the oxidative damage initiated by increased ROS production. The moderate recovery of GPx and GR activities in the serum and organs of the DCAH group indicates the protective effect of CAAF on the in vivo anti-oxidants (Figure 5.24 and 5.24).
5.4.6 Conclusion

In conclusion, oral administration of the fraction especially at higher dose demonstrates strong anti-oxidant activities via reduction of lipid peroxidation, improving glutathione levels and endogenous anti-oxidants enzymes in serum and organs mostly affected by diabetic-complications. Therefore, CAAF is considered as excellent anti-oxidant agent with no considerable side effects. Further clinical study is required to confirm its effects in human subjects. The isolation and partial characterization of the active principle from CAAF has been conducted to find the compounds responsible for this activity.
CHAPTER 6

6.1 Anti-diabetic action of some African medicinal plants
(Aframomum melegueta, Xylopia aethiopica and Capsicum annuum)
in vitro and isolation of bioactive compounds

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6.1.1 Abstract

Objective: The use of Aframomum melegueta K. Schum. (Zingiberaceae), Xylopia aethiopica (Dunal) A. Rich. (Annonaceae) and Capsicum annuum L. (Solanaceae) fruits for treatment of diabetes and related metabolic disorders have been reported. This has recently been established in vivo. However, compounds responsible for the anti-diabetic action have not been identified. The present study is aimed at carrying out the bioassay-guided isolation of possible compounds responsible for the anti-diabetic action of the selected plants.

Methods: Plant parts were extracted in organic solvents and the secondary metabolites, isolated and purified before being identified by spectroscopic analysis. Their α-glycosidase and α-amylase inhibitory activities were investigated.

Results: Three arylalkanes, 6-paradol (1), 6-shagaol (2) and 6-gingerol (3) and a pentacyclic triterpene, oleanolic acid (4) were isolated from A. melegueta fruit. Compounds (3) and (4) were also isolated from the fruit of X. aethiopica. A mixture of aromatic compounds and a fatty acid (unable to be identified) was also isolated from the active subfraction of C. annuum and labeled as (5). All the compounds exhibited considerable inhibitory activity against α-amylase and α-glucosidase. Compounds (3) and (4) showed the best activity against α-amylase (IC50: (3): 156.96 ± 12.23 µM; (4): 91.72 ± 1.63 µM) and α-glucosidase (IC50: (3): 104.34 ± 3.99 µM; (4): 47.10 ± 0.42 µM) compared to the standard acarbose and the other isolated compounds. The kinetics of the enzyme action shown by the compounds showed a non-competitive mode of inhibition.
Conclusion: 6-Gingerol (3) oleanolic acid (4) present in the fruit of both *A. melegueta* and *X. aethiopica* are possibly responsible for the anti-diabetic action attributed to these plants. Both 3 and 4 could be potential anti-diabetic plants.

Keywords: *Aframomum melegueta*, *Capsicum annuum*, *Xylopia aethiopica* Anti-diabetic, α-amylase, α-glucosidase

6.1.2 Introduction

Type 2 diabetes (T2D) is a metabolic disorder characterized by chronic insulin resistance and loss of functional pancreatic β-cells due to relative (rather than absolute) deficiency of insulin (ADA, 2015). The inability of the pancreatic β-cell to secrete enough insulin disrupts the regulation of hepatic glucose synthesis, muscles glucose uptake and lipolysis in adipose tissue. The consequence is postprandial hyperglycemia which subsequently causes T2D (Gastaldelli, 2011). This postprandial hyperglycemia must be controlled in order to prevent diabetic complications (Rahati et al. 2014). One therapeutic approach to reducing postprandial hyperglycemia in T2D is delaying glucose absorption via inhibition of carbohydrate-digesting enzymes, such as α-glucosidase and α-amylase located at the intestinal brush border (Holman et al. 1999). Acarbose and miglitol are the major available glucosidase inhibitors currently used in treatment of T2D. However these drugs have side-effects major drawback with the use of the existing drugs has been the adverse consequences such as diarrhea, bloating, flatulence, cramping and abdominal pain (Fujisawa et al. 2005). Thus anti-diabetic leads with similar or better activity are sought after with fewer side effects (Hung et al. 2012). The use of plant based approaches is emerging as alternatives to these nasty drugs and is currently receiving much attention. Plant-derived natural products are becoming popular as alternatives to synthetic drugs as they are considered to be safer with fewer side-effects (Taveira et al. 2010).

*Aframomum melegueta* K. Schum. (Zingiberaceae) commonly known as guinea or alligator pepper is abundantly found in the central and western part of Africa (Iwu, 2014). The fruit, leaf or seeds are consumed as flavoring agents in various traditional foods and are used locally to treat diseases including diabetes, microbial infections, diarrhea, abdominal pain, snakebite and wound healing in many parts of Africa (Iwu, 2014; Soladoye et al. 2012; Gbolade, 2009; Akendengue and Louis, 1994; Kokwaro, 1993). Previous studies have shown that *A. melegueta* possessed anti-microbial (Nneka and Jude, 2013; Doherty et al. 2010), hepato-protective (Nwozo and Oyinloye, 2011), anti-inflammatory (Ilic et al. 2014; Umukoro and Ashorobi, 2008), anti-oxidative and potent inhibitory effects against α-glucosidase activities (Mohammed et al. 2015; Onoja et al. 2014; Adefegha and Oboh, 2012a; Kazeem et al. 2012; Adefegha and Oboh, 2011; Etoundi et al. 2010). In a recent study, the diterpenoids isolated from the organic seed extract of *A. melegueta* showed bactericidal activity in vitro against *Escherichia coli*, *Listeria moncyogenes* and...
**Staphylococcus aureus** strains (Ngwoke et al. 2014). In another study, paradol (1), shogaol (2) and gingerol (3) isolated from the seed have showed anti-inflammatory activity in a rat paw edema model (Ilic et al. 2014). El-Halawany et al. (2014) have also reported that diarylheptanoid, 8-dehydrogingerdione, 6-dehydroparadol, dihydrogingerol, 6-gingerol, dihydroparadol, paradol and 6-shogaol demonstrated hepato-protective and anti-oxidative actions in CCl$_4$ induced acute liver injury in rats.

*Xylopia aethiopica* (Dunal) A. Rich. (Annonaceae) also known as Ethiopian pepper is an indigenous spice widely distributed in almost all parts of Africa. The fruit is popularly used as a condiment in many local dishes by different traditions in Africa and Asia (Kingsley, 2012). In addition, *X. aethiopica* fruit is widely used locally in the treatment of various ailments and as an excipient to many other medicines (Freiesleben et al. 2015). In Nigerian, Guinean, Togolese and Senegalese traditional medicines, the fruit decoction is widely used in the treatment of diabetes (Soladoye et al. 2012; Diallo et al. 2012; Karou et al. 2011; Diéye et al. 2008). Moreover, previous studies have shown that various extracts from *X. aethiopica* fruit possessed anti-cancer (Kuete et al. 2013; 2011; Choumessi et al. 2012), anti-fertility (Nwangwa, 2012), anti-sickling (Uwakwe and Nwaoguikpe, 2008), anti-microbial (Eskehia and et al. 2009), anti-oxidative actions and inhibits the activities of carbohydrate hydrolyzing enzymes in vitro (Adefegha and Oboh, 2012a,c; 2011; Etoundi et al. 2010; Odukoya et al. 2005; George and Osiama, 2011). Previously, diterpene kaurenoids isolated from *X. aethiopica* fruit exhibited hypotensive and diuretic effects in rats (Somova et al. 2001).

*Capsicum annuum* L. (Solanaceae) also known as red pepper is a spice that is readily available in almost all parts of the world. The fruit is widely consumed in vast quantities as a flavoring agent in various traditional foods (Khan et al. 2014). The fruit is locally used as a blood tonic, stimulant and in the treatment of nerve weakness or pain due to diabetes in some parts of Africa and Asia (Iwu 2014; Jayakumar et al. 2010). Studies reported earlier indicated that *C. annuum* fruit extracts exhibited potent anti-microbial (Santos et al. 2012; Careaga et al. 2003), anti-diabetic (Monsereenison, 1980), anti-nociceptive and anti-inflammatory (Hernández-Ortega et al. 2012), anti-oxidative and inhibitory effects against α-glucosidase activities (Tundis et al. 2011; 2013; Kwon et al. 2007; Materska and Perucka, 2005; Reddy and Lokesh, 1992). In addition, consumption of the whole fruit extract or capsaicin was reported to attenuate dyslipidemia, improve satiety and have an impact on the thermogenic effect in hyperlipidemic animals (Kempaiah and Srinivasan 2006; Kawada et al. 1986; Sambaiah et al. 1978). Capsaicin and dihydrocapsaicin isolated from *C. annuum* fruit have demonstrated α-amylase and α-glucosidase inhibitory actions (Tundis et al. 2013). Moreover, the volatile oil derived from *C. annuum* fruit also exhibited excellent anti-parasitic effects against *Anopheles gambiae* (Dadji et al. 2011).

Our recent findings have shown that, *A. melegueta* ethyl acetate, *X. aethiopica* acetone and *C. annuum* acetone fractions from the fruit exhibited anti-hyperglycemic and anti-hyperlipidemic actions,
ameliorate β-cell dysfunction and other major diabetes-related parameters in a T2D model of rats (data not shown). However, the compounds responsible for the excellent anti-diabetic action of the above-mentioned fractions remain speculative.

This study is therefore aimed at carry out the bioassay-guided isolation of possible compounds responsible for the anti-diabetic action of the selected plants.

6.1.3 Materials and methods

For sample collection, identification and preparation of the extracts, including the detail experimental protocols, please refer to chapter 2, sub-sections 2.3 and 2.4.

6.1.4 Results

The phytochemical investigation of the fruits *A. melegueta*, *X. aethiopica* and *C. annuum* led to the isolation of compounds 1-4 and the mixture 5 (Figure 6.1) which were characterized by 1D (¹H and ¹³C) and 2D NMR and confirmed by comparison with data in the literature as 6-paradol (1) (Ma et al., 2004), 6-shogoal (2) (Lee et al., 2011), 6-gingerol (3) (Lee et al., 2011), oleanolic acid (4) (Mahato and Kundo, 1994). The mixture 5 consisted of a fatty acid and aromatic compounds; however the structures could not be determined due to the mixture of compounds. Compounds 1-4 were isolated from *A. melegueta*, 3 and 4 were also obtained from *X. aethiopica* and the mixture 5 from *C. annuum*.

![Figure 6.1: Structures of compounds 1-4 isolated from A. melegueta and X. aethiopica](image)

The data from the α-amylase inhibition of the compounds from the fruits of the three plants are presented in Figure 6.2 and Table 6.1 (IC₅₀ values). It was observed that 6-gingerol (3) isolated from *A.
*A. melegueta* and *X. aethiopica* exhibited the lowest IC₅₀ value (156.96±12.23 µM), significantly lower (*p*<0.05) compared to 1, 2 and the mixture 5 from *C. annuum* (Table 6.1). However, the IC₅₀ of oleanolic acid (4) (91.72 ± 1.63 µM) was significantly lower (*p*<0.05) than the 6-gingerol (3). The IC₅₀ values of compounds 2-4 were significantly lower (*p*<0.05) than the standard acarbose (580.77 ± 20.71 µM), when the IC₅₀ value (664.56 ± 11.58 µM) of 6-paradol (1) was higher compared to the acarbose (Table 6.1). In addition, the IC₅₀ value depicted by the mixture (5) was found to be 124.95±6.38 µg/ml.

![Figure 6.2: α-amylase Inhibition (%) of compounds isolated from A. melegueta, X. aethiopica and C. annuum fruits. Data are presented as mean ± SD of triplicate determinations. a-e Values with different letters presented for a given concentration for each extract are significantly different from each other.](image)

**Table 6.1:** IC₅₀ values of bioactive compounds isolated from the *A. melegueta* and *X. aethiopica* fruit in anti-diabetic models.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>IC₅₀ (µM)</th>
<th>α-amylase inhibition</th>
<th>α-glucosidase inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. melegueta</em>/<em>X. aethiopica</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6-Paradol (1)</td>
<td>664.56 ± 11.58&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
<td>243.32 ± 6.65&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>6-Shagaol (2)</td>
<td>443.17 ± 1.38&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
<td>326.11 ± 5.60&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>6-Gingerol (3)</td>
<td>156.96 ± 12.23&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td>104.34 ± 3.99&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Oleanolic acid (4)</td>
<td>91.72 ± 1.63&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td>47.10 ± 0.42&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Acarbose</td>
<td>580.77 ± 20.71&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
<td>327.74 ± 9.88&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SD values of triplicate determinations. a-d Different superscript letters presented within a column for a given parameter are significantly different from each other (Tukey’s-HSD multiple range post hoc test, *p* < 0.05).
The results of the α-glucosidase inhibition of the compounds from the fruits of *A. melegueta*, *X. aethiopica* and *C. annuum* are presented in Figure 6.3 and Table 6.1 (IC$_{50}$ values). 6-Gingerol (3) and oleanolic acid (4) showed the lowest calculated IC$_{50}$ values; 104.34 ± 3.99 µM for 3 and 47.10 ± 0.42 µM for 4, significantly lower ($p<0.05$) compared to 1, 2 and the mixture 5 as well as the standard, acarbose (Table 6.1). Following these two compounds (3 and 4), 6-paradol (1) (243.32 ± 6.65 µM) had a lower IC$_{50}$ than 6-shogaol (2) (326.11 ± 5.60 µM), and the acarbose (327.74 ± 9.88 µM) was the least active (Table 6.1). It was also observed that the mixture (5) showed the IC$_{50}$ value of 51.03 ± 2.73 µg/ml for the α-glucosidase inhibition.

![Figure 6.3: α-glucosidase Inhibition (%) of compounds isolated from *A. melegueta*, *X. aethiopica* and *C. annuum* fruits. Data are presented as mean ± SD of triplicate determinations. a-e Values with different letters presented for a given concentration for each extract are significantly different from each other.](image)

The kinetics data of the inhibitory mode of the α-amylase and α-glucosidase enzymes by the isolated compounds are presented in Figure 6.4, 6.5 and Table 6.2. Compounds 1, 3 and the mixture 5 exhibited a non-competitive inhibition for α-amylase. The $K_M$ value remains unchanged (0.06%), and the $V_{max}$ was found to decrease from 35.67 µmol/min to 16.73 µmol/min (3), 24.10 µmol/min (1) and 15.60 µmol/min (5). Oleanolic acid (4) and compound (2) showed a mixed inhibition type for α-amylase action were both the $K_M$ and $V_{max}$ values are altered (Figure 6.4 and Table 6.2). For the α-glucosidase action all the isolated bioactive compounds from *A. melegueta*, *X. aethiopica* and *C. annuum* demonstrated a non-competitive inhibition mode. The $K_M$ value remains unchanged (1.10 mmol/L), and $V_{max}$ was found to decreases from 471.56 µmol/min to 123.10 µmol/min (3), 55.70 µmol/min (2), 81.20 µmol/min (1), 64.20 µmol/min (4) and 88.50 µmol/min (5) (Figure 6.5 and Table 6.2). In addition, the equilibrium constant for
inhibitor binding ($K_i$) of the compounds for $\alpha$-glucosidase were lower compared to that of $\alpha$-amylase (Table 6.2).

**Table 6.2:** Kinetic analysis of $\alpha$-amylase and $\alpha$-glucosidase inhibition by compounds isolated from the fruit of *A. melegueta, C. annuum* and *X. aethiopica* in anti-diabetic models.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>$\alpha$-Amylase inhibition</th>
<th>$\alpha$-Glucosidase inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_M$ (µmol/min)</td>
<td>$V_{max}$ (µmol/min)</td>
</tr>
<tr>
<td>Control</td>
<td>0.06</td>
<td>35.65</td>
</tr>
<tr>
<td><em>A. melegueta/ X. aethiopica</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6-Paradol (1)</td>
<td>0.06</td>
<td>15.60</td>
</tr>
<tr>
<td>6-Shagaol (2)</td>
<td>0.09</td>
<td>24.10</td>
</tr>
<tr>
<td>6-Gingerol (3)</td>
<td>0.06</td>
<td>16.73</td>
</tr>
<tr>
<td>Oleanolic acid (4)</td>
<td>0.13</td>
<td>273.00</td>
</tr>
<tr>
<td><em>C. annuum</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Compound (5)</td>
<td>0.06</td>
<td>20.40</td>
</tr>
</tbody>
</table>
Figure 6.4. Lineweaver-Burke plot for α-amylase in the absence and presence of the inhibitors (bioactive compounds).
6.1.5 Discussion

In our present study, three of the isolated compounds (1-3) from *A. melegueta* are arylalkanes common to the Zingeberaceae. These arylalkanes all have a common 4-hydroxy-3-methoxyphenyl moiety and differ in the side chain due to the biosynthetic conversion of 6-paradol (1) to 6-gingerol (3) by hydroxylation and dehydration to 6-shogoal (2) (Figure 6.1). These compounds were previously isolated from *A. melegueta* seed and reported to possess a variety of interesting pharmacological properties including hepato-protective and anti-oxidant activity (El-Halawany et al. 2014), as well as insecticidal (Escoubas et al. 1995) and anti-inflammatory activity (Illic et al. 2014; Dugasani et al. 2010; Young et al. 2005). 6-Gingerol (3) was isolated from *X. aethiopica* for the first time and establishes a chemotaxonomic link between the two plants, *A. melegueta* and *X. aethiopica*. Oleanolic acid (4), a common phytocompound was isolated from *X. aethiopica* fruit previously (Bernhoft, 2008). Previous reports on the phytochemistry of *A. melegueta* and *X. aethiopica* indicated these plants to contain monoterpenoids, sesquiterpenoids and
diterpenoids (Moreira et al. 2013; Tane et al. 2005). The isolation of oleanolic acid (4) a pentacyclic triterpene from *A. melegueta* fruit is not surprising since it is found quite commonly in the plant kingdom. Oleanolic acid is quite active pharmacologically, having anti-oxidant, anti-inflammatory and anti-diabetic activity (Pollier and Goossens, 2012).

The preferred method for screening anti-diabetic agents used recently involves the inhibition of carbohydrate hydrolyzing enzymes such as α-amylase and α-glucosidase with the aim of controlling postprandial hyperglycemia. Inhibitors of these enzymes decrease the breakdown of complex carbohydrates to absorbable monosaccharides such as glucose (Imam, 2015). The isolated arylalkanes have not been tested previously on α-amylase and α-glucosidase, however previous reports have shown that compound 6-shogoal (2) and 6-gingerol (3) might play a crucial role in the treatment and control of T2D. Singh et al. (2009) reported that treatment of 6-gingerol (3) (100 mg/kg bw) to db/db mice for 12 days resulted in anti-hyperglycemic, anti-hyperlipidemic and anti-oxidant action. In another study, 6-gingerol (3) (10 µM) was shown to attenuate β-amyloid-induced oxidative cell death in SH-SY5Y cell lines (Lee et al. 2011). Chakraborty et al. (2012) have shown that, 6-gingerol (3) (50 and 75 mg/kg bw) down-regulate the over-expression of TNF-α and IL6 in sodium arsenate intoxicated mice via up-regulation of GLUT4, IRS-1, IRS-2, PI3K, AKT, PPARα signaling molecules. 6-Shogoal (2) and 6-gingerol (3) (10 µM) were reported to inhibit the TNF-α mediated downregulation of adiponectin expression in 3T3-L1 adipocytes (Isa et al. 2008). Interestingly, our present data support the studies mentioned above and further show the contribution of 1-3 to T2D treatment as they exhibited considerable inhibitory action on α-amylase and α-glucosidase (*Table 6.1*). The lowest calculated IC_{50} values of (3) (α-amylase: 156.96 ± 12.23 µM; α-glucosidase: 104.34 ± 3.99 µM) indicates that this is the most suitable candidate for an anti-diabetic drug compared to the other isolated compounds.

Oleanolic acid (4) was shown to have an inhibitory effect on α-amylase and α-glucosidase. Ali et al. (2002) reported an IC_{50} value of 11.16 ± 0.49 µM for 4 on α-glucosidase. Most recently, an IC_{50} value of 3.8 ± 0.2 µM on α-glucosidase was repoted by Guo et al. (2013). Komak et al. (2003) reported inhibitory activity of 4 against α-amylase with an IC_{50} of 154.89 µM. The IC_{50} values of oleanolic acid obtained in this study was 59.22 ± 1.05 µg/mL for α-amylase and 91.72 ± 1.63 µM for α-glucosidase. These values were lower than the standard acarbose (α-amylase: 580.77 ± 20.71 µM; α-glucosidase: 327.74 ± 9.88 µM) and that reported by Komak et al. (2003) for α-amylase action (154.89 µM). These results support previous findings, which have shown the ability of oleanolic acid (4) to improve blood glucose tolerance in mice fed a high-fat diet as well as enhance insulin secretion in pancreatic β-cells (Castellano et al. 2013; de Melo et al. 2010; Teodoro et al. 2008).

The kinetic parameters derived from the double reciprocal plot revealed that the mode of α-amylase and α-glucosidase inhibitory activity exhibited by 1-5 is either non-competitive or of the mixed inhibition
type (Figure 6.4, 6.5 and Table 6.2). It is therefore suggested that these compounds bind to other site(s),
apart from the active site of the enzyme, which induces a conformational change in the three-dimensional
structure of the enzymes and ultimately slows down its activity (Mahomoodally and Muthoora, 2014). The
equilibrium constant for inhibitor binding ($K_i$) of the compounds were lower for $\alpha$-glucosidase than $\alpha$-
amylase, indicating greater stability of the enzyme-substrate complex for $\alpha$-glucosidase compared to $\alpha$-
amylase (Table 6.2).

Structure-activity relationship reported in the literature for the arylalkanes state that the 4-hydroxy-
3-methoxyphenyl moiety and C-3 carbonyl group of the aliphatic side chain are crucial for the activity of
these compounds (El-Halawany et al. 2014). It is also suggested that the presence of the $\beta$-OH at C-5 in 6-
 gingerol (3) or $\alpha,\beta$-unsaturated double bond in 6-shogoal (2) of the aliphatic side chain significantly
increases the $\alpha$-amylase and $\alpha$-glucosidase inhibitory activity. In oleanolic acid (4), the hydroxyl at C-3 and
the carboxyl group (C-28) are essential for its activity (Matsuda et al. 1998).

6.1.6 Conclusion

In conclusion, the results of the present study indicate that 6-gingerol (3) and oleanolic acid (4)
isolated from the fruit of $A$. melegueta and $X$. aethiopica demonstrate better $\alpha$-amylase and $\alpha$-glucosidase
inhibitory activity compared to the other isolated compounds and could be a good lead for an anti-diabetic
drug. The anti-diabetic activity of the fruit of $A$. melegueta and $X$. aethiopica is attributed to these active
ingredients. Further clinical and detailed toxicological studies are required to fully establish the potency of
the isolated bioactive compounds.
CHAPTER 7

7.0 General discussion, conclusion and recommendations

7.1 General discussion

Africa is among the regions endowed with the richest biodiversity in the world, with an abundance of many plants that could provide a new lead for treatment of several diseases including diabetes mellitus (DM). In our present study, three (3) African medicinal plants \[Aframomum melegueta\] K. Schum., \[Xylopia aethiopica\] (Dunal.) A. Rich. and \[Capsicum annuum\] L. were selected based on their traditional uses in the treatment of DM.

According to the literatures, several extraction methods of plant-derived products are available that include hot percolation, supercritical fluid extraction, soxhlet extraction and cold extraction among others (Brusotti et al. 2014; Harborne, 1998; Hostettmann et al. 1991). In our present study, the cold extraction technique was employed due to easeful extraction method and no heating is required. This allows most ingredients present in the plant samples to be extracted with low possibility of degradation (Heinrich et al. 2012). This is of enormous interest as the bioactivity of plant-derived extracts depends entirely on the presence of compounds such terpenoids, alkaloids and polyphenols to mention a few. Additionally, the amount of the bioactive compounds depends on the polarity of the extracting solvents as well.

In a number of previous studies, the most polar solvent extracts such as ethanolic (EtOH) and aqueous from the fruit of \[A. melegueta, X. aethiopica\] and \[C. annuum\] were reported to be rich in phenolic contents (Tundis et al. 2013; Adefegha and Oboh, 2012a, c; 2011; Etoundi et al. 2010; Deepa et al. 2007). This is in line with our present data (Tables 3.1, 4.1 and 5.1). In addition, the higher phenolic contents of the fruits than other parts (leaves, stems and roots) could be influenced by several factors including genetic and environmental factors (nature of the soil, high temperature and rain fall) (Mamphiswana et al. 2010; Pandey and Rizvi, 2009). Furthermore, it was observed from the results that higher phenolic content were available in the most polar solvent fractions (acetone and ethyl acetate (EtOAc)) after partitioning of the crude EtOH extracts (Tables 3.4, 4.4 and 5.4). This could be due to the greater polarity of these solvents than other less polar solvents (hexane and dichloromethane). While not discounting the contributions of other compounds, our interest in determining the phenolic contents was due to the fact that several therapeutic potentials of plant-derived products such as anti-oxidative action are attributed to their rich-phenolic contents (Gülçin, 2012; Ferrazzano et al. 2011). Therefore, phenolics may play a significant role in the amelioration of oxidative damages associated with many disease conditions such as T2D.
In the pathogenesis of T2D, increased generation of reactive oxygen species (ROS) due to hyperglycemia is considered as a major contributor for oxidative as well as associated complications (Pazdro and Burgess, 2010). A number of previous studies supported the contribution of anti-oxidants in attenuating the complications associated with diabetes (McCune and Johns, 2007; Rahimi et al. 2005; McCune and Johns, 2002). However, the increasing risk associated with the use of existing synthetic anti-oxidants has been an issue of concern. This cause a shift to the use of plant-derived anti-oxidants in preventing oxidative damage either in disease condition or food preservation. Moreover, several methods are used to assess the anti-oxidative potential of plant-derived products due to the diverse nature of the ROS. In our present study, DPPH radical scavenging activity, ferric (Fe³⁺) reducing anti-oxidant power and inhibition of hemoglobin glycation were used to evaluate the anti-oxidative action of the extracts and/or fractions derived from the plants under this study. The anti-oxidative effectiveness of various solvent extracts was assessed based on the calculated IC₅₀ values.

In some previous studies, various extracts from these plants demonstrated anti-oxidative activity. The *A. melegueta* seed acetone and aqueous extracts (Adefegha and Oboh, 2012a, b; Kazeem et al. 2012), *X. aethiopica* fruit aqueous extract (Adefegha and Oboh, 2012a, c; 2011; Odukoya et al. 2005) and *C. annuum* fruit aqueous, methanolic and EtOH extracts (Azeez et al. 2012; Chen et al. 2012; Tundis et al. 2012; Perucka and Materska, 2007; Conforti et al. 2007; Kwon et. 2007) were reported to demonstrate excellent anti-radical (IC₅₀: 100-400 µg/mL) as well as anti-glycation (IC₅₀ >100 µg/mL) activities *in vitro*. However, most of the above-mentioned studies did not evaluate the anti-oxidative activities of the extracts and fractions from the different parts of the plants which have been done in our study. The results of above-mentioned studies are also in line with the results of our present data (Tables 3.5, 4.5, and 5.5). Therefore, the data of our study suggest that most active extracts or fractions contained active ingredients with high redox potentials that could reasonably quench the activities of ROS and other radicals released due to the disease condition.

On the other hand, plant-derived products have been reported to demonstrate inhibitory effect against α-glucosidase and α-amylase activities (Xiao et al. 2013a,b; Elya et al. 2012; Rastija et al. 2012; Sudha et al. 2011). These are key enzymes involved in the metabolism of complex carbohydrates to absorbable monosaccharides. Inhibition of the activities of these enzymes limit the release of dietary monosaccharide (glucose) and subsequent absorption via the intestinal brush border, thereby controlling postprandial hyperglycemia in T2D. Hence, this strategy is recently employed as a treatment option of T2D. According to the previous data, *A. melegueta* seed aqueous extract (Adefegha and Oboh, 2012a, b), *X. aethiopica* fruit aqueous extract (Adefegha and Oboh, 2012a; Etoundi et al. 2010) and *C. annuum* fruit EtOH extract (Tundis et al. 2013; 2012; Kwon et. 2007) exhibited inhibitory effect against α-glucosidase (IC₅₀: 0.005-5 mg/mL) and α-amylase (IC₅₀: 0.05-3 mg/mL) actions, which agree with the data of our
present study (Tables 3.2, 4.2, and 5.2). Interestingly, the inhibitory action against α-glucosidase (IC\textsubscript{50}: 40-90 µg/mL) and α-amylase (IC\textsubscript{50}: 60-170 µg/mL) actions exhibited by the fractions in our present study were the least (Tables 3.5, 4.5, and 5.5) compared to the previously published studies (Tundis et al. 2013; 2012; Adefegha and Oboh, 2012a, b; Etoundi et al. 2010; Kwon et. 2007). This shows that fractionation of the crude extracts further increases the activities of the extracts.

Moreover, analysis of the mode of inhibition exhibited by the most active fractions revealed that EtAOc fraction from \textit{A. melegueta} and acetone fractions from \textit{X. aethiopica} and \textit{C. annuum} fruits demonstrated non-competitive or mixed inhibitions towards α-glucosidase and α-amylase actions (Figures 3.13, 4.12, 5.14). This again is in accordance with the mode of inhibition exhibited by several phenolic compounds reported previously (Rastija et al. 2012). In these types of inhibition the inhibitor binds to other site(s), apart from the active site of the enzyme, and causes some conformational changes in the three-dimensional structure of the enzyme that slows down the activity of the enzyme (Mahomoodally and Muthoora, 2014).

Based on the higher anti-oxidative and anti-diabetic effects of the most active extracts and fractions, they were subjected to GC-MS analysis. The objective of conducting GC-MS analysis was to identify the small organic secondary metabolites and possibly to find a link between these organic molecules and the \textit{in vitro} anti-diabetic and anti-oxidative actions of the extracts and fractions. In addition, the data revealed the type of compounds present in the fractions under this study. Most small molecular weight compounds detected were volatile and identified by their fragmentation pattern based on the data from the NIST library (Tables 3.2, 3.5, 4.2, 4.5, 5.2, and 5.5). Interestingly, 4-hydroxy-3-methoxyphenyl derivatives including some terpenoids were observed to be available from the GC-MS analysis of the most active fractions, which confirmed the type of bioactive compounds present in these fractions. Finally, the fractions with higher \textit{in vitro} anti-diabetic and anti-oxidative actions (\textit{A. melegueta} EtAOc fraction (AMEF) and \textit{X. aethiopica} acetone fractions (XAAF) and \textit{C. annuum} acetone fractions (CAAF) from fruits) were selected for the \textit{in vivo} studies to further confirm their potentials.

The \textit{in vivo} anti-diabetic and anti-oxidative actions were investigated in a fructose-fed streptozotocin (STZ)-induced T2D model of rats through 4 weeks oral intervention period. The high fructose feeding followed by low-dose STZ (40 mg/kg bw) injection was reported to induce the 2 major pathological features of T2D, insulin resistance and β-cell failure (Wilson and Islam, 2012). It is postulated that the fructose feeding stimulate lipolysis from the adipocytes, which promote the activity of HMG-CoA reductase and decrease lipoprotein lipase action (De Silva and Frayling, 2010; Hotamisligil, 2000). This leads to the elevation of TG and TC levels that induce insulin resistance. Furthermore, the selective affinity of STZ to the pancreatic β-cells leads to DNA alkylation that induces free radical formation, which target
the DNA sugar residue and result in DNA strand breakage (KunduSen et al. 2011). This leads to pancreatic β-cell damage as well as hyperglycemia in T2D.

It has been reported that polyphagia, polydipsia and weight loss are classical symptoms of T2D, possibly attributed to the increased energy expenditure, urinary excretion and catabolic processes in diabetic condition (ADA, 2015; Kamalakkannan and Prince, 2006). These effects were evidently observed in the diabetic group (DBC) compared to the normal control (NC) group. However, oral treatment of fractions at both dosages (150 and 300 mg/kg bw) significantly decreased these alterations which was comparable to diabetic metformin (DMF) treated group (Figures 3.15, 3.16, 4.14, 4.15, 5.16 and 5.17). This indicates a recovery from the diabetes-induced alterations in the feeding habit of the diabetic-treated animals, which was further supported by the significant decrease in hyperglycemia in diabetic treated groups.

Persistent hyperglycemia and lack of glucose tolerance ability are the major contributors of most of the complications associated with T2D (Monnier et al. 2006). In some preliminary studies, it has been reported that the treatment of aqueous extracts from A. melegueta seed (200-400 mg/kg bw/2 weeks), X. aethiopica fruit (400 mg/kg bw/2 hours) and C. annuum fruit (500-700 mg/kg bw/2 hours) demonstrated blood glucose lowering effects in alloxan-induced diabetic, high sucrose-fed and normal rats, respectively (Etoundi et al. 2013; Adesokan et al. 2010; Monsereenusorn, 1980). However, the major limitations with above-mentioned previous studies include short treatment periods, lack of proper control groups and the type of animal models used, which have been considered in our present study. Our data showed better anti-hyperglycemic action (Figures 3.17, 3.18 and 4.16) and glucose tolerance ability (Figures 5.18 and 5.19) in the diabetic treated groups compared to the previously reported studies (Etoundi et al. 2013; Adesokan et al. 2010; Monsereenusorn, 1980). Additionally, the better anti-hyperglycemic action of the fractions might be correlated to potent α-glucosidase and α-amylase inhibitory effects exhibited by these fractions that limit the absorption of dietary glucose in to the blood stream and thus, reduced hyperglycemia. Another assumption is that the active ingredients from the fractions might stimulate the regeneration of pancreatic β-cells in the diabetic animals due to their strong anti-oxidative nature. This speculation was supported by the increased serum insulin levels and improved pancreatic morphology and β-cell function in the diabetic treated animals.

In prolonged uncontrolled T2D, loss of pancreatic β-cell function and the insulin resistance lead to decrease in serum insulin level as well as damage to the pancreatic architecture (Bonner-Weir and O’Brien, 2008; Nattrass and Bailey, 1999). These effects were prominent in the DBC group than the NC group (Figures 3.19, 4.18 and 5.20; Tables 3.7, 4.7 and 5.7). The DBC group had a smaller islet and morphologically deformed β-cells with higher fatty infiltrations (Figures 3.19, 4.18 and 5.20). However, oral administration of the fractions especially at the higher dose (300 mg/kg bw/day) significantly decreased insulin resistance, improved β-cell function and serum insulin level to near normal and were comparable to
the DMF group. This further display the anti-diabetic action of the plants used in our present study that was not yet previously reported. Therefore, the ability of AMEF, XAAF and CAAF to increase serum insulin levels might facilitate the passage of glucose across the membrane into the target cells and thereby attenuate hyperglycemia in diabetic treated animals. Furthermore, these effects might also be linked to the excellent *in vitro* anti-oxidative actions of the fractions, which is again supported by the potent reduced serum fructosamine levels in the diabetic treated groups (Tables 3.7, 4.7 and 5.7).

On the other hand, long term pathological features of T2D may include elevation of hepatic glycogen content and increased liver weight (Paschos and Paletas, 2009). In our present study, these kind of alterations observed in the DBC group might be attributed to fructose feeding that triggers hepatic glycogenesis via stimulation of glucose-6-phosphatase activity and cause higher accumulation of glycogen in the liver despite low circulating serum insulin (Ciudad et al. 1988). However, AMEF and XAAF treatment to diabetic animals reverted these effects near to normal, when no significant alterations were observed in the treated non-diabetic animals (Tables 3.8, 4.8 and 5.8). The results were further supported by the excellent attenuation of dyslipidemia in the diabetic treated animals (Tables 3.9, 4.9 and 5.9).

Previous data have strongly correlated insulin resistance in the etiology of dyslipidemia in T2D. Decrease in circulating serum insulin lead to lipoprotein lipase inactivation thereby causing lipid abnormalities (Verma et al. 2013). The most frequently observed diabetes-induced lipid alterations include elevation of serum TG and TC levels with concomitant reduction of HDL-cholesterol level. Furthermore, according to some previous studies, aqueous extracts from *A. melegueta* seed, *X. aethiopica* and *C. annuum* fruits showed lipid lowering potentials ethanol-induced hepatotoxic, hypercholesterolemic and normal rats, respectively (Nwozo and Oyinloye, 2011; Nwozo et al. 2011; Monsereenusorn, 1983), with no data on any diabetic-induced animal models. However according to our present data, the potent anti-hyperlipidemic effect in the diabetic treated groups further contribute to the anti-diabetic action of AMEF, XAAF and CAAF in diabetic-induced animal model. In addition, the reduction was more pronounced in groups treated with the higher dose of the fractions and comparable to the DMF group (Tables 3.9, 4.9 and 5.9). Moreover, the excellent decrease of the coronary risk index (CRI) and atherogenic index (AI) further support the effects of the fractions in ameliorating dyslipidemia in type 2 diabetic animals (Tables 3.9, 4.9 and 5.9). Therefore, it is suggested that the elevation of serum insulin level observed in the diabetic treated groups might be directly activated lipoprotein lipase activity, which promoted lipolysis and thus attenuated dyslipidemia. Our data are again supported by the reduction of serum indices of liver, heart and kidney in the diabetic treated groups compared to the DBC group (Tables 3.10, 4.10 and 5.10).

It has been highlighted that in some disease conditions such as T2D, elevation of serum ALT, AST, ALP, creatinine, urea, LDH and CK-MB into blood circulation induces hepatic injuries, myocardial infarction and functional disturbance of hepatic and renal tissues (Abolfathi et al. 2012; Jaramillo-Juarez et
al. 2008). These effects were evidently observed in the DBC group of our study (Tables 3.10, 4.10 and 5.10). However, oral administration of the A.ME, XAAF and CAAF (300 mg/kg bw) significantly ameliorated the serum elevations of ALT, ALP, creatinine, urea, LDH and CK-MB indicating their possible hepato-protective, cardio-protective and reno-protective effects (Tables 3.10, 4.10 and 5.10). It was observed from our data that serum AST level was not affected by the diabetes. In comparison with ALT, AST is less specific for the detection of hepatic injury (Center, 2007). This is partly due to the fact that the half-life of ALT is much longer compared to AST in experimental animals (Ramaiah, 2007; Meyer and Harvey, 2004). Therefore, in a condition of acute liver injury, there will be an increase in both serum ALT and AST levels. However, the serum AST level may likely return to normal more rapidly compared to the level of serum ALT, which makes ALT more sensitive in assessing hepatic damage in disease conditions (Ramaiah, 2007; Meyer and Harvey, 2004). The excellent hepato-protective, cardio-protective and reno-protective effects observed might be also attributed to the potent anti-oxidative action in vitro as exhibited by the fractions.

Moreover, in vivo anti-oxidative action of the various fractions was extended to further support the in vitro anti-oxidative effects. Our present data are the first to comprehensively reveal the anti-oxidative action of the extracts or fractions from A. melegueta seed, X. aethiopica and C. annuum in vivo. It has been highlighted that chronic uncontrolled hyperglycemia leads to increased production of ROS as well as declined of in vivo anti-oxidant defense system referred as oxidative stress. This results to increase lipid peroxidation that impaired cellular function by inducing osmotic stress and thereby alters the activity of membrane-bound enzymes and receptors (Verma et al. 2013). The observed elevation of lipid peroxidation (expressed as MDA equivalent) in DBC group were attenuated in the diabetic treated groups in the serum and organs (liver, kidney, heart and pancreas) in dose-depended manner (Figure 3.20, 4.19 and 5.21), attributed to the potent anti-oxidative action of the fractions in vitro. The data support the improved pancreatic β-cell morphology in the diabetic treated animals (Figures 3.19 4.18 and 5.20) that again correlated to the increase in both enzymatic and non-enzymatic in vivo anti-oxidants defense system in the diabetic treated groups.

The α-tocopherol, ascorbic acid, bilirubin and reduced glutathione (GSH) are considered as non-enzymatic in vivo anti-oxidant molecules. The GSH plays a crucial role in modulating the oxidative damages induced due to increased lipid peroxidation (Rahimi et al. 2005). According to our present data, the marked reduction of GSH levels in serum and organs of the DBC group were augmented in the diabetic treated groups (Figure 3.21, 4.20 and 5.22). Therefore, it is suggested that A.MEF, XAAF and CAAF improve the anti-oxidant activity by stimulating the biosynthesis of GSH that prevented and terminated the lipid peroxidation in the treated diabetic animals.
The levels of the enzymatic anti-oxidants, superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR) of the DBC group were observed to decrease in the serum and the organs as well. This might be due to the hyperglycemia-induced oxidative stress in the diabetic condition as reported earlier (Verma et al. 2013; Sreelatha and Inbavalli, 2012; Gokce and Haznedaroglu, 2008). However, the increased activities of these enzymes in the diabetic treated groups indicated the possible contribution of the constituents of the fractions in attenuating the deleterious action of the lipid peroxidation and consequently reduced the MDA levels (Figure 3.22-3.25, 4.21-4.24 and 5.23-5.26). Therefore, as suggested earlier, the potent anti-diabetic and anti-oxidative activities observed in both in vitro and in vivo conditions in our present study are attributed to the bioactive compounds present in the AMEF, XAAF and CAAF.

In our present study, three of the isolated compounds (1-3) from A. melegueta are arylalkanes common to the Zingeberaceae. These arylalkanes all have a common 4-hydroxy-3-methoxyphenyl moiety and differ in the side chain due to the biosynthetic conversion of 6-paradol (1) to 6-gingerol (3) by hydroxylation and dehydration to 6-shogoal (2) (Figure 6.1). These compounds were previously isolated from A. melegueta seed and reported to possess a variety of interesting pharmacological properties including hepato-protective and anti-oxidant activity (El-Halawany et al. 2014), as well as insecticidal (Escoubas et al. 1995) and anti-inflammatory activity (Ilic et al. 2014; Dugasani et al. 2010; Young et al. 2005). 6-Gingerol (3) and oleanolic acid (4) were isolated from X. aethiopica for the first time and establishes a chemotaxonomic link between the two plants, A. melegueta and X. aethiopica (Bernhoft, 2008). Previous reports on the phytochemistry of A. melegueta and X. aethiopica indicated these plants to contain monoterpenoids, sesquiterpenoids and diterpenoids (Moreira et al. 2013; Tane et al. 2005). The isolation of oleanolic acid (4) a pentacyclic triterpene from A. melegueta fruit is not surprising since it is found quite commonly in the plant kingdom. Oleanolic acid is quite active pharmacologically, having anti-oxidant, anti-inflammatory and anti-diabetic activity (Pollier and Goossens, 2012).

The isolated arylalkanes have not been tested previously on α-amylase and α-glucosidase, which have been done in our present study. However previous reports have shown that compound 6-shogoal (2) and 6-gingerol (3) might play a crucial role in the treatment and control of T2D (Chakraborty et al. 2012; Lee et al. 2011; Singh et al. 2009; Isa et al. 2008). Interestingly, our present data support the studies mentioned above and further show the contribution of 1-3 to T2D treatment as they exhibited considerable inhibitory action on α-amylase and α-glucosidase (Table 6.1). The lowest calculated IC\textsubscript{50} values of (3) (α-amylase: 156.96 ± 12.23 μM; α-glucosidase: 104.34 ± 3.99 μM) indicates that this is the most suitable candidate for an anti-diabetic drug compared to the other isolated compounds.

According to some previous studies, oleanolic acid (4) was shown to have an inhibitory effect on α-amylase and α-glucosidase (Guo et al. 2013; Komak et al. 2003; Ali et al. 2002). The IC\textsubscript{50} values of
oleanolic acid obtained in our present study for \( \alpha \)-amylase (91.72 ± 1.63 \( \mu \)M) and \( \alpha \)-glucosidase (47.10 ± 0.42 \( \mu \)M) were significantly lower compared to the standard acarbose (\( \alpha \)-amylase: 580.77 ± 20.71 \( \mu \)M; \( \alpha \)-glucosidase: 327.74 ± 9.88 \( \mu \)M). The results support previous findings, which have shown the ability of oleanolic acid (4) to improve blood glucose tolerance in mice fed a high-fat diet as well as enhance insulin secretion in pancreatic \( \beta \)-cells (Castellano et al. 2013; de Melo et al. 2010; Teodoro et al. 2008).

The kinetic parameters derived from the double reciprocal plot revealed that the mode of \( \alpha \)-amylase and \( \alpha \)-glucosidase inhibitory activity exhibited by 1-5 is either non-competitive or of the mixed inhibition type (Figure 6.4, 6.5 and Table 6.2). It is therefore suggested that these compounds bind to other site(s), apart from the active site of the enzyme, which induces a conformational change in the three-dimensional structure of the enzymes and ultimately slows down its activity (Mahomoodally and Muthoora, 2014). The equilibrium constant for inhibitor binding (\( K_i \)) of the compounds were lower for \( \alpha \)-glucosidase than \( \alpha \)-amylase, indicating greater stability of the enzyme-substrate complex for \( \alpha \)-glucosidase compared to \( \alpha \)-amylase (Table 6.2).

Structure-activity relationship reported in the literature for the arylalkanes state that the 4-hydroxy-3-methoxyphenyl moiety and C-3 carbonyl group of the aliphatic side chain are crucial for the activity of these compounds (El-Halawany et al. 2014). It is also suggested that the presence of the \( \beta \)-OH at C-5 in 6-gingerol (3) or \( \alpha \), \( \beta \)-unsaturated double bond in 6-shogoal (2) of the aliphatic side chain significantly increases the \( \alpha \)-amylase and \( \alpha \)-glucosidase inhibitory activity. In oleanolic acid (4), the hydroxyl at C-3 and the carboxyl group (C-28) are essential for its activity (Matsuda et al. 1998).

### 7.2 Overall conclusion

In conclusion, the results of our present study have shown that *A. melegueta* ethyl acetate fraction (AMEF), *X. aethiopica* acetone fraction (XAAF) and *C. annuum* acetone fraction (CAAF) from fruits demonstrated potent anti-diabetic and anti-oxidative actions *in vitro* and *in vivo*. The improved glucose tolerance ability, insulin sensitivity and *in vivo* anti-oxidants status as well as amelioration of pancreatic \( \beta \)-cell histology and \( \beta \)-cell functions are mechanisms via which AMEF, XAAF and CAAF exhibited anti-diabetic action that were not previously reported. Hence, our findings suggest that AMEF, XAAF and CAAF may provide an excellent anti-diabetic remedy with no considerable side effects. Moreover, our present study establish that 6-paradol (1), 6-shagaol (2), 6-gingerol (3) and oleanolic acid (4) isolated and purified from AMEF and XAAF are potential anti-diabetic drugs and the anti-diabetic action of *A. melegueta* and *X. aethiopica* fruits is attributed to the presence of these compounds. This study also confirmed the use of these plants as natural anti-diabetic medicine by African traditional healers.
7.3 Further research

On the basis of the findings of this study, it is recommended that:

i. Detail toxicological studies of the most active fractions (AMEF, XAAF and CAAF) and the isolated bioactive compounds (6-gingerol, 6-shagaol, 6-paradol and oleanolic acid) are required to further validate their safety.

ii. Mechanistic-based *in vitro* studies are required to evaluate the mode of action of the active fractions and the isolated compounds as well.

iii. Clinical studies are necessary to further confirm the effects in human subjects.
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Appendix I

PUBLICATIONS
Anti-diabetic effect of *Xylopia aethiopica* (Dunal) A. Rich. (Annonaceae) fruit acetone fraction in a type 2 diabetes model of rats

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**Chemical compounds studied in this article:**
Absolute ethanol (CD 702)
Citric acid (CD 311)
Dichloromethane (CD 6344)
Ethyl acetate (CD 8057)
Fumaric acid (CD 5084)
Glucose (CD 5783)
Hexane (CD 8058)
Methanol (CD 1891)
Methanol (CD 1897)
Sodium citrate (CD 6224)
Streptozotocin (CD 28327)

**Abstract**

**Ethnopharmacological relevance:** In traditional medicine from West Africa, the fruit decoction of *Xylopia aethiopica* (Dunal) A. Rich. is widely used for the treatment of diabetes mellitus (DM) either alone or in combination with other plants. The present study is designed to investigate the anti-diabetic effects of *X. aethiopica* acetone fraction (XAAF) from fruit ethanolic extract in a type 2 diabetes (T2D) model of rats. Materials and methods: T2D was induced in rats by feeding a 15% fructose solution ad libitum for 2 weeks followed by a single intraperitoneal injection of streptozotocin (40 mg/kg body weight) and the animals were orally treated with 150 or 300 mg/kg body weight (bw) of the XAAF once daily for four weeks. Results: After 4 weeks study period, diabetic untreated controls (DRC) exhibited significantly higher serum glucose, serum fructoseamine, LDH, CK-MB, serum lipids, liver glycogen, insulin resistance (HOMA-IR), AI, CRI and lower serum insulin, β-cell function (HOMA-β) and glucose tolerance ability compared to the normal animals. Histopathological examination of their pancreas revealed a pathological change in the islets and β-cells. These alterations were reverted to near-normal after the treatment of XAAF at 150 (OXAA) and 300 (OXAAX) mg/kg bw with the effects being more pronounced in the OXAH group compared to the OXAL group. Moreover, the effects in the animals of OXAH group were comparable to the diabetic metformin (DMF) treated animals. In addition, no significant alterations were observed in non-diabetic animals treated with 300 mg/kg bw of XAAF (NXAH).

**Conclusion:** The results of our study suggest that XAAF treatment showed excellent anti-diabetic effects in a T2D model of rats.

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1. **Introduction**

The progressive increase in the prevalence of diabetes mellitus (DM) constitutes a global public health problem. In a recent report by the International Diabetes Federation (IDF), it has been estimated that DM affects more than 378 million people worldwide, with this figure most likely to be doubled by 2035 (IDF, 2014). Type 2 diabetes (T2D), the most prevalent form of diabetes, is a heterogeneous disorder characterized by insulin resistance and partially dysfunctional pancreatic β-cell to properly secrete insulin in response to hyperglycemia (Hui et al., 2007). Despite the currently available conventional drugs (sulfonylureas, biguanides, α-glucosidase inhibitors, thiazolidinediones and dipeptidyl peptidase-4 (DPP-4) inhibitors), the treatment of T2D has been difficult. This is due to the unwanted side effects associated with the use of the conventional drugs, which include hypoglycemia, weight gain, hypoglycemia, gastrointestinal discomfort, nausea, liver and heart failure, and diarrhea (Hung et al., 2012). Therefore, in order to curb these problems, the search for anti-diabetic agents has been extended to plant-derived products, since fewer side
effects have been reported with the use of plants in the treatment of several diseases (Singh, 2001; Cakab, 2000).

*Xylopia aethiopica* (Dunal) A. Rich. (Annonaceae) also known as Ethiopian pepper is an indigenous spice widely distributed in almost all parts of Africa. It is an aromatic, evergreen plant, 20 m height, fruits in carpels forming a dense cluster, twisted bean-like pods, dark brown, cylindrical, 1.5-6 cm long and 4-7 mm thick (Kwu, 2014; Orwa et al., 2009). The fruit is popularly used as a condiment in many local dishes by different traditions in Africa and Asia (Kingley, 2012). In addition, *X. aethiopica* fruit is widely used locally in the treatment of various ailments and also as an excipient to many other medicines (Freiseksb et al., 2015). In Nigerian, Guinean, Togolese and Senegalese traditional medicines, the fruit decoction is widely used in the treatment of diabetes (Soladoye et al., 2012; Diallo et al., 2012; Karou et al., 2011; Diéye et al., 2008). Additionally, there are some anti-diabetic recipes such as Osun diabetes, in Nigeria and Togo that are used for the treatment of diabetes containing *X. aethiopica* fruit as one of the ingredients of the poly-herbal drug (Soladoye et al., 2012; Karou et al., 2011; Ogbuosa et al., 2006). Moreover, previous studies have shown that various extracts from *X. aethiopica* fruit possessed anti-cancer (Kute et al., 2013, 2011; Choumelli et al., 2012), anti-fertility (Nwawara, 2012), anti-sickling (Uwakwe and Nwaguike, 2008), anti-microbial (Esichagbe et al., 2008), anti-oxidative actions and inhibits the activities of carbohydrate hydrolyzing enzymes in vitro (Adefegha and Omoj, 2012a, 2011; Etoundi et al., 2010; Obolgeta et al., 2005; George and Osiloma, 2011).

Furthermore, the toxicological studies so far conducted have indicated that oral administration of *X. aethiopica* fruit aqueous extract for 2 weeks was found not to pose any side effects in rats (Johnkennedy et al., 2011; Taiwo et al., 2009). Xylopia acid (15β-acetoxyp-)-kaure-16-en-19-oic acid), kaurenic, 15-oxo-kaurenic acid, and kauran-16-ol are some of the compounds isolated from *X. aethiopica* fruit (Kwu, 2014; Ekong and Ogan, 1968). Moreover, *X. aethiopica* seed extract was reported to exhibit hypolipidemic and anti-oxidant actions in hypercholesterolemic rats (Nwonz et al., 2011). In a more recent study, Etoundi et al. (2013) have indicated that *X. aethiopica* fruit ethanolic extract improved glucose tolerance ability in high-surplus containing diet-fed animals, when the dose anti-diabetic action of either extract or fraction from *X. aethiopica* fruit has yet been conducted either in humans or in experimental animals.

Therefore, the present study was designed to investigate the in vivo anti-diabetic effects of the fraction of the extract derived from the fruit ethanolic extract of *X. aethiopica* in a T2D model of rats.

2. Materials and methods

2.1. Plant material

The fruit sample of *X. aethiopica* was freshly collected during December, 2012 from Ibadan, Oyo State, Nigeria. The plant as well as fruit was identified and authenticated at the herbarium unit of the Biological Science Department, Ahmadu Bello University, Zaria, Nigeria by Mr. Umar Gallah and a voucher specimen number 1026 was deposited accordingly. The fruit sample was immediately washed and shade-dried for two weeks at 25 °C to constant weight. The dried fruit sample was ground to a fine powder and then stored in an airtight container to transport to the University of KwaZulu-Natal, Westville campus, Durban, South Africa for further investigation.

2.2. Extraction and fractionation of the sample

The extraction and subsequent fractionation of *X. aethiopica* fruit sample was carried out according to the method as reported previously (Ibrahim and Islam, 2014). Briefly, the fine powdered sample (3 kg) was separately defatted with 10 l hexane. The defatted sample was sequentially extracted with ethanol by soaking for 48 h and filtered through Whatmann filter paper (No. 1). The resultant extract was evaporated under vacuum, using a rotary evaporator (Buchi Rotavapor II, Buchi, Germany) at 40 °C under reduced pressure to obtain the crude ethanolic extract with a yield of 7.05%. Forty grams of the crude ethanolic extract of the fruit was dissolved in 500 ml of distilled water; methanol (9:1) and successively partitioned with hexane (2 x 500 ml), dichloromethane (2 x 500 ml), ethyl acetate (2 x 500 ml) and acetone (2 x 500 ml). The fractions were evaporated to dryness under vacuum at 40 °C whereas the remaining aqueous fraction was dried in a water bath at 50 °C. The dried fractions were transferred to micro tubes and stored at 4 °C until further analysis. The *X. aethiopica* acetone fraction (XAAF) was a dark brownish residue and demonstrated the highest in vitro α-glucosidase (*IC_{50} 86.23 ± 0.30 g/mL*) and amylose (*IC_{50} 155.41 ± 1.83 g/mL*) inhibitory effect amongst the fractions and thus selected for the in vivo study.

2.3. Gas chromatography-mass spectrometric (GC-MS) analysis of XAAF

The gas chromatography-mass spectrometric (GC-MS) analysis of XAAF was conducted by an Agilent technology 6890 GC coupled with an Agilent 5973 Mass Selective Detector and driven by Agilent Chemstation software. Compounds were identified by direct comparison of the retention times and mass spectral data with those in the National Institute of Standards and Technology (NIST) library.

2.4. Experimental animals

Six-week-old male Sprague-Dawley (SD) rats were obtained from the Biomedical Resource Unit (BRU) located at the University of KwaZulu-Natal (Westville Campus), South Africa with initial mean bw 183.25 ± 13.67 g. Animals were housed as 2-3 in one medium size poly-carbonated cage in a temperature and humidity controlled room with a 12 h light-dark cycle. A standard rat pellet diet was supplied ad libitum to all animals during the entire experimental period. Animals were maintained according to the rules and regulations of the Experimental Animal Research Ethics Committee of the University of KwaZulu-Natal, South Africa (Ethical approval number: 018/14/Animal).

2.5. Animal grouping

Animals were randomly divided into six groups namely: NC: Normal Control (n=5), DRC: Diabetic Control (n=8), DXAL: Diabetic + low dose (150 mg/kg bw) of XAAF (n=8), DXAH: Diabetic + high dose (300 mg/kg bw) of XAAF (n=8), DMF: Diabetic + metformin (300 mg/kg bw) (n=8), NXAH: Non-diabetic + high dose (300 mg/kg bw) of XAAF (n=5) according to the equal mean body weight in each group. The animals were allowed to acclimatize for one week before starting the experiment.

2.6. Induction of type 2 diabetes

The insulin resistance and partial pancreatic beta-cell dysfunction are two major pathogenesis of T2D. During the first two weeks of the experiment, the animals in the DRC, DXAL, DXAH and DMF groups were supplied with a 10% fructose solution for
induction of insulin resistance and the animals in the NC and NAXH groups were supplied with normal drinking water. After this period, a low dose of STZ (40 mg/kg bw) dissolved in citrate buffer (pH 4.5) was intraperitoneally injected to the animals in the DBC, DXAL, DXAH and DMF groups to induce partial pancreatic β-cell dysfunction. The animals in the NC and NAXH groups were injected with a similar volume of vehicle buffer only (Wilson and Islam, 2012). One week after the STZ injection, the non-fasting blood glucose (NFBG) level of all animals was measured by using a portable glucometer (Glucoplus Inc., Saint-Laurent, Quebec, Canada) in the blood collected from the tail vein. Animals with a NFBG level $\geq$11.1 mmol/l were considered as diabetic (Davidson et al. 2011) while the animals with a NFBG level $<$11.1 mmol/l were considered as nondiabetic. The study was not diabetogenic from different groups (DXAL, DXAH, DMF) and one animal from the DBC group died during the intervention period due to the severity of diabetes. Hence, the final animal number in each group was as: NC (n=5), DBC (n=7), DXAL (n=7), DXAH (n=7), DMF (n=7) and NAXH (n=5).

2.7. Intervention period

After the confirmation of diabetes, a respective dose of the fraction was orally administered once daily for 4 weeks using a gastric gavage needle to the animals in DXAL, DXAH and NAXH groups while the animals in controls (NC and DBC) and DMF groups were treated with similar volume of the vehicle and metformin, respectively. Throughout the experimental period, food and fluid intake were measured every morning by subtracting the remaining amount of feed and fluid respectively from the amount given on the previous morning. Moreover, the weekly bw and NFBG levels were measured in all animal groups during the entire intervention period.

2.8. Oral glucose tolerance test (OGTT)

To measure the glucose tolerance ability of each animal, the OGTT was performed in the last week of the 4-week intervention period. To perform this test, a single dose of glucose solution (2 g/kg bw) was orally administered to each animal and the subsequent levels of blood glucose were measured at 0 (just before glucose ingestion), 30, 60, 90 and 120 min after the ingestion of glucose.

2.9. Collection of blood and organs

At the end of the experimental period, animals were euthanized by halothane anesthesia and blood and organ samples were collected. The whole blood of each animal was collected via cardiac puncture and immediately preserved in a refrigerator until further processing. The blood samples were centrifuged at 3000 rpm for 15 min and serum from each blood sample was separated and preserved at $-30^\circ$ C for further analysis. The liver was collected from each animal, washed with normal saline, wiped with filter paper, weighed and preserved at $-30^\circ$ C until subsequent analysis. A small piece of pancreatic tissue from each animal was cut and placed in a 10% neutral buffered formalin solution and preserved at room temperature for the histopathological study. The neutral buffered formalin of each pancreatic tissue sample was replaced weekly during the entire preservation period.

2.10. Analytical methods

The serum insulin concentration was measured by an enzyme-linked immunosorbent assay (ELISA) method using an ultra-sensitive rat insulin ELISA kit (Merckodia, Uppsala, Sweden) in a multiplate reader (MR-96A, Vacuette Pvt. Ltd., Durban, South Africa). The serum lipid profile, fructosamine, urea, uric acid, lactate dehydrogenase (LDH), creatine kinase (CK-MB) and creatine concentrations, as well as liver function enzymes, aspartate and alanine transaminases (AST and ALT) and alkaline phosphatase (ALP) were measured using an Automated Chemistry Analyzer (Lammax Plero, Labtest Co. Ltd., Iagoa Santa, Brazil) with commercial assay kits from the same company. Homeostatic model assessment (HOMA-IR and HOMA-%) scores were calculated at the end of the intervention period according to the following formula:

\[\text{HOMA-IR}=\left(\frac{\text{Fasting serum insulin in U/l} \times \text{Fasting blood glucose in mmol/l}}{22.5}\right)\]

\[\text{HOMA-IR}=\left(\frac{\text{Fasting serum insulin in U/l} \times \text{Fasting blood glucose in mmol/l}}{1.25}\right)\]

Conversion factor: insulin (1 U/l=7.174 pmol/l).

LDL-cholesterol was calculated according to Friedewald et al. (1972) equation as shown below:

\[\text{LDL-Cholesterol [mmol/l]}=\text{TC - HDL - (TG/5)}\]

Where, TG/5 is equivalent to the concentration of VLDL-cholesterol.

Atherogenic index (AI) was calculated according to the method described by Liu et al. (1999) and expressed as:

\[\text{AI}=\left(\frac{\text{Total cholesterol - HDL-cholesterol}}{\text{HDL-cholesterol}}\right)\]

Coronary artery risk index (CRI) was also calculated using the following formula (Boen et al., 2003):

\[\text{Coronary artery risk index (CRI)}=\left(\frac{\text{Total cholesterol [mmol/l]}}{\text{HDL-cholesterol [mmol/l]}}\right)\]

Liver glycogen concentrations were measured by phosol-sulfuric acid method as described by Lo et al. (1970).

2.11. Histopathological examination of pancreatic tissue

A standard laboratory protocol for paraffin embedding was used to treat the formalin preserved pancreatic tissues. Tissue sections were cut into a size of 4 mm before fixing on the slides. Then, the slides were deparaffinized in xylene and rehydrated in ethanol gradient (100%, 80%, 70% and 50%) and rinsed with water. Slides were stained in hematoxylin for 5 min and rinsed with water, and were counter stained in eosin, mounted in DPX, covered-slipped and viewed with a Leica slide scanner (SCN 4000, Leica Biosystems, Wetzlar, Germany).

2.12. Statistical analysis

All data are presented as the mean±SD of 5–7 animals. Data were analyzed by using a statistical software package (SPSS for Windows, version 22, IBM Corporation, NY, USA) using Tukey’s-HSD multiple range post-hoc test. Values were considered significantly different at p<0.05.

3. Results

The results of the phytochemical analysis using GC-MS analysis of the X. aethiopica acetone fraction (XAAF) are presented in Fig. 1 and Table 1. Several peaks were visible in the chromatogram and were identified by their fragmentation pattern in conjunction with the NIST library (Fig. 1). The available constituents were smaller volatile molecules that include basically terpenes [1-methyl-4-(1-methyl-ethyl)-1,4-cyclohexadiene; trans-2-nerialdehyde epoxide; terpinen-4-ol; cuminolic aldehyde; androst-5-ene-4-one; laraan-16-ol], benzene derivatives [1,2-dimethoxy-4-(2-propenyl) benzene; 2-methoxy-(1-propenyl)-phenol; butyl isobutyl phtalate; 4-methoxy-(2-propenyl)-1,3-benzodioxole; 1,2,3-trimethoxy-(2-propenyl) benzene; 2,6-dimethoxy-(2-propenyl)-phenol; isoelemicin; 4,4′-di hydroxy-7-methoxy-3-methyl-5-(1-propenyl)-2-benzofuranyll-2-methoxy-phenol; 1,2-benzenedi-carboxylic acid,
mono(2-ethylhexyl) ester and 1-(4-methoxyphenyl)-2-hydroxy-

The data of weekly body weight (bw) change of all the animals are presented in Fig. 2. It was observed from the result that before the STZ injection, fructose feeding did not affect the bw of the animals. However, after one week of STZ injection, the bw of the diabetic animals were significantly (p < 0.05) decreased compared to non-diabetic animals. Treatment of XAAF to diabetic animals for 4-weeks significantly (p < 0.05) increased the bw to near normal and comparable to metformin treated animals (Fig. 2). The effect was more pronounced in the DXAH group compared to the DXAL group when the observed increase in bw of the NXAH group did not differ significantly (p < 0.05) compared to the NC group (Fig. 2).

The results of the daily feed and fluid intake of all the animals are presented in Fig. 3. According to the data, the feed and fluid intake of the DXAH group were significantly (p < 0.05) increased compared to the NC group. However, oral administration of the XAAF to diabetic animals significantly decreased the feed and fluid intake compared to the DXC group, when the reduction of fluid intake of the DXAH group was significantly (p < 0.05) lower compared to the DXAL group (Fig. 3). Furthermore, the daily feed and fluid intake of NXAH group were not affected throughout the study period.

The weekly blood glucose levels (NFRG and FGR) of all the animals throughout the study period are presented in Fig. 4. It was observed that, one week after the confirmation of diabetes (week 0), the blood glucose level of the diabetic animals was significantly (p < 0.05) deviated compared to non-diabetic animals. But the treatment of XAAF for four weeks significantly (p < 0.05) reduced the elevated blood glucose compared to the DXC group and this reduction was comparable with the metformin-administered DMF group [Fig. 4]. Although not significant, better reduction was observed in the DXAH group compared to the DXAL group. It was also observed that, the NXAH group maintained the blood glucose level within the normal range throughout the study period.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Identified compounds from the acetone fraction from fruit ethanolic extract of X. arctos by GC-MS.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Compounds</strong></td>
<td><strong>Retention time (min)</strong></td>
</tr>
<tr>
<td>1-Methyl-4-(1-methylhexyl)-1,4-benzoxazaine</td>
<td>4.446</td>
</tr>
<tr>
<td>1,2-Dimethoxy-4-(2-propenyl) benzene</td>
<td>6.855</td>
</tr>
<tr>
<td>2-Methoxy-4-(1-propenyl) phenol</td>
<td>7.335</td>
</tr>
<tr>
<td>4-Methoxy-6-(2-propenyl)-1,3-benzodioxole</td>
<td>3.818</td>
</tr>
<tr>
<td>1,3,5-Trimethoxy-5-(2-propenyl) benzene</td>
<td>7.906</td>
</tr>
<tr>
<td>2,6-Dimethoxy-4-(2-propenyl) phenol</td>
<td>8.250</td>
</tr>
<tr>
<td>Iodobenem</td>
<td>8.585</td>
</tr>
<tr>
<td>Iodobenem</td>
<td>9.150</td>
</tr>
<tr>
<td>Tetraphen-4-ol</td>
<td>9.340</td>
</tr>
<tr>
<td>Guanidino acrylate</td>
<td>10.225</td>
</tr>
<tr>
<td>Iodobenem phosphate</td>
<td>10.262</td>
</tr>
<tr>
<td>Oleic acid</td>
<td>11.110</td>
</tr>
<tr>
<td>Andros-5-ene-4-one</td>
<td>12.347</td>
</tr>
<tr>
<td>Kazerin-16-gal</td>
<td>13.166</td>
</tr>
<tr>
<td>12-Oxinode-3,6,9-trimethyl-5-(1-propenyl)-2-benzoxazoline</td>
<td>14.671</td>
</tr>
<tr>
<td>12-Benzenoic acid, mono(2-ethylhexyl) ester</td>
<td>14.675, 14.819</td>
</tr>
<tr>
<td>N-(4-Methoxyphenyl)-2-hydroxyxminoacetamide</td>
<td>15.249</td>
</tr>
</tbody>
</table>
The results of the oral glucose tolerance test (OGTT) are presented in Fig. 5. It was observed that induction of the diabetes significantly \((p<0.05)\) affected the glucose tolerance of the DBC group compared to the NC group. On the other hand, significantly \((p<0.05)\) better glucose tolerance ability was observed in the DXAH and DXAL groups compared to the DBC group, when the effect was more pronounced in the DXAH group compared to the DXAL group, which is comparable to the DMF group (Fig. 5).

The results of serum insulin and fructosamine levels including the calculated HOMA-IR and HOMA-β scores are presented in Table 2. From the results, the serum insulin level and the calculated HOMA-β scores were decreased significantly \((p<0.05)\) whereas serum fructosamine as well as HOMA-IR scores were increased significantly \((p<0.05)\) in the DBC group compared to the NC group (Table 2). After 4-week treatment of XAAF, the serum insulin level was significantly \((p<0.05)\) increased and the serum fructosamine and HOMA-IR scores were significantly \((p<0.05)\) decreased in DXAH and DXAL groups compared to the DBC group (Table 2). The calculated HOMA-β scores of the diabetic treated animals was significantly \((p>0.05)\) decreased in the DXAH and DMF group compared to the DBC group when no significant difference was observed between the DXAH and DMF groups (Table 2). The above-mentioned parameters were not affected in the NXAH group throughout the test period (Table 2).

The histopathological slides of the pancreas for all the groups are presented in Fig. 6. The slides revealed that, there were fatty infiltrations, reduce number of pancreatic β-cells and smaller size of the pancreatic islet of the DBC group compared to the NC group. However, treatment with XAAF to diabetic animals reduced fatty infiltrations, improved the number of pancreatic β-cells and the pancreatic islet size in the DXAL and DXAH groups (Fig. 6). Additionally, the fatty infiltrations in the DXAH group were less compared to that of DXAL group. Furthermore, the pancreatic morphology was not affected by the higher dose of XAAF in the NXAH group (Fig. 6).

The results of absolute and relative liver weight including liver glycogen contents are presented in Table 3. According to the data, although the absolute liver weight was not affected by the induction of diabetes, the significantly \((p<0.05)\) increased relative liver weight and glycogen content was observed in the DBC group compared to the NC group. Oral administration of the XAAF significantly \((p<0.05)\) restored the relative liver weight and glycogen content in the DXAH and DXAL groups, which is comparable to the NC and DMF groups (Table 3). The liver weights and glycogen content of the NXAH group were not affected by the administration of XAAF throughout the experimental period.

In Table 4, the serum lipids profile and calculated atherogenic index \((AI)\) and coronary risk index \((CRI)\) are presented. The increased serum total cholesterol \((TC)\), triglycerides \((TG)\) and low density lipoprotein \((LDL)\) cholesterol as well as calculated \(AI\) and \(CRI\) along with the reduction of serum high density lipoprotein...
Table 2

<table>
<thead>
<tr>
<th>Group</th>
<th>NC</th>
<th>DBC</th>
<th>DXAL</th>
<th>DXAH</th>
<th>DMF</th>
<th>NXAH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin (μmol/l)</td>
<td>76.50 ± 6.26**</td>
<td>60.50 ± 9.30*</td>
<td>56.46 ± 6.01*</td>
<td>67.07 ± 10.00**</td>
<td>75.80 ± 12.25</td>
<td>75.00 ± 6.14*</td>
</tr>
<tr>
<td>Fructosamine (μmol/l)</td>
<td>234.20 ± 47.49*</td>
<td>304.43 ± 48.34**</td>
<td>301.00 ± 56.15*</td>
<td>288.00 ± 23.40**</td>
<td>203.14 ± 32.46**</td>
<td>225.00 ± 24.99**</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>2.50 ± 0.27**</td>
<td>6.30 ± 0.15*</td>
<td>3.43 ± 0.71*</td>
<td>3.17 ± 0.48**</td>
<td>3.82 ± 0.86*</td>
<td>2.33 ± 0.20**</td>
</tr>
<tr>
<td>HOMA-β</td>
<td>127.66 ± 43.74**</td>
<td>72.3 ± 12.69**</td>
<td>25.00 ± 7.13**</td>
<td>46.50 ± 12.00**</td>
<td>12.97 ± 20.76**</td>
<td>12.00 ± 32.44**</td>
</tr>
</tbody>
</table>

Data are presented as the mean ± SD of 6-7 animals. **Values with different superscript letters within a row for a given parameter are significantly different from each other group of animals and * significantly different from the DBC group (Tukey's LSD multiple range post hoc test; p < 0.05). NC, Normal Control; DBC, Diabetic Control; DXAL, Diabetic X. anthopica low dose; DXAH, Diabetic X. anthopica high dose; DMF, Diabetic Meftinmin; NXAH, Normal X. anthopica high dose.

(HDL) cholesterol were observed in the DBC group compared to the NC group. After 4-week of oral intervention with XAAF, the serum TC, TG, LDL-cholesterol, calculated AI and CRI were significantly (p < 0.05) decreased in the DXAL and DXAH groups compared to the DBC group in a dose-dependent manner (Table 4) when the serum HDL-cholesterol level of diabetic treated groups was not significantly (p < 0.05) different compared to the DBC group. Administration of the high dose of the fraction to non-diabetic animals did not affect these parameters in the NXAH group compared to the NC group (Table 4).

The results of serum ALT, AST, ALP, urea, creatinine, LDH and CK-MB are presented in Table 5. It was observed from the data that, the serum ALT, ALP, urea, creatinine, LDH and CK-MB levels were elevated in the DBC group compared to the NC group, when the serum ALP and uric acid levels were not affected by the induction of diabetes (Table 5). Treatment with XAAF to diabetic animals reduced serum ALT, ALP, urea, creatinine, LDH and CK-MB levels compared to the DBC group (Table 5). Moreover, treatment to non-diabetics with high dose of XAAF did not alter the serum levels of these parameters compared to the NC group.

4. Discussion

According to the GC-MS analysis of XAAF, the chemical constituents detected were mostly terpenes or smaller aromatic derivatives (Table 1). The presence of these compounds such as trans-Z-α-bisabolene epoxide, terpinen-4-ol, cummamic aldehyde and kauran-16-ol were previously linked to the potent anti-oxidative action of αl derived from the X. anthopica fruit (Karino et al., 2004). In another study, treatment of butyl isobutyl phthalate (20-500 mg/kg bw/day) for 3 days showed anti-hyperglycemic action in STZ-induced diabetic rats (Bu et al., 2010). Additionally, butyl isobutyl phthalate and cummamic aldehyde (cloppealdehyde) were reported to exhibit α-glucosidase inhibitory action (Iiu et al., 2011; Lee, 2005). Therefore, the promising anti-diabetic potential of XAAF observed in our present study might be attributed to the presence of these compounds as well as other aromatic molecules detected.

Grounds et al. (2013) have reported that administration of X. anthopica fruit ethanol extract (400 mg/kg bw/day) reversed the gained bw in high-sucrose fed rats, when no significant effect was observed in the normal animals treated with fruit powder (2-4%) (Adefeha and Otoh, 2012a). However, the effect of X. anthopica
fruit ethanolic extracts or fruit itself may be different from the X. aethiopica acetone fraction of the fruit ethanolic extract. Additionally, the effects of X aethiopica fruit extracts or fraction may not be similar in non-diabetic and diabetic animals. Hence, the effects XAAF in our study are not exactly consistent with the above-mentioned studies. However, despite lower feed and fluid intake (Fig 3) the significantly improved body weight in the XAAF administered groups (DVAL and DXAH) compared to the DBC group (Fig 2) revealed its potent anti-diabetic effects. Additionally, no significant alterations were observed in the eating behavior as well as body weight gain of the animals in the XAH group compared to the animals in the NC group (Fig 2). This result was further supported by the significantly reduced blood glucose level in the DVAL and DXAH groups compared to the DBC group (Fig 4).

In our present study, persistent hyperglycemia was observed in the DBC group throughout the experimental period (Fig 4), which is an indication of successful induction of diabetes. At the same time, significantly higher HOMA-B scores and significantly lower HOMA-β scores in the DBC group compared to the NC group (Table 2) confirmed the induction of insulin resistance and partial pancreatic beta-cell dysfunction. However, a dose-dependent reduction of blood glucose level was observed in the diabetic treated animals (Fig 4), which was further supported by the OGTT data (Fig 5) and is in line with the earlier study (Etondu et al., 2015). This could be due to the potent inhibitory effects of X aethiopica fruit on the activities of carbohydrate-hydrolyzing enzymes as reported previously (Adelegba and Obih, 2012a; Etondu et al., 2010). The inhibitors of these enzymes cause a decrease in the digestion of carbohydrates and resultant absorption of glucose and therefore reduced the hyperglycemia in T2D (Adelegba and Obih, 2017). Furthermore, the blood glucose level in the XAH group, which remained within normal levels and is of pharmaceutical interest, as hypoglycemia is among the side effects associated with the current synthetic anti-diabetic drugs (Hug et al., 2012).

It has been reported that chymic uncontrolled hyperglycemia in T2D causes an impairment or decline in insulin secretion (Nattrass and Bailey, 1999), with subsequent distortion of pancreatic architecture, β-cell integrity and function as well (Bonne-Weir and O'Brien, 2008). These effects were fully manifested in the DBC group in our present study (Table 2 and Fig 6). On the other hand, the oral treatment of XAAF dose-dependently improved serum insulin levels, decreased insulin resistance and restored the pancreatic morphology to near normal in diabetic animals, when no considerable alterations were observed in the XAH group. In one of our previous studies, the XAAF was also found to possess high in vitro anti-oxidative activity (data not shown) in addition to other studies conducted on the crude extracts of X. aethiopica fruit (Adelegba and Obih, 2012a; 2011; Etondu et al., 2010; Odokoya et al., 2003; George and Osamie 2011). Therefore, we may propose that the protection of the pancreatic duct morphology could be mediated through the anti-oxidant potentials because oxidative stress is the major causative factor for pancreatic β-cell damage in T2D (Huang et al., 2011). Interestingly, the above hypothesis was further supported by the significant attenuation of the serum fructosamine level in diabetic treated animals (Table 2). This is an indication of a decrease in protein glycosylation, a major contributor to the production of advanced glycation end products in diabetic condition.

Consistent with earlier reports, the absolute liver weight was not affected but the relative liver weight and liver glycogen content were significantly increased by the induction of diabetes (Cobre et al., 1992; Oliveira et al., 2008). The elevation of hepatic glycogen content could possibly be linked to fructose feeding that triggers hepatic glycogenesis via stimulation of glucose-6-phosphatase activity and causes higher accumulation of glycogen in the liver despite low circulating insulin (Ciudad et al., 1988). However, these effects were completely attenuated to near normal in the DXAH and DVAL groups and were comparable to the DMF group (Table 3). In addition, no significant alterations were evident observed in the XAH group. This suggests that XAAF may modulate glycogen biosynthesis by suppressing the activity of glucose-6-phosphatase and therefore decreases the hepatic glycogen synthesis and storage (Ciudad et al., 1988). Though the diabetic treated animals have shown insulinitropic ability, which invariably mobilizes hepatic glucose input for glycogenesis, the hepatic glycogen level was maintained within the normal range. This has demonstrated that treatment of XAAF to diabetic animals restored the normal metabolic processes altered by the induction.

---

### Table 3

<table>
<thead>
<tr>
<th></th>
<th>NC</th>
<th>DBC</th>
<th>DVAL</th>
<th>DXAH</th>
<th>DMF</th>
<th>XAH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver weight (g)</td>
<td>9.25 ± 0.14</td>
<td>10.34 ± 0.31</td>
<td>9.25 ± 0.14</td>
<td>10.85 ± 0.84</td>
<td>10.51 ± 0.53</td>
<td>9.36 ± 0.12</td>
</tr>
<tr>
<td>Relative liver weight (%)</td>
<td>2.89 ± 0.24**</td>
<td>4.55 ± 0.34A</td>
<td>3.75 ± 0.25**</td>
<td>4.36 ± 0.42**</td>
<td>3.46 ± 0.45**</td>
<td>2.93 ± 0.14**</td>
</tr>
<tr>
<td>Liver glycogen (mg/g tissue)</td>
<td>5.92 ± 0.81**</td>
<td>8.25 ± 0.58A</td>
<td>7.14 ± 0.46**</td>
<td>6.93 ± 0.45**</td>
<td>6.82 ± 0.56A</td>
<td>6.58 ± 0.42A</td>
</tr>
</tbody>
</table>

Data are presented as the mean ± SD of 5-7 animals. Values with different superscript letter within a row for a given parameter are significantly different from each other group of animals and significantly different from the DBC group (Tukey’s-HSD multiple range post hoc test, p < 0.05). NC: Normal Control; DBC: Diabetic Control; DVAL: Diabetic X aethiopica low dose; DXAH: Diabetic X aethiopica high dose; DMF: Diabetic Metformin; XAH: Normal X aethiopica high dose.

---

### Table 4

<table>
<thead>
<tr>
<th></th>
<th>NC</th>
<th>DBC</th>
<th>DVAL</th>
<th>DXAH</th>
<th>DMF</th>
<th>XAH</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC (mmol/L)</td>
<td>1.91 ± 0.19**</td>
<td>2.97 ± 0.38**</td>
<td>2.02 ± 0.23**</td>
<td>2.05 ± 0.19**</td>
<td>2.03 ± 0.16**</td>
<td>1.90 ± 0.21**</td>
</tr>
<tr>
<td>TG (mmol/L)</td>
<td>0.75 ± 0.29**</td>
<td>1.66 ± 0.31**</td>
<td>1.06 ± 0.26**</td>
<td>1.05 ± 0.19**</td>
<td>1.22 ± 0.29**</td>
<td>1.26 ± 0.21**</td>
</tr>
<tr>
<td>LDL-Cholesterol (mmol/L)</td>
<td>0.34 ± 0.16**</td>
<td>1.43 ± 0.14**</td>
<td>0.91 ± 0.23**</td>
<td>0.87 ± 0.14**</td>
<td>1.01 ± 0.17**</td>
<td>1.17 ± 0.22**</td>
</tr>
<tr>
<td>HDL-Cholesterol (mmol/L)</td>
<td>1.21 ± 0.22**</td>
<td>0.29 ± 0.17**</td>
<td>0.63 ± 0.24**</td>
<td>0.70 ± 0.12**</td>
<td>0.76 ± 0.24**</td>
<td>0.32 ± 0.21**</td>
</tr>
<tr>
<td>AI</td>
<td>0.57 ± 0.13**</td>
<td>3.30 ± 0.18**</td>
<td>2.04 ± 0.20**</td>
<td>1.81 ± 0.16**</td>
<td>1.86 ± 0.20**</td>
<td>1.27 ± 0.24**</td>
</tr>
<tr>
<td>CR</td>
<td>4.30 ± 0.16**</td>
<td>3.04 ± 0.20**</td>
<td>2.81 ± 0.14**</td>
<td>2.81 ± 0.20**</td>
<td>3.00 ± 0.16**</td>
<td>2.27 ± 0.12**</td>
</tr>
</tbody>
</table>

Data are presented as the mean ± SD of 5-7 animals. **Values with different superscript letter within a row for a given parameter are significantly different from each other group of animals and significantly different from the DBC group (Tukey’s-HSD multiple range post hoc test, p < 0.05). NC: Normal Control; DBC: Diabetic Control; DVAL: Diabetic X aethiopica low dose; DXAH: Diabetic X aethiopica high dose; DMF: Diabetic Metformin; XAH: Normal X aethiopica high dose; TC, Total cholesterol; TG, Triglyceride; LDL, Low density lipoprotein-cholesterol; HDL, High density lipoprotein-cholesterol; AI, Atherogenic index; CR, Coronary risk index.”
of diabetes, which was further supported by the anti-hyperlipidemic effect of XAAF (Table 4).

Considerable evidence has been accumulated to correlate insulin resistance in the etiology of dyslipidemia in T2D. It has been postulated that insulin resistance stimulates lipolysis from the fat depots, which promote the activity of HMG-CoA reductase and decrease lipoprotein lipase, which lead to the elevation of TG and TC levels [De Silva and Frayling, 2010; Hotamisilgil, 2000]. In previous studies, treatment of X. aethiopica fruit aqueous extract (200 mg/kg bw) and powder (2–4%) to non-diabetic animals for 2 weeks exhibited hypolipidemic action [Adieghe and Oboh (2012a)]. In another study, administration of X. aethiopica aqueous extract (250 mg/kg bw) for 8 weeks demonstrated potent anti-hyperlipidemic effects in hypercholesterolemic rats [Nwozo et al., 2011]. Conversely, in a more recent study, Etoundi et al. (2013) have shown that the ethanolic extract (400 mg/kg bw) did not show any significant anti-hyperlipidemic action after 2-day post-treatment period in hyperlipidemic rats. However, in our study, the X. aethiopica fruit acetone fraction showed potent anti-hyperlipidemic ability with no alterations in serum lipid profile of the NXAH group (Table 4). Therefore, the lipid-lowering effect of X. aethiopica fruit can only be achieved after prolonged treatment (sub-chronic or chronic) against the acute treatment as recently reported by Etoundi et al. (2013). In addition, the anti-hyperlipidemic action of XAAF observed in our present study could be attributed to the reduction of insulin resistance in the diabetic treated animals.

On the other hand, Etoundi et al. (2013) have also reported the hepato-protective ability of X. aethiopica fruit (400 mg/kg bw) in hyperlipidemic rats via reduction of serum ALT and AST levels in the treated animals. Additionally, no significant alterations of serum ALT, AST and AUP were observed in non-diabetic animals treated with X. aethiopica fruit (3% and 4%) in the diet for 2 weeks (Adieghe and Oboh, 2012a). This was apparently in line with our present data (Table 4). Treatment of the fraction protects the integrity of the liver as well as heart via reductions of serum ALT, AST, urea, creatinine, LDH and CK-MB in the treated diabetic animals (Table 5). Interestingly, no sign of alterations were observed in the levels of these parameters in the NXAH group throughout the study period, when at a dose of 400 mg/kg bw the extract was reported to induce some hepatic injury in normal animals (Taiwo et al., 2009). This could be due to the dose variation, which indicates that the safety of the extract above 300 mg/kg bw is still questionable. The serum AST was found not to be affected by the diabetes throughout the study period. In comparison with ALT, AST is less specific for the detection of hepatic injury due to its shorter half-life compared to ALT, particularly in experimental animals (Centeno, 2007).

In conclusion, oral intervention of the X. aethiopica fruit acetone fraction (150 and 300 mg/kg bw) have demonstrated anti-diabetic actions via improving BW gain, reducing food and fluid intake and hyperglycemia, improving glucose tolerance ability, insulin sensitivity, amelioration of pancreatic β-cell histology and β-cell function and improving dyslipidemia in T2D model of rats. Hence, our findings suggest that the acetone fraction derived from X. aethiopica fruit may be a potent and possible anti-diabetic remedy with no considerable side effects at the dosages used. However, further clinical and more detailed toxicological studies are required to confirm the effects and safety in human subjects. The isolation and partial characterization of the active principle from XAAF is currently underway to find the compound (s) responsible for this activity.

Declaration of interest

We declare that we have no conflict of interest with this article.

Acknowledgments

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References


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Ethyl acetate fraction of *Aframomum melegueta* fruit ameliorates pancreatic β-cell dysfunction and major diabetes-related parameters in a type 2 diabetes model of rats

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Absolute ethanol (CD 702)
Glicolic acid (CD 318)
Dichromethane (CD 8544)
Dilinoleic acid (DMS) (CD 1187)
Ethyl acetate (CD 8837)
Hexane (CD 8058)
Metformin (CD 4081)
2-nitrophenyl-4d-glycopyranoside (pNPG) (CD 119754)
Sodium citrate (CD 6224)
Streptomycin (CD 3032)

ABSTRACT

Ethnopharmacological relevance: In West Africa, various preparations of the fruit seed and leaf of *Aframomum melegueta* K. Schum. are reputedly used for the management of diabetes mellitus (DM) and other metabolic disorders. The present study evaluated the anti-diabetic effects of *A. melegueta* ethyl acetate fraction (AMEF) from fruit ethanolic extract in a type 2 diabetes (T2D) model of rats.

Materials and methods: T2D was induced in rats by feeding a 10% fructose solution ad libitum for two weeks followed by a single intraperitoneal injection of streptozotocin (40 mg/kg body weight) and the animals were orally treated with 150 or 300 mg/kg body weight (bw) of the AMEF once daily for four weeks.

Results: At the end of the intervention, diabetic untreated animals showed significantly higher serum glucose, serum fructosamine, LDL, CR, CR-MB, serum lipids, liver glycogen, insulin resistance (HOMA-IR), AI CR and lower serum insulin, pancreatic β-cell function (HOMA-β) and glucose tolerance ability compared to the normal animals. Histopathological examination of their pancreas revealed corresponding pathological changes in the islets and β-cells. These alterations were reverted to near-normal after the treatment of AMEF at 150 and 200 mg/kg bw when, the effects were more pronounced at 300 mg/kg bw compared to the 150 mg/kg bw.

Conclusion: The results of our study suggest that AMEF treatment at 300 mg/kg bw showed potent anti-diabetic effect in a T2D model of rats.

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1. Introduction

Diabetes mellitus (DM) is a condition that causes hyperglycemia due to either decreased insulin secretion or insulin sensitivity of target tissues (Panini, 2013). Recent data indicate that more than 371 million people have DM and this figure is likely to be doubled by 2035 (IDF, 2013). Among the two major types of diabetes (type 1 and type 2), type 2 is the most prevalent one, accounting for more than 90% of all diabetic cases. Type 2 diabetes (T2D) is a heterogeneous disorder characterized by insulin resistance and partially dysfunctional pancreatic β-cells which cannot properly secrete insulin in response to hyperglycemia (Hu...
et al., 2007). Insulin resistance usually occurs in the early stage of T2D after which a further decline of β-cells is induced, resulting in hyperglycemia and hyperlipidemia (Roberston et al., 2004).

The current treatment option for T2D include mainly oral anti-diabetic drugs, such as sulfonylureas, biguanides, α-glucosidase inhibitors, thiazolidinediones and dipeptidyl peptidase-4 (DPP-4) inhibitors among others. But these drugs do not adequately improve the consequences of T2D and have caused characteristic profiles of serious side effects, which include hypoglycemia, weight gain, hypersensitivity, gastrointestinal discomfort, nausea, liver and heart failure, and diarrhea (Hung et al., 2012). Therefore, search for novel molecules has been extended to natural plant-based remedies, as they are considered more effective, safe, natural and cause less adverse effects compared to modern synthetic drugs.

Aframomum melegueta K. Schum. (Zingiberaceae) commonly known as guinea pepper is an herbaceous plant that grows up to 1500 cm height, with purple flowers that develop into 5–7 cm long pods containing small, reddish-brown aromatic and pungent seeds. The fruits are ovoid with reddish color and numerous small brownish angular seeds with a cardamom flavor (Iwu, 2014). Fruit seed and leaf of A. melegueta are commonly used as spices in various food preparations in different parts of Africa. The fruit preparation (mostly in alcoholic solution) has been locally used in the treatment of DM in Nigeria (Abo et al., 2008). In another study, pap containing A. melegueta seed and African giant snail is employed in treatment of DM in Nigeria (Gbolade, 2009). Additionally, a mixture containing Allium cepa dried leaf, Carica papaya root and A. melegueta leaf was also used locally to treat DM and other metabolic disorders in some parts of Nigeria (Gbolaide, 2009). A recent study by Sugita et al. (2013) reported that A. melegueta seed alcoholic extract stimulates brown adipose tissue and increases whole-body energy expenditure in human subjects which is directly linked with the pathogenesis of T2D. In a very recent study, we showed that different solvent extracts from the various parts (fruit, leaf and stem) of A. melegueta demonstrated anti-oxidative and anti-diabetic potentials in vitro (Mohammed et al., in press). Similarly, in vitro anti-diabetic as well as antioxidant potentials of the aqueous seed extracts of A. melegueta have been reported in many recent studies (Adegbegah and Oboh, 2012; Kareem et al., 2012; Adegbegah and Oboh, 2011; Etoundi et al., 2010). In another study, Cooja et al. (2014) have reported the anti-oxidative potentials of seed methanolic extracts of A. melegueta both in vitro and in vivo. In addition, phenolics such as ginkgol, shagard and paradols are compounds reported to be rich in the crude ethanolic extract of the seed which showed minimum toxicity after a four week oral treatment in rats (Bil et al., 2010). In preliminary studies, seed (Adegbegah and Oboh, 2010) and leaf (Mojekwu et al., 2011) aqueous extracts of A. melegueta have demonstrated blood glucose lowering ability in allin-induced diabetic rats. However, the detail in vivo anti-diabetic study on any part, extract or fraction of this plant has not yet been conducted either in humans or in experimental animals till today.

Therefore, the present study was designed to investigate the in vivo anti-diabetic effects of the ethyl acetate fraction derived from the fruit ethanolic extract of A. melegueta in a T2D model of rats.

2. Materials and methods

2.1. Plant material

The fruit sample (pod and seed) of A. melegueta was freshly collected in December, 2012 from Ibadan, Oyo State, Nigeria. The plant was identified and authenticated at the herbarium unit of the Biological Science Department, Ahmadu Bello University, Zaria, Nigeria by Mr. Umar Galah and a voucher specimen number 1511 was deposited accordingly. The plant sample was immediately washed and shade-dried for two weeks to constant weights. The dried sample was ground to a fine powder, and then stored in an airtight container to transport to the University of KwaZulu-Natal, Westville campus, Durban, South Africa for further investigation.

2.2. Extraction and fractionation of the sample

The extraction and subsequent fractionation was carried out according to the method as reported previously (Ibrahim and Islam, 2014). Briefly, the fine powdered sample (3 kg) was separately defatted with 10 l hexane. The defatted sample was sequentially extracted with ethanol by soaking for 48 h and filtered through Whatman filter paper (No. 1). The resultant extract was evaporated under vacuum, using a rotary evaporator (Buchi Rotavapor II, Buchi, Germany) at 40 °C under reduced pressure to obtain the crude ethanolic extract with a yield of 5.25%. Forty grams of the crude ethanolic extract of the fruit was dissolved in 500 ml of distilled water: methanol (9:1) and successively partitioned with hexane (2 × 500 ml), dichloromethane (2 × 500 ml), ethyl acetate (2 × 500 ml) and acetone (2 × 500 ml). The fractions were evaporated to dryness in vacuum at 40 °C under reduced pressure whereas the remaining aqueous fraction was dried in a water bath at 50 °C. The dried fractions were transferred to micro tubes and stored at 4 °C until further analysis. The A. melegueta ethyl acetate fraction (AMEF) was a greenish residue.

2.3. Gas chromatography-mass spectroscopic (GC-MS) analysis of AMEF

The gas chromatography mass spectroscopic (GC-MS) analysis of AMEF was conducted by an Agilent technique 6890 GC, coupled with an Agilent 5973 Mass Selective Detector and driven by Agilent Chemstation software. Compounds were identified by direct comparison of the retention times and mass spectral data with those in the National Institute of Standards and Technology (NIST) library.

2.4. α-amylase (E.C. 3.2.1.1) inhibitory effect of the solvent fractions

The α-amylase inhibitory activities of solvent fractions were measured by using a method as described by McCabe and Shetty (2004) after slight modification. Briefly, a 250 μl aliquot of fraction at different concentrations (30, 60, 120 and 240 μg/ml) was placed in a series of tubes and 250 μl of 0.02 M sodium phosphate buffer (pH 6.9) containing α-amylase solution was added. This solution was pre-incubated at 25 °C for 10 min, after which 250 μl of 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9) was added at timed intervals and then further incubated at 25 °C for 10 min. The reaction was terminated after incubation by adding 1 ml of dinitrosalicylic acid (DNS) reagent. The tubes were then boiled for 10 min and cooled to room temperature. The reaction mixture was diluted with 5 ml distilled water and the absorbance was measured at 540 nm using Shimadzu UV mini 1240 spectrophotometer. A control was prepared using the same procedure replacing the extract with distilled water. The α-amylase inhibitory activity was calculated as percentage inhibition according to the following formula:

% Inhibition = (Abs control–Abs extracts)/Abs control) × 100.

2.5. α-Glucosidase (E.C. 3.2.1.20) inhibitory effect of the solvent fractions

The effect of the fractions on α-glucosidase activity was determined according to the method described by Kim et al. (2005),
using α-glucosidase from Saccharomyces cerevisiae. The substrate solution p-nitrophenyl glucopyranoside (pNPG) was prepared in 20 mM phosphate buffer, pH 6.9. Then 500 μL of α-glucosidase (E. C. 3.2.1.20) was pre-incubated with 250 μL of the different concentrations of the fractions (30, 60, 120 and 240 μg/ml) for 10 min. Then 250 μL of 5 mM (pNPG) dissolved in 20 mM phosphate buffer (pH 6.9) was added as a substrate to start the reaction. The reaction mixture was incubated at 37 °C for 30 min. The α-glucosidase activity was determined by measuring the yellow colored p-nitrophenol released from pNPG at 405 nm. The results were expressed as percentage of the blank control. The percentage inhibition was calculated according to the following formula:

\[ \% \text{Inhibition} = \left( \frac{\text{Abs control} - \text{Abs extract}}{\text{Abs control}} \right) \times 100 \]

2.6. Experimental animals

Six-week-old male Sprague–Dawley (SD) rats were obtained from the Biomedical Resource Unit (BRU) located at the University of KwaZulu-Natal (Westville Campus), South Africa with initial mean bw 163.25 ± 13.67 g. Animals were housed as two in one medium size poly-carbonated cage in a temperature and humidity controlled room with a 12 h light–dark cycle. A standard rat pelleted diet was supplied ad libitum to all animals during the entire experimental period. Animals were maintained according to the rules and regulations of the Experimental Animal Research Ethics Committee of the University of KwaZulu-Natal, South Africa (Ethical approval number: 018/14/Animal).

2.7. Animal grouping

Animals were randomly divided into six groups of 5–7 animals namely, NC: Normal Control; DBC: Diabetic Control; DAMH: Diabetic+low dose (150 mg/kg bw) of AMEF; DAMH: Diabetic+high dose (300 mg/kg bw) of AMEF; DMF: Diabetic+metformin (300 mg/kg bw); NAMH: Non-diabetic+high dose (300 mg/kg bw) of AMEF. The animals were allowed to acclimatize for one week before starting the experiment.

2.8. Induction of type 2 diabetes

In order to induce the two major pathogeneses of T2D, insulin resistance and partial pancreatic β-cell dysfunction, during the first two weeks of the experiment, the animals in the DBC, DAMH, NAMH and DMF groups were supplied with a 10% fructose solution for the induction of insulin resistance when the animals in the NC and NAMH groups were supplied with normal drinking water. After this period, a low dose of STZ (40 mg/kg bw) dissolved in citrate buffer (pH 4.5) were intraperitoneally injected to the animals in the DBC, DAMH and DMF groups to induce partial pancreatic β-cell dysfunction, whereas the animals in the NC and NAMH groups were injected with a similar volume of vehicle buffer only (Wilson and Islam, 2012). One week after the STZ injection, the non-fasting blood glucose (NFBG) of all animals were measured in the blood collected from the tail vein by using a portable glucometer (Glucopix Inc., Saint-Laurent, Quebec, Canada). Animals with a NFBG level > 200 mg/dL were considered as diabetic (Davidson et al., 2011) while animals with a NFBG level < 200 mg/dL were excluded from the study.

2.9. Intervention period

After the confirmation of diabetes, a respective dose of the fraction was orally administered once daily for four weeks using a gastric gavage needle to the animals in DMF and DAMH and NAMH groups while the animals in controls (NC and DBC) and DMF groups were treated with similar volume of the vehicle and metformin, respectively. Throughout the experimental period, feed and fluid intake were measured every morning by subtracting the remaining amount of feed and fluid respectively from the amount given on the previous morning. Moreover, the weekly bw and NFBG levels were measured in all animal groups during the entire intervention period.

2.10. Oral glucose tolerance test (OGTT)

To measure the glucose tolerance ability of each animal, the OGTT was performed in the last week of the four-week intervention period. To perform this test, a single dose of glucose solution (2 g/kg bw) was orally administered to each animal and the subsequent levels of blood glucose were measured at 0 (just before glucose ingestion), 30, 60, 90 and 120 min after the ingestion of glucose.

2.11. Collection of blood and organs

At the end of the experimental period, animals were euthanized by halothane anesthesia and blood and organ samples were collected. The whole blood of each animal was collected via cardiac puncture and immediately preserved in a refrigerator until further processing. The blood samples were centrifuged at 3000 rpm for 15 min and serum from each blood sample was separated and preserved at –30 °C for further analysis. The liver was collected from each animal, washed with normal saline, wiped with filter paper, weighed and preserved at –30 °C until subsequent analysis. A small piece of pancreatic tissue from each animal was cut and placed in a 10% neutral buffered formalin solution and preserved at room temperature for histopathological study. The neutral buffered formalin of each pancreatic tissue sample was replaced weekly during the entire preservation period.

2.12. Analytical methods

The serum insulin concentrations were measured by an enzyme-linked immunosorbent assay (ELISA) method using an ultrasensitive rat insulin ELISA kit (Mercodia, Uppsala, Sweden) in a multiplate reader (MR-96A, Vacuettic Pvt. Ltd., Durban, South Africa). The serum lipid profile, fructoseamine, urea, uric acid, lactate dehydrogenase (LDH), creatine kinase (CK-MB) and creatinine concentrations as well as liver function enzymes, aspartate and alanine aminotransferases (AST and ALT) and alkaline phosphatase (ALP) were measured using an Automated Chemistry Analyzer (Labmax Pienoo, Labtest Co. Ltd., Lagoa Santa, Brazil) with commercial assay kits from the same company. Homeostatic model assessment (HOMA-IR and HOMA-β) scores were calculated at the end of the intervention according to the following formula:

HOMA-IR = \left( \frac{\text{Fasting serum insulin in } \mu \text{U/mL} \times \text{Fasting blood glucose in mmol/L}}{22.5} \right)

HOMA-β = \left( \frac{\text{Fasting serum insulin in } \mu \text{U/mL} \times 20}{\text{Fasting blood glucose in mmol/L} - 3.5} \right)

Conversion factor: insulin (\mu U/mL = 7.174 pmol/L).

LDL-cholesterol was calculated according to Friedewald et al. (1972) equation as shown below:

LDL-Cholesterol (mg/dL) = \left( \frac{\text{TC-HDL}}{\text{TG/5}} \right)

where TG/5 is equivalent to the concentration of VLDL-cholesterol.
Atherogenic index (AI) was calculated according to the method described by Liu et al. (1999) and expressed as:

Atherogenic index (AI) = (TC−HDL cholesterol)/HDL−cholesterol.

Coronary artery risk index (CRI) was also calculated using the following formula (Boers et al., 2003):

Coronary artery risk index (CRI) = TC/(mg/dl−HDL cholesterol)/(mg/dl)

Liver glycogen concentrations were measured by phenol−sulfuric acid method as described by Ito et al. (1970).

2.13. Histopathological examination of pancreatic tissue

A standard laboratory protocol for paraffin embedding was used to treat the formalin preserved pancreatic tissues. Tissue sections were cut into a size of 4 mm before fixing on the slides. Then, the slides were deparaffinized in p-xylene and rehydrated in ethanol gradient (100%, 80%, 70% and 50%) and rinsed with water. Slides were stained in hematoxylin for 5 min and rinsed with water, and were counter stained in eosin, mounted in DPX, coverslipped and viewed with Leica slide scanner (SCN 4000, Leica Biosystems, Wetzlar, Germany).

2.14. Statistical analysis

Data are presented as the mean ± SD (n=5−7). Data were analyzed by using a statistical software package (SPSS for Windows, version 18, IBM Corporation, NY, USA) using Tukey's HSD multiple range post hoc test. Values were considered significantly different at p<0.05.

3. Results

The data of the α-amylase and α-glucosidase inhibitory activities of the fractions derived from ethanolic extract of A. melageta fruit are shown in Fig. 1. It was observed that the A. melageta ethyl acetate fraction (AMEF) exhibited a significantly (p<0.05) higher α-amylase (Fig. 1A) and α-glucosidase (Fig. 1B) inhibitory activities compared to other fractions as well as acarbose. Furthermore, the inhibitory actions of dichloromethane and acetone fractions were higher compared to the hexane fraction.

The possible compounds detected from AMEF via GC-MS analysis are shown in Table 1 and the respective chromatograms are shown in the NIST library. According to the data, 10 bioactive principles were identified by their fragmentation pattern and in conjunction with the NIST library. According to the data, 10 bioactive principles were identified in the AMEF. These compounds include, 4-methylphenol (1), 2,6-dimethylphenol (2), isobornyl propionate (3), eugenol (4), 5-ethyl-2-methyl thiazole (5), 4-hydroxy-3-methoxyphenyl butan-2-one (6), benzyl benzoate (7), 1,2-benzene dicarboxylic acid, butyl 2-methylpropyl ester (8), 4-hydroxy-3-methoxyphenyl decan-3-one (9) and 1,2-benzene dicarboxylic acid, mono(2-ethyl) ester (10). Moreover, with the exception of compounds 3 and 5, the remaining compounds are aromatic in nature when compounds 1, 4, 6 and 9 are phenolic derivatives. Additionally, compound 6 was observed to elute in more than one retention time.

The results of weekly mean body weight (bw) are shown in Fig. 3. Although fructose feeding for two weeks did not significantly affect the mean bw of the treated animals compared to the untreated animals, after STZ injection, the bw of DBC group was decreased for the entire intervention period. On the other hand, AMEF oral treatment ameliorated this reduction when significant (p<0.05) amelioration was observed after the 2nd, 3rd and 4th week of intervention. In addition, no significant difference of bw was observed between the AMEF treated groups (DALM and DAIMH) and NC and DMF groups (Fig. 3).

The results of feed and fluid intake in different group of animals are shown in Fig. 4. The results of DBC group suggest that the induction of diabetes significantly (p<0.05) increased feed and fluid intake compared to NC group (Fig. 4). Treatment with various dosages of AMEF for weeks to diabetic animals significantly (p<0.05) ameliorated the alterations of feed and fluid intake which are comparable to the DMF group (Fig. 4).

The results of weekly FGBG and FEX (last week only) are shown in Fig. 5. The data showed that the induction of T2D significantly (p<0.05) increased FGBG in diabetic animals compared to the normal animals (NC). On the other hand, the oral administration of AMEF significantly (p<0.05) decreased FGBG in DALM and DAMH groups compared to the DBC group when no significant difference was observed between the AMEF (DALM and DAIMH) and metformin (DMF) treated groups. This effect was more pronounced in the DAMH group compared to the DALM group. Additionally, no significant difference was observed between the last week FGBG of DMH and NC group (Fig. 5).

The data of oral glucose tolerance test (OGTT) along with calculated area under the curve (AUC) are shown in Fig. 6. According to the results, the glucose tolerance ability of the animals in AMEF treated groups was significantly better than the DBC group for the entire period of the test when the results were significantly better in the AMEF treated groups compared to the DMF group from 60 to 120 min of the test period. No significant difference was observed between the glucose tolerance ability of AMEF treated groups and NC group during this period as well (Fig. 6). The
Table 1

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Compounds</th>
<th>Retention time (min)</th>
<th>Mass/charge</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethyl acetate</td>
<td>4-Methylphenol</td>
<td>4.44</td>
<td>109 [M]+</td>
</tr>
<tr>
<td></td>
<td>2,6-Dimethylphenol</td>
<td>5.07</td>
<td>122 [M]+</td>
</tr>
<tr>
<td></td>
<td>Isobutyl proprionate</td>
<td>6.22</td>
<td>210 [M]+</td>
</tr>
<tr>
<td></td>
<td>Eugenol</td>
<td>6.67</td>
<td>164 [M]+</td>
</tr>
<tr>
<td></td>
<td>5-ethyl-2-methyl thiadiazole</td>
<td>7.65</td>
<td>127 [M]+</td>
</tr>
<tr>
<td></td>
<td>4-hydroxy-3-methoxyphenyl butan-2-one</td>
<td>8.58, 12.45</td>
<td>154 [M]+</td>
</tr>
<tr>
<td></td>
<td>Benzoic lactone</td>
<td>9.36</td>
<td>212 [M]+</td>
</tr>
<tr>
<td></td>
<td>1,2-Benzene dicarboxylic acid, butyl (2-methylpropyl) ester</td>
<td>10.26</td>
<td>275 [M]+</td>
</tr>
<tr>
<td>9</td>
<td>4-Hydroxy-3-methoxyphenyl deca-3-one</td>
<td>11.63</td>
<td>278 [M]+</td>
</tr>
<tr>
<td>10</td>
<td>1,2-Benzene dicarboxylic acid, mono(2-methylb) ester</td>
<td>13.17</td>
<td>279 [M]+</td>
</tr>
</tbody>
</table>

Fig. 2. GC-MS chromatogram of ethyl acetate fraction from fruit ethanolic extract of A. melanges.

Fig. 3. Mean body weight change in all animal groups during the entire study period. Data are presented as the mean ± SD of 8–12 animals. a–b Values with different letters over the bars for a given parameter 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; DAM, Diabetic A. melanges low dose; DMM, Diabetic A. melanges high dose; DMF, Diabetic Metformin; NAM, Normal A. melanges high dose.

Fig. 4. Mean food and fluid intake of different animal groups during 4-week intervention period. Data are presented as the mean ± SD of 8–12 animals. a–d Values with different letters over the bars for a given parameter 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; DAM, Diabetic A. melanges low dose; DMM, Diabetic A. melanges high dose; DMF, Diabetic Metformin; NAM, Normal A. melanges high dose.

The calculated AUC of DBC group was significantly higher compared to the NC and NAMH groups when the AUC of DAM, DAMH and DMF groups were significantly lower than the DBC group (Fig. 6).

The data for serum insulin, fructosamine and calculated HOMA-IR and HOMA-β scores are shown in Table 2. Serum insulin level and the calculated HOMA-β scores were decreased significantly (p < 0.05) whereas serum fructosamine as well as HOMA-IR were increased significantly (p < 0.05) in the DBC group compared to the NC group (Table 2). Oral intervention of AMF for four weeks to diabetic animals elevated serum insulin and HOMA-β scores with subsequent attenuation of serum fructosamine level and HOMA-IR scores in DAM and DAMH groups compared to the DBC
dosages of AMEF as well as standard drug attenuated the diabetes induced pancreatic damage and restored the pancreatic morphology to near normal when the number of \( \beta \)-cells were significantly higher in the DAMH group compared to the DAML group (Fig. 7).

The results of liver weight, relative liver weight and liver glycogen levels are shown in Table 3. The induction of T2D did not affect the absolute liver weight but significantly \((p < 0.05)\) increase the relative liver weight and liver glycogen level in the DBC group compared to the NC group. A significant \((p < 0.05)\) reduction of relative liver weight and liver glycogen levels was observed in the DAML and DAMH groups compared to the DBC group when the results were not significantly different compared to the NC and DMEF groups (Table 3).

The data for serum lipid profile and calculated atherogenic index (AI) and coronary risk index (CRI) are shown in Table 4. Elevated serum concentrations of TC, TG and LDL-cholesterol levels as well as calculated AI and CRI with subsequent reduction on serum HDL-cholesterol were observed in the DBC group compared to the NC group (Table 5). Treatment with AMEF to diabetic animals significantly \((p < 0.05)\) and dose-dependently reduced TC, TG and LDL-cholesterol, AI and CRI in DAML and DAMH groups compared to the DBC group. Although dose-dependent increase in serum HEI-cholesterol level was observed in the AMEF treated groups compared to the DBC and DMEF groups, the data were not significantly different (Table 4).

The data for serum ALT, AST, ALP, urea, uric acid, creatinine, LDH and CK-MB are shown in Table 5. At the end of the experimental period, the concentrations of all of the above-mentioned serum parameters were increased in the DBC group compared to the NC group, when serum AST level was not affected by the induction of diabetes. On the other hand, oral administration of AMEF to diabetic animals significantly \((p < 0.05)\) ameliorated these alterations in DAML and DAMH groups. The effects were more pronounced in the DAMH group compared to the DBC group and which did not differ significantly \((p > 0.05)\) compared to the DMEF group regarding most of the parameters. Similarly, treatment of AMEF to non-diabetic animals demonstrated no significant \((p > 0.05)\) effect on these serum parameters compared to the NC group (Table 5).

4. Discussion

In the present study, we have investigated the effect of oral administration of a low and a high dose of \( A. \) melanogaster ethyl acetate fraction (AMEF) of fruit ethanol extract in a T2D model of rats through four weeks post-treatment period. This is obviously the first study that comprehensively reported the anti-diabetic effect of \( A. \) melanogaster despite the reported ethnobotanical potentials of \( A. \) melanogaster in the management of diabetes. According to

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Table 2: Serum insulin and fructoseamine levels, HOMA IR and HOMA \( \beta \) scores of different animal groups at the end of the experimental period.

<table>
<thead>
<tr>
<th></th>
<th>NC</th>
<th>DBC</th>
<th>DAML</th>
<th>DAMH</th>
<th>DMEF</th>
<th>NAMH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin (ng/ml)</td>
<td>76.70 ± 6.20*</td>
<td>47.05 ± 9.02*</td>
<td>55.85 ± 11.45*</td>
<td>64.81 ± 8.77*</td>
<td>75.88 ± 14.23*</td>
<td>65.49 ± 11.38*</td>
</tr>
<tr>
<td>Fructoseamine (mM)</td>
<td>239.20 ± 47.67*</td>
<td>305.43 ± 44.34*</td>
<td>298.00 ± 23.39*</td>
<td>247.86 ± 28.03*</td>
<td>283.14 ± 32.49*</td>
<td>274.00 ± 19.95*</td>
</tr>
<tr>
<td>HOMA IR</td>
<td>2.50 ± 0.27*</td>
<td>6.30 ± 1.64</td>
<td>3.75 ± 0.53*</td>
<td>3.11 ± 0.81</td>
<td>3.82 ± 0.80*</td>
<td>2.11 ± 0.44*</td>
</tr>
<tr>
<td>HOMA ( \beta )</td>
<td>1.27 ± 0.31*</td>
<td>7.33 ± 1.26</td>
<td>2.67 ± 0.38 *</td>
<td>4.32 ± 0.80 *</td>
<td>5.27 ± 0.52 *</td>
<td>10.60 ± 0.24 *</td>
</tr>
</tbody>
</table>

Data are presented as the mean ± SD of 5–7 animals. a–c Values with different letters along the rows for a given parameter 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; DAML, Diabetic A. melanogaster low dose; DMEF, Diabetic A. melanogaster high dose; HOMA IR, Homeostasis model assessment-insulin resistance; HOMA \( \beta \), Homeostasis model assessment-\( \beta \).

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\* HOMA IR =\( \frac{\text{Fasting serum insulin in U/ml}}{\text{Fasting blood glucose in mg/dl}} \) (52.55).

\* HOMA \( \beta \) =\( \frac{\text{Fasting serum insulin in U/ml}}{\text{Fasting blood glucose in mg/dl}} \) (32.5).

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Fig. 5: Weekly NFFG of all animal groups during the entire experimental period. Data are presented as the mean ± SD of 5–7 animals. a–c Values with different letters among the bars for a given parameter 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; DAML, Diabetic A. melanogaster low dose; DMEF, Diabetic A. melanogaster high dose; NAMH, Normal A. melanogaster high dose; NFG, non-fasting blood glucose; FPG, fasting blood glucose.

Fig. 6: Oral glucose tolerance test (OGTT) in all animal groups in the last week of the 4-week experimental period. Data are presented as the mean ± SD of 5–7 animals. a–c Values with different letters among the bars for a given parameter 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; DAML, Diabetic A. melanogaster low dose; DMEF, Diabetic A. melanogaster high dose; NAMH, Normal A. melanogaster high dose. The area under the curve (AUC) was calculated according to the following formula: \( \text{AUC} = \sum_{i=1}^{n} \left( \frac{T_{i} - T_{i-1}}{2} \right) \times \text{C}_{i} \) where \( \text{C}_{i} \) is the concentration of blood glucose at time \( T_{i} \).
the present data, the higher α-amylase and α-glucosidase inhibitory actions of AMEF than other fractions indicated the possible anti-diabetic potential of the fraction (Fig. 1). Therefore, AMEF fraction was selected for the in vivo studies. These inhibitory activities might be attributed to the possible chemical constituents present in the AMEF.

The possible bioactive compounds in AMEF were detected using GC-MS analysis. In some previous studies, it has been reported that A. melegueta fruit and seed ethanolic extract were rich in phenolic compounds such as gingerol, paradol, shagaoal and eugenol (Mohammed et al., in press; Illic et al., 2010). The compounds identified in the AMEF in our study (Table 1, Fig. 2) are in line with the results of the above-mentioned studies. Additionally, the potent anti-diabetic action of AMEF in our study could possibly be attributed to the presence of these compounds as well.

It is evident from the results of our study that induction of T2D decreased bw and caused polyphagia and polydipsia, the classical symptoms of DM (Figs. 3 and 4). The observed polydipsia could possibly be linked to polyuria due to the excessive fluid retention in uncontrolled diabetic condition (Kitabchi et al., 2000). Interestingly, post-treatment of AMEF for four weeks ameliorated these alterations even at a lower dose administered (150 mg/kg bw), indicating possible recovery from the diabetic state (Figs. 3 and 4). Our findings are also in line with the results of a number of previously published studies (Omar et al., 2014; Ibrahim and Islam, 2014; Olcan et al., 2012) which has been further supported by the significant reduction in hyperglycemia in AMEF treated groups (Fig. 5).

It is well known that the successful treatment of T2D requires an effective control of hyperglycemia and hence search for active anti-hyperglycemic agents cannot be underscored (Bakarava and Narasimhacharya, 2008). In our study, the marked appearance of hyperglycemia in DBC group throughout the study period is an indication of successful induction of diabetes. On the other hand, the AMEF treated diabetic animals responded positively to the treatment and significantly reduced diabetes-induced hyperglycemia to near normal just after a four-week post-administration period (Fig. 5). Similar reduction on blood glucose level was also reported previously when alloucan-induced diabetic rats were treated with seed aqueous extract (Adesokan et al., 2010). Moreover, in our present study, the more pronounced anti-hyperglycemic effect in the DAML group compared to the DAMH group suggests the dose-dependent effects AMEF, which has been further supported by the significantly better glucose tolerance ability of the animals in treated groups compared to the DBC group (Fig. 6). Additionally, the AMEF treatment at higher dose (300 mg/kg bw) to normal animals did not affect the blood glucose levels and therefore confirmed the anti-hyperglycemic ability of AMEF but not hyperglycemic effect (Fig. 3), which has been again supported by the pancreatic β-cell ameliorating as well as insulin secreting effects of AMEF in this study (Fig. 7, Table 2).

T2D is a heterogeneous disorder characterized by insulin resistance and pancreatic β-cell dysfunction that cannot properly secrete insulin in response to hyperglycemia (Hui et al., 2007). In this study, decreased insulin levels and loss of β-cell integrity were observed in the DBC group compared to the NC group (Table 2). In

Table 3
<table>
<thead>
<tr>
<th>Condition</th>
<th>NC</th>
<th>DBC</th>
<th>DAMH</th>
<th>DMMH</th>
<th>NAMH</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW (g)</td>
<td>20.2 ± 1.0</td>
<td>14.3 ± 0.31</td>
<td>20.7 ± 0.20</td>
<td>20.7 ± 0.40</td>
<td>20.7 ± 0.20</td>
</tr>
<tr>
<td>Relative BW (%)</td>
<td>2.80 ± 0.29</td>
<td>6.50 ± 0.34</td>
<td>3.36 ± 0.14</td>
<td>3.31 ± 0.40</td>
<td>3.10 ± 0.45</td>
</tr>
<tr>
<td>Glycogen (mg/g tissue)</td>
<td>6.78 ± 0.81</td>
<td>8.25 ± 0.83</td>
<td>7.83 ± 0.53</td>
<td>6.86 ± 0.61</td>
<td>6.92 ± 0.50</td>
</tr>
</tbody>
</table>

Data are presented as the mean ± SD of 5–7 animals. * and ** Values with different letter along a row for a given parameter 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; DAMH: Diabetic A. melegueta low dose; DMMH: Diabetic A. melegueta high dose; NAMH: Normal A. melegueta high dose.

* Relative liver weight (%) = Weight of the liver / Body weight (g) × 100%.
the diabetic AMEF treated as well as DMF groups, these effects were significantly ameliorated and the morphology of pancreas was restored near to normal. The improved serum insulin levels in the AMEF treated diabetic animals (DMAL and DMAMH) (Table 2) could be due to the regeneration of pancreatic islets observed in the pancreatic histology (Fig. 7), which has been additionally supported by the significantly higher HOMA-β index (β-cell function) in the treated groups compared to the DBC group (Table 2). The significantly lower HOMA-IR index in the DMAL and DMAMH groups compared to the DBC group also confirmed the improvement in insulin sensitivity as well as the stimulation of peripheral glucose absorption in these groups (Table 2). Therefore, the anti-hyperglycemic effect of AMEF could partly be mediated via decreasing insulin resistance and not only by hyperinsulinemic mechanism. The anti-hyperglycemic effect of AMEF is further supported by the significantly lower serum fructosamine levels in the DMAL and DMAMH groups compared to the DBC group which did not differ significantly with the NC and DMF groups (Table 2). This could be attributed to some of the phenolic compounds such as gingerols, shogaols and paradols that were reported to possess anti-oxidative potentials (Ilic et al., 2014, 2010).

On the other hand, increase liver weight as well as accumulation of liver glycogen are some common pathological features of metabolic disorders including T2D which apparently lead to hepatomegaly (Pachos and Paletas, 2009). In our study, the relative liver weight was found to decrease in AMEF treated diabetic rats whereas no significant effect was observed in the normal treated animals (Table 3), which is contradictory with an earlier report (Ilic et al., 2010). Ilic et al. (2010) highlighted an increase on the absolute and relative liver weight in normal animals after the supplementation of seed ethanolic extract, which could be attributed to the higher dosages (450 and 1500 mg/kg bw) used in the reported study when the lower dosage (120 mg/kg bw) did not cause any significant increase on absolute and relative liver weight of normal treated animals (Ilic et al., 2010). Similarly, a reduction on glycogen content in the liver of treated diabetic animals was observed, which apparently indicates the attenuation of hepatic glycogenesis. This could possibly be another anti-hyperglycemic mechanism exhibited by AMEF and has been a possible mode of action by several anti-diabetic plant-derived formulations and compounds (Vats et al., 2004; Brouham et al., 2006; Grover et al., 2002) which was further supported by the anti-hyperglycemic effect of AMEF (Table 4).

There has been a significant elevation on serum lipid profiles and calculated AI and CRI indices with a reduction on serum HDL-cholesterol in DBC group compared to the NC group (Table 4). This might be due to the effect of fructose feeding on DBC group. Fructose metabolism has been shown to be independent of insulin action, which is an important factor for the regulation of fat production as well as energy utilization by the cells (Bassano et al., 2005). In addition, fructose induced more hyperlipidemic condition by increasing the levels of LDL, VLDL and total cholesterol in experimental animals compared to other carbohydrate molecules (Benado et al., 2004). Oral intervention of AMEF at lower and higher dosages as well as the standard drug prevents diabetes-induced dyslipidemia, reduced the risk of atherogenesis and coronary artery disease and augmented serum HDL-cholesterol level (Table 4). Our result is in line with the results of a number of recently published studies (Adeneye, 2012; Adeyi et al., 2012; Sharmi et al., 2012).

In a previous study, co-administration of seed aqueous extract (100 and 150 mg/kg bw) and CCl4 showed hepatoprotective action in rats (Rokou et al., 2013). Nwozo and Oyinloye (2011) have also reported the protective effect of seed aqueous extract (100 and 200 mg/kg bw/day) to ethanol-induced liver toxicity in rats. In contrast, the treatment with higher dosages of seed ethanolic extract (450 and 1500 mg/kg bw) for 4 weeks induced liver damage in normal rats via elevation of the levels of liver-function

<table>
<thead>
<tr>
<th>Table 4</th>
<th>Serum lipid profiles atherogenic and coronary risk indices of different animal groups at the end of the experimental period.</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td>DBC</td>
</tr>
<tr>
<td>TC (mg/dl)</td>
<td>76.29 ± 7.40</td>
</tr>
<tr>
<td>TG (mg/dl)</td>
<td>70.29 ± 10.34</td>
</tr>
<tr>
<td>LDL-Cholesterol (mg/dl)</td>
<td>12.96 ± 4.16</td>
</tr>
<tr>
<td>HDL-Cholesterol (mg/dl)</td>
<td>40.29 ± 8.35</td>
</tr>
<tr>
<td>Atherogenic index (AI)</td>
<td>0.57 ± 0.53</td>
</tr>
<tr>
<td>Coroniary risk index (CRI)</td>
<td>1.57 ± 0.53</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SD of 5-7 animals. a,b Values with different superscript letters along a row for a given parameter 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; DMAL, Diabetic A. melgeon low dose; DMAMH, Diabetic A. melgeon high dose; DMF, Diabetic Metformin; NAMH, Normal A. melgeon high dose; TC, Total cholesterol; TG, Triglycerides; LDL-cholesterol, Low density lipoprotein-cholesterol; HDL-cholesterol, High density lipoprotein-cholesterol.

<table>
<thead>
<tr>
<th>Table 5</th>
<th>Serum ALT, AST, AIP and other biochemical parameters different animal groups at the end of the experimental period.</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td>DBC</td>
</tr>
<tr>
<td>ALT (UI)</td>
<td>77.40 ± 20.38</td>
</tr>
<tr>
<td>AST (UI)</td>
<td>86.40 ± 11.41</td>
</tr>
<tr>
<td>ALP (mg/dl)</td>
<td>140.40 ± 33.13</td>
</tr>
<tr>
<td>Creatinine (mg/dl)</td>
<td>2.52 ± 0.78</td>
</tr>
<tr>
<td>Urea (mg/dl)</td>
<td>22.00 ± 6.09</td>
</tr>
<tr>
<td>Uric acid (mg/dl)</td>
<td>2.00 ± 0.97</td>
</tr>
<tr>
<td>LDH (U/L)</td>
<td>264.60 ± 52.65</td>
</tr>
<tr>
<td>CK-MB (UI)</td>
<td>737.54 ± 82.58</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SD of 5-7 animals. a,b Values with different superscript letters along a row for a given parameter 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; DMAL, Diabetic A. melgeon low dose; DMAMH, Diabetic A. melgeon high dose; DBC, Diabetic Metformin; NAMH, Normal A. melgeon high dose; ALT, Alanine transaminase; AST, Aspartate transaminase; AIP, Alkaline phosphatase; LDH, Lactate dehydrogenase; CK-MB, Creatine kinase.
enzymes in serum (Iw et al., 2010). On the other hand, treatment with 150 or 300 mg/kg bw AMEF showed significant hepatoprotective effects without any toxicity (Table 5), which is in line with the results of a number of previously published studies (Kokou et al., 2013; Nwoza and Oyinboye, 2011). Furthermore, in comparison with ALT, AST is less specific for the detection of hepatic injury (Center, 2007). This is partly due to the fact that the half-life of ALT is much longer compared to AST in experimental animals (Ramaiah 2007; Meyer and Harvey, 2004). Therefore, in a condition of acute liver injury, there will be an increase in both serum ALT and AST levels. However, the serum AST level may likely return to normal more rapidly compared to the level of serum ALT, which makes ALT more sensitive in assessing hepatic damage in disease conditions (Ramaiah, 2007; Meyer and Harvey, 2004). Therefore, the unaffect serum ALT level in our present result could be linked to the above-stated factor. Hence, the hepatoprotective ability of AMEF is supported by the reduced serum ALT, ALP, and uric acid levels in the DAML and DAMH groups compared to the DBC group (Table 5). Moreover, in addition to the amelioration of hepatic injury as well as the risk of arteriosclerosis and coronary artery diseases, diabetic AMEF treated animals demonstrated lower serum LDH and CK-MB levels, suggesting the cardioprotective effects of AMEF (Table 5). The CK-MB offers a much greater extent in cardiac muscle than skeletal muscle and is thus a useful diagnostic marker in myocardial infarction (Baird et al., 2012). The significantly lower serum creatinine, uric acid in the AMEF treated groups compared to the DBC group suggested its possible effects on the amelioration of diabetic nephropathy (Table 5).

In conclusion, various doses of A. melegueta ethyl acetate fraction from fruit possess strong anti-T2D activity via improving both the reduction of blood glucose level and hyperglycemia, improving glucose tolerance ability, insulin sensitivity, amelioration of pancreatic β-cell and β-cell function, improving dyslipidemia and number of organ specific diabetic complications related parameters in type 2 diabetic rats when α-amylase and α-glucosidase inhibitory activity of this fraction may be partly responsible for some of these effects. Hence, ethyl acetate fraction of A. melegueta fruit from crude ethanolic extract may be a potential anti-diabetic natural product with no considerable side effects. The isolation and partial characterization of the active principle from AMEF is currently underway to find the compounds responsible for this activity in addition to its molecular mechanisms behind the anti-diabetic activity.

Declaration of interest

We declare that we have no conflict of interest.

Acknowledgments

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NATURAL DRUGS

PHYTOCHEMISTRY, ANTIOXIDATIVE AND ANTIDIABETIC EFFECTS OF VARIOUS PARTS OF EUGENIA CARYOPHYLLATA THUNB. IN VITRO

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Abstract: Eugenia caryophyllata Thunb. possesses a wide variety of therapeutic potential and has been recognized as a source of antioxidant and antidiabetic agents. This study was designed to investigate and compare the antioxidant and antidiabetic effects of different parts (bud, leaf, stem and root) of E. caryophyllata. Samples were sequentially extracted using solvents of increasing polarity and investigated for 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity, total reducing power, inhibition of hemoglobin glycosylation, α-amylase and α-glucosidase activities as markers of in vitro antidiabetic effects. Phytochemicals were analyzed using gas chromatography-mass spectrometry (GC-MS) analysis. The ethanol (EtOH) extracts of the bud, leaf and stem exhibited a higher total polyphenol and flavonoid content compared to other extracts, with the EtOH extracts of the bud and leaf exhibiting lower IC50 values than the extracts of the other plant parts for all the models used in this study (DPPH bud: 0.17 ± 0.02 mg/mL; leaf: 0.03 ± 0.01 mg/mL; hemoglobin glycosylation: bud: 0.17 ± 0.02 mg/mL; leaf: 0.83 ± 0.04 mg/mL; α-amylase: bud: 0.20 ± 0.02 mg/mL; 322.27 ± 73.29 mg/mL; α-glucosidase: bud: 0.03 ± 0.01 mg/mL; 0.74 ± 0.02 mg/mL). A similar result was observed for the reducing potentials of Fe2+ to Fe3+ by the extract. The GC-MS analysis of these parts indicated several aromatic phenols, acids, carophyllene and long chain aliphatic acids. Conclusively, various solvent extracts from the leaf, bud and stem of E. caryophyllata showed higher antioxidant and antidiabetic effects in comparison to common standards used in these assays.

Keywords: α-amylase, α-glucosidase, antidiabetic, antioxidative, Eugenia caryophyllata

With the increasing cost of drugs and the accessibility of these drugs to African countries, especially in rural and remote areas, the popularity of using medicinal plants for therapeutic purposes has increased significantly during the past decade. In Africa, about 80% of the population depend almost entirely on traditional medicine or herbal medicine, for their primary health care needs (1, 2). This is not surprising as several modern conventional drugs were originally obtained from plant sources and caused minimal or no side effects compared to synthetic drugs. For instance, metformin, an antidiabetic drug originated from Galega officinalis (3), quinine and quinidine, antirhymic drugs were phytochemicals from Cinchona spp (4). In addition, the perceived effectiveness of the herbal therapies as well as the availability of these medicinal plants makes them a popular source of medicines. Moreover, the African continent accounts for about 25% of the total number of higher plants in the world, where more than 5400 medicinal plants are reported to have over 16300 medicinal uses (5). Consequently, the World Health Organization (WHO) has encouraged researchers to investigate and validate the folk uses of plants used in the treatment of various diseases such as diabetes mellitus, hypertension, malaria and microbial infections among others (6).

Eugenia caryophyllata Thunb. (Syn. Syzygium aromaticum (Linn.) Merr. & L.M. Perry) or clove is an aromatic plant that belongs to the Myrtaceae family (7) and is widely available in Africa, Asia and North America. The bud and leaf are locally used as spice in various food preparations and possess a wide variety of therapeutic potential (8). E. caryophyllata parts or extracted oils are traditionally used in the treatment of toothache (9) and also been reported to have a strong antimicrobial effect (10). It is locally utilized in the treatment of asthma in Asia (11), disorders associated with respiratory

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and digestive systems (12) and in diarrheal and sexual disorders (13).

Previous studies reported that E. caryophyllata bud and its essential oils possessed strong antioxidative properties and thus terminate lipid peroxidation and other oxidative processes (14, 15). Adefegha and Oboh (16) reported the ability of the bud to inhibit α-amylase and α-glucosidase activities in vitro. The insulinoactive effect (17) and hepatoprotective effect against ethanol-induced liver cell injury (18) of the bud has also been reported. More recently, antihyperglycemic, hypolipidemic, hepatoprotective and antioxidative effects of clove powder have also been reported (19). Active principles identified in the bud or its essential oils include eugenol, β-caryophyllene, fatty acids, triterpenes, alcohols, flavonoids and other phenolics (20). It is hypothesized that since E. caryophyllata buds have been reported to have antioxidative and antidiabetic effects, other parts of the plant may also have similar activities and possess similar bioactive compounds. However, till now, the majority of scientific investigations had only focused on the buds and its essential oils. No data is available on the validity or potential of extracts of the other parts of the plant. Thus, this study was designed to investigate and compare the antioxidative and antidiabetic effects of various parts (including bud) of E. caryophyllata using several in vitro models. Additionally, phytochemical analysis of the possible bioactive compounds present in most active extracts was also carried out using GC-MS analysis.

MATERIALS AND METHODS

Chemicals and reagents

Ascorbic acid, quercetin, hemoglobin (human lyophilized powder), gallic acid, aluminum chloride, α-amylase from porcine pancreas, α-glucosidase from Saccharomyces cerevisiae and 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) were purchased from Sigma-Aldrich through Capital Lab Supplies, New Germany, South Africa. Folin Ciocalteau reagent was purchased from Merck Chemical Company, South Africa. Gentamycin was purchased from EMD Chemicals, San Diego, CA, USA.

Plant material

The bud, leaf, stem and root samples of E. caryophyllata were identified and authenticated at the herbarium unit of the Biological Science Department, Ahmadu Bello University, Zaria, Nigeria by Mr. Umar Gallah and a voucher specimen number 13209 was deposited accordingly. The plant samples were immediately washed and shade-dried to constant weights for two weeks. The dried samples were ground to a fine powder, and then stored individually in airtight containers to transport to the University of KwaZulu-Natal, Westville campus, Durban, South Africa for subsequent analysis.

Preparation of the plant extracts

Forty (40) grams of each of the fine powdered plant parts were separately defatted with 200 mL of n-hexane. The defatted material was sequentially extracted with ethyl acetate, ethanol and water by soaking for 48 h in 200 mL of the relevant solvent followed by a 2 h orbital shaking at 200 rpm. After filtration through Whatmann filter paper (No. 1), respective solvents were evaporated under vacuum, using a rotary evaporator (Buchi Rotavapor II, Buchi, Germany) at 40°C under reduced pressure to obtain the different solvent extracts with the exception of the aqueous extracts which were dried on a water bath at 45°C. The extracts in each case were weighed, transferred to micro tubes and stored in a refrigerator at 4°C until further analysis.

Estimation of total polyphenol content

The total polyphenol content of each extract was determined (as gallic acid equivalent) according to the method described by McDonald et al. (21) with slight modifications. Briefly, 200 μL of the extract (240 μg/mL) was incubated with 1 mL of 10 times diluted Folin Ciocalteau reagent and 800 μL of 0.7 M Na₂CO₃ for 30 min at room temperature. The absorbance values were then determined at 765 nm in a Shimadzu UV mini 1240 spectrophotometer (Shimadzu Corporation, Kyoto, Japan). All measurements were done in triplicate.

Determination of total flavonoid content

The total flavonoid content of the plant extracts were determined using a method reported by Chang et al. (22) with slight modification. Briefly, 500 μL (240 μg/mL) of each sample was mixed with 500 μL methanol, 50 μL of 10% AlCl₃, 50 μL of 1 mol/L potassium acetate and 1.4 mL water, and allowed to incubate at room temperature for 30 min. The absorbance of the reaction mixture was subsequently measured at 415 nm using the spectrophotometer mentioned above. The total flavonoid content was calculated as quercetin equivalent (QE) in μg per mg dry extract.

DPPH radical scavenging activity

The total free radical scavenging activity of the extracts was determined and compared to that of
ascorbic and gallic acids by using a slightly modified method described by Tuba & Guler (23). An aliquot of 500 µL of a 0.3 mM solution of DPPH in methanol was added to 1 mL of the extracts at different concentrations (30, 60, 120 and 240 µg/mL). These solutions were mixed and incubated in the dark for 30 min at room temperature. The absorbance was measured at 517 nm against blank samples lacking the free radical scavengers.

**Ferric (Fe³⁺) reducing antioxidant power assay**

The ferric reducing antioxidant power method of Oyaizu (24) was used with slight modifications to measure the reducing capacity of the extracts. To perform this assay, 1 mL of each extract (30, 60, 120 and 240 µg/mL) was incubated with 1 mL of 0.2 M sodium phosphate buffer (pH 6.6) and 1% potassium ferricyanide at 50°C for 30 min. After 30 min incubation, the reaction mixtures were acidified with 1 mL of 10% trichloroacetic acid. Thereafter, 1 mL of the acidified sample of this solution was mixed with 1 mL of distilled water and 200 µL of FeCl₃ (0.1%). The absorbance of the resulting solution was measured at 700 nm in a spectrophotometer. Increased absorbance of the reaction mixture indicated greater reductive capability of the extracts (15).

**Inhibition of hemoglobin glycosylation**

Inhibition of non-enzymatic glycosylation of hemoglobin by various extracts was investigated by the modified method of Pal & Dutta (25). Glucose (2%), hemoglobin (0.06%) and gentamycin (0.02%) solutions were prepared in phosphate buffer 0.01 M, pH 7.4. An aliquot of 1 mL of each of the solutions were mixed with 1 mL of different concentration of the extracts (30, 60, 120 and 240 µg/mL) in dimethyl sulfoxide (DMSO). These mixtures were incubated in the dark at room temperature for 72 h. The percentage inhibition of glycosylation of hemoglobin was calculated from the absorbance measured at 520 nm. Gallic acid was used as a standard.

**α-Amylase inhibitory effect**

The α-amylase inhibitory effect of the extracts was carried out using a modified method of McCue and Shetty (26). Briefly, a 250 µL aliquot of extract at different concentrations (30, 60, 120 and 240 µg/mL) was placed in a tube and 250 µL of 0.02 M sodium phosphate buffer (pH 6.9) containing α-amylase solution was added. This solution was preincubated at 25°C for 10 min, after which 250 µL of 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9) was added at a time interval of 10 s and then further incubated at 25°C for 10 min. The

<table>
<thead>
<tr>
<th>Samples</th>
<th>Yield (%)</th>
<th>Total polyphenols content (mg/g GAE)</th>
<th>Total flavonoids content (mg/g QE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bud</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>2.75</td>
<td>9.68 ± 0.26⁸</td>
<td>1.08 ± 0.29⁸</td>
</tr>
<tr>
<td>Ethanol</td>
<td>5.00</td>
<td>27.23 ± 1.45⁶</td>
<td>10.64 ± 0.59⁶</td>
</tr>
<tr>
<td>Aqueous</td>
<td>1.25</td>
<td>11.17 ± 0.11⁴</td>
<td>3.19 ± 0.41⁴</td>
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<tr>
<td>Leaf</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Ethyl acetate</td>
<td>0.85</td>
<td>7.45 ± 0.11⁴</td>
<td>1.11 ± 0.24⁴</td>
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<td>Ethanol</td>
<td>4.05</td>
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<tr>
<td>Aqueous</td>
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<td>6.94 ± 0.10⁸</td>
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<tr>
<td>Root</td>
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<tr>
<td>Ethyl acetate</td>
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<td>2.65 ± 0.05⁸</td>
<td>0.58 ± 0.12⁸</td>
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<td>Stem</td>
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<td></td>
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<tr>
<td>Ethyl acetate</td>
<td>0.62</td>
<td>0.66 ± 0.05⁶</td>
<td>0.17 ± 0.12²</td>
</tr>
<tr>
<td>Ethanol</td>
<td>2.77</td>
<td>6.12 ± 0.11⁴</td>
<td>2.47 ± 0.12²</td>
</tr>
<tr>
<td>Aqueous</td>
<td>1.45</td>
<td>1.40 ± 0.12⁸</td>
<td>0.33 ± 0.12²</td>
</tr>
</tbody>
</table>

Data are presented as the mean ± SD values of triplicate determinations. ** Different superscripted letters within a column are significantly different from each other (Tukey's-HSD multiple range post hoc test, p < 0.05).
Figure 1. DPPH radical scavenging activity (%) of bud (A), leaf (B), root (C) and stem (D) extracts of *E. caryophyllata*. Data are presented as the mean ± SD of triplicate determinations. **Different letters presented over the bars for a given concentration of each extract are significantly different from each other (Tukey’s HSD multiple range post hoc test, p < 0.05).**
reaction was terminated after incubation by adding 1 mL of dinitrosalicylic acid (DNS) reagent. The tube was then boiled for 10 min and cooled to room temperature. The reaction mixture was diluted with 5 mL of distilled water and the absorbance was measured at 540 nm using a Shimadzu UV mini 1240 spectrophotometer. A control was prepared using the same procedure, replacing the extract with distilled water.

**α-Glucosidase inhibitory effect**

The inhibitory effect of the plant extracts on α-glucosidase activity was determined according to the method described by Kim et al. (27) using α-glucosidase from *Saccharomyces cerevisiae*. The substrate solution p-nitrophenyl glucopyranoside (pNPG) was prepared in 20 mM phosphate buffer, pH 6.9. An aliquot of 500 μL of α-glucosidase was then preincubated with 250 μL of the different concentrations of the extracts (30, 60, 120 and 240 μg/mL) for 10 min. Thereafter, 250 μL of 5.0 mM pNPG was dissolved in 20 mM phosphate buffer (pH 6.9) as a substrate to start the reaction. The reaction mixture was incubated at 37°C for 30 min. The α-glucosidase activity was determined by measuring the yellow colored p-nitrophenol released from pNPG at 405 nm. The results of the DPPH, inhibition of hemoglobin glycosylation, α-amylase and α-glucosidase assays were expressed as a percentage of the control (blank) according to the following formula:

\[
\% \text{ Inhibition} = \left(\frac{\text{Abs. of control} - \text{Abs. of extract}}{\text{Abs. of control}}\right) \times 100
\]

Concentrations of extracts resulting in 50% inhibition of enzyme activity (IC₅₀) were calculated from the data as well.

**Gas chromatography-mass spectroscopic (GC-MS) analysis**

Based on the results of antioxidative and antidiabetic studies, the most active extracts (EtOH bud, leaf and stem) were subjected to GC-MS analysis. The GC-MS analysis was conducted with an Agilent Technology 6890 gas chromatograph cou-

<table>
<thead>
<tr>
<th>Samples</th>
<th>DPPH scavenging activity</th>
<th>Non-enzymatic glycosylation of hemoglobin</th>
<th>α-Amylase inhibitory effect</th>
<th>α-Glucosidase inhibitory effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bud</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>0.06 ± 0.01*</td>
<td>535.62 ± 372.42*</td>
<td>320.36 ± 167.03*</td>
<td>0.68 ± 0.03*</td>
</tr>
<tr>
<td>Ethanol</td>
<td>0.02 ± 0.01*</td>
<td>0.17 ± 0.02*</td>
<td>0.20 ± 0.02*</td>
<td>0.03 ± 0.01*</td>
</tr>
<tr>
<td>Aqueous</td>
<td>0.62 ± 0.14*</td>
<td>559.96 ± 377.16*</td>
<td>386.36 ± 97.28*</td>
<td>5260.41 ± 54.99f</td>
</tr>
<tr>
<td>Leaf</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>0.16 ± 0.03*</td>
<td>301.92 ± 195.09*</td>
<td>505.79 ± 32.95*</td>
<td>414.04 ± 75.44f</td>
</tr>
<tr>
<td>Ethanol</td>
<td>0.03 ± 0.01*</td>
<td>0.83 ± 0.04*</td>
<td>322.27 ± 73.29*</td>
<td>0.74 ± 0.02*</td>
</tr>
<tr>
<td>Aqueous</td>
<td>0.07 ± 0.02*</td>
<td>584.56 ± 234.39*</td>
<td>983.49 ± 62.64*</td>
<td>596.30 ± 7.32f</td>
</tr>
<tr>
<td>Root</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>296.73 ± 117.28f</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Ethanol</td>
<td>3.66 ± 1.16f</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Aqueous</td>
<td>222980.33 ± 815.45f</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Stem</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>230.68 ± 99.66f</td>
<td>1019.48 ± 75.97f</td>
<td>1110.51 ± 108.53f</td>
<td>89149.67 ± 107.99f</td>
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<tr>
<td>Ethanol</td>
<td>0.99 ± 0.32f</td>
<td>1.03 ± 0.42f</td>
<td>349.53 ± 282.83f</td>
<td>6.89±0.11c</td>
</tr>
<tr>
<td>Aqueous</td>
<td>527647.61 ± 684.74f</td>
<td>650.06 ± 276.14f</td>
<td>2561.78 ± 109.69f</td>
<td>83528.18 ± 1001.29f</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>0.03 ± 0.02*</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>0.05 ± 0.01*</td>
<td>0.20 ± 0.01*</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Acarbose</td>
<td>ND</td>
<td>4.91 ± 0.80*</td>
<td>0.34 ± 0.02*</td>
<td></td>
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</table>

Data are presented as the mean ± SD values of triplicate determinations. **Different superscript letters presented within a column for a given parameter are significantly different from each other (Tukey’s-HSD multiple range post hoc test, p < 0.05). ND = Not determined.
plied with an Agilent 5973 Mass Selective Detector and driven by Agilent Chemstation software. Compounds were identified by direct comparison of the retention times and mass spectral data with those in the National Institute of Standards and Technology (NIST) library.

Statistical analysis

All data are presented as the mean ± SD of triplicates determination. Data were analyzed by using a statistical software package (SPSS for Windows, version 18, IBM Corporation, NY, USA) using Tukey’s HSD multiple range post-hoc test. Values were considered significantly different at p < 0.05.

RESULTS

The yield recovered from different solvent extracts of various parts of *E. canophyllata* indicated that higher yields are obtained from the bud and leaf extracts compared to roots and stem (Table 1). Furthermore, different parts showed variable amounts of polyphenols and flavonoid contents. EtOH extracts of various parts of the plant possessed a significantly (p < 0.05) higher total polyphenol and flavonoid content with the bud, leaf and stem containing the highest (Table 1). It was also observed that the aqueous extracts from the bud and stem showed a higher polyphenolic and flavonoid content compared to the ethyl acetate extracts. In the leaf, the aqueous extract had lower polyphenolic content than the ethyl acetate extract and in the root, the aqueous extract exhibited a lower flavonoid content compared to the ethyl acetate extract.

The ability of various solvent extracts to scavenge the DPPH radical were investigated and compared with ascorbic acid and gallic acid. The results are presented in Figure 1. It is evident from the results that the EtOH extracts from various parts of *E. canophyllata* exhibited lower *IC*₅₀ values compared to other solvent extracts. The bud (A) and leaf (B) EtOH extracts demonstrated significantly (p < 0.05) lower *IC*₅₀ values of 0.02 ± 0.01 mg/mL and 0.03 ± 0.01 mg/mL, respectively, compared to other

<table>
<thead>
<tr>
<th>Extracts</th>
<th>Compounds</th>
<th>Retention time (min)</th>
<th>Mass [a.m.u.]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bud</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Eugenol</td>
<td>6.67</td>
<td>164 [M⁺]</td>
</tr>
<tr>
<td>3</td>
<td>Caryophyllene</td>
<td>7.21</td>
<td>204 [M⁺]</td>
</tr>
<tr>
<td>4</td>
<td>2-Acetyl-4(2-propenyl)anisole</td>
<td>7.72</td>
<td>206 [M⁺]</td>
</tr>
<tr>
<td>5</td>
<td>1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester</td>
<td>11.33, 13.16</td>
<td>279 [M + H⁺]</td>
</tr>
<tr>
<td>6</td>
<td>n-Hexadecanoic acid</td>
<td>10.04-10.22</td>
<td>256 [M⁺]</td>
</tr>
<tr>
<td>7</td>
<td>9,12-Octadeicadienal</td>
<td>11.12, 14.40</td>
<td>264 [M⁺]</td>
</tr>
<tr>
<td>8</td>
<td>n-Octadecanoic acid</td>
<td>11.21</td>
<td>284 [M⁺]</td>
</tr>
<tr>
<td>9</td>
<td>9,12-Octadecadienoic acid</td>
<td>11.50</td>
<td>280 [M⁺]</td>
</tr>
<tr>
<td>10</td>
<td>Heneicosanoic acid</td>
<td>11.96</td>
<td>326 [M⁺]</td>
</tr>
<tr>
<td>Leaf</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Eugenol</td>
<td>6.67</td>
<td>164 [M⁺]</td>
</tr>
<tr>
<td>3</td>
<td>Caryophyllene</td>
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<td>279 [M + H⁺]</td>
</tr>
<tr>
<td>Stem</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Eugenol</td>
<td>6.66</td>
<td>164 [M⁺]</td>
</tr>
<tr>
<td>2</td>
<td>2-Methoxy-3-(2-propenyl)phenol</td>
<td>6.66</td>
<td>164 [M⁺]</td>
</tr>
<tr>
<td>3</td>
<td>Caryophyllene</td>
<td>7.72</td>
<td>204 [M⁺]</td>
</tr>
</tbody>
</table>

* isomers co-eluted at the same retention time

Table 3. Phytochemicals identified in the EtOH extracts of the bud, leaf and stem by GC-MS
Figure 2. Total reducing power (relative to gallic acid) of bud (A), leaf (B) and stem (C) extracts of E. caryophyllata. Data are presented as the mean ± SD of triplicate determinations. **Different letters presented over the bars for a given concentration of each extract are significantly different from each other (Tukey’s HSD multiple range test; p < 0.05)**.
Figure 3. Inhibition of hemoglobin glycosylation (%) of basil (A), leaf (B) and stem (C) extracts of *E. caryophyllata*. Data are presented as the mean ± SD of triplicate determinations. Different letters presented over the bars for a given concentration of each extract are significantly different from each other (Tukey’s HSD multiple range post hoc test, p < 0.05).
Figure 4. α-Amylase inhibition (%) of bud (A), leaf (B) and stem (C) extracts of E. caryophylla. Data are presented as the mean ± SD of triplicate determinations. Different letters presented over the bars for a given concentration of each extract are significantly different from each other (Tukey's HSD multiple range post hoc test, p < 0.05).
Figure 5. α-Glucosidase inhibition (%) of bud (A), leaf (B) and stem (C) extracts of *E. cariophyllata*. Data are presented as mean ± SD of triplicate determinations. Different letters presented over the bars for a given concentration of each extract are significantly different from each other (Tukey's HSD multiple range post hoc test, p < 0.05).
Figure 6. GC-MS chromatograms of ethanolic extracts of bud (A), leaf (B) and stem (C) extracts of *E. caryophyllata*. 
Figure 7. Structures of identified compounds from bud, leaf and stem of E. caryophyllana [Aromatic phenols, acids and caryophyllene (A), long chain aliphatic acids (B)]
solvent extracts (Table 2). The effect was comparable to that of ascorbic acid (0.03 ± 0.02 mg/mL) and gallic acid (0.05 ± 0.01 mg/mL).

Furthermore, our results indicated that the extracts of all the parts with the exception of the root, possessed the ability to reduce Fe^{3+} to Fe^{2+} (in terms of percentage gallic acid equivalent), which is comparable to that of ascorbic acid (Fig. 2). The EtOH extracts demonstrated a significantly (p < 0.05) higher activity compared to other solvent extracts in a dose-dependent manner. Bud (A), leaf (B) and stem (C) EtOH extracts showed higher reducing power compared to other solvent extracts. Similarly, the highest activity was exhibited by the EtOH extract of the bud, which is significantly higher compared to ascorbic acid. The extent of the reducing power (Fe^{3+} to Fe^{2+}) by different parts of *E. caryophyllata* is in the order: bud > leaf > stem > root.

In addition, the EtOH extracts of the bud (A), leaf (B) and stem (C) exhibited significantly (p < 0.05) higher inhibitory effects toward hemoglobin glycosylation (Fig. 3). The IC_{50} values recorded were 0.17 ± 0.02, 0.83 ± 0.04 and 1.03 ± 0.42 mg/mL for the bud, leaf and stem, respectively (Table 2). Similarly, various extracts from the root showed no inhibitory effect towards hemoglobin glycosylation. The leaf and stem EtOH extracts recorded higher IC_{50} values than the bud EtOH extracts although not significantly different (p < 0.05) in comparison to gallic acid (IC_{50} values: 0.20 ± 0.01 mg/mL).

Figure 4 shows the α-amylase inhibitory effect of various extracts from *E. caryophyllata* parts. Only the bud EtOH extract exhibited a significantly (p < 0.05) lower IC_{50} value (0.20 ± 0.02 mg/mL) compared to acarbose (IC_{50} value: 4.91 ± 0.80 mg/mL). The inhibitory effects observed on the leaf and stem EtOH extracts as well as the bud ethyl acetate and aqueous extracts did not differ significantly. The IC_{50} values demonstrated by these extracts were significantly higher compared to acarbose (4.91 ± 0.80 mg/mL). In addition, ethyl acetate extracts from various parts showed lower IC_{50} values compared to the aqueous extracts (Table 2). No activity was recorded with various solvent extracts from the root.

Similarly, all the solvent extracts with the exception of the root extracts demonstrated dose-dependent inhibition of α-glucosidase (Fig. 5). Significantly (p < 0.05) lower IC_{50} values were exhibited by the EtOH extracts of the bud (0.03 ± 0.01 mg/mL), leaf (0.74 ± 0.02 mg/mL), and stem (6.89 ± 0.11 mg/mL) and by the ethyl acetate extract (0.68 ± 0.03 mg/mL) of the bud compared to other solvent extracts (Table 2). The IC_{50} values demonstrated by different parts are in the order of bud > leaf > stem. The ethyl acetate extract of the stem showed the least α-glucosidase inhibitory effect, having the highest IC_{50} value.

Based on the results obtained, the EtOH extracts of the bud, leaf and stem showed consistently higher activity compared to other solvent extracts and was thus subjected to GC-MS analysis to determine the phytochemicals present in this extract. From the results obtained, several peaks were observed in the chromatograms (Fig. 6). Peaks detected were compared with the data available in the NIST library and the compounds detected correspond to aromatic phenols (1, 2), caryophyllene (3), aromatics containing ether, ester and acid moieties (4, 5) and long chain aliphatic acids (6-10) (Table 3; Fig. 7). Eugenol (1) and caryophyllene (3) were detected in all the extracts analyzed and 2-acetyl-4-(2-propenyl)anisole (4) and 1,2-benzenedicarboxylic acid mono (2-ethylhexyl) ester (5) were detected in the EtOH extracts of the bud and leaf. The long chain aliphatic acids (6-10) were present in the EtOH extract of the bud alone.

**DISCUSSION**

The present study investigated and compared, for the first time, the antioxidantive and antidiabetic effects of various solvent extracts of *E. caryophyllata* parts in vitro. This is the first report of the potential of plant parts other than the buds (leaf, root and stem) as antidiabetic and antioxidantive agents. From the results of this study, it is evident that EtOH extracts have higher yields and contain a higher total polyphenol and flavonoid content in comparison to other solvent extracts. This is consistent with previous findings that ethanol is the best solvent for the extraction of a maximum yield of polyphenols compared to other solvents (28). The amount of total polyphenols and flavonoid content was in the order of bud > leaf > stem > root (Table 1). A possible explanation could be linked to several factors including genetic and environmental factors (nature of the soil, high temperature and rainfall) in addition to growth or maturation stages (29, 30). Although no correlation analysis was carried out in this study, previous studies strongly correlate antioxidantive effect to total polyphenol contents (31).

Methods adopted to assess the antioxidantive effect of various parts of *E. caryophyllata* include among others, the DPPH radical scavenging assay, a widely used method for assessing the antioxidant status of compounds or plant products. In addition,
calculated IC₅₀ values were used to demonstrate the extent of scavenging power for different parts of the plant. The lower the IC₅₀ values the higher the scavenging activity. More importantly, the consistently lower IC₅₀ value exhibited by the EtOH extracts, comparable to standard antioxidants (ascorbic acid and gallic acid) (Fig. 2) suggest that the extracts possess compounds with high radical-quenching ability that could terminate free radical activities. This is consistent with previous studies (32-34).

The ferric reducing power which reflects electron donating capacity of various extracts has been used to assess the antioxidative status of several natural products. In this study, the EtOH extracts from the bud, leaf and stem demonstrated higher activity and therefore possessed phytochemicals that cause the reduction of Fe³⁺ to Fe²⁺, which is monitored by measuring the formation of Perls prussian blue at 700 nm.

To further explore the antioxidative potential of various extracts, their ability to inhibit glycosylation of hemoglobin was determined. Glycosylation is a term used to describe the non-enzymatic reaction between reducing sugars and proteins (hemoglobin, albumin) and usually contributes enormously to the formation of advanced glycation end products (35). Consequently, it is evident from the results obtained that the EtOH extracts depicted lower IC₅₀ values, comparable to that of standard antioxidants used, indicating higher radical scavenging and anti-glycosylation activity (Table 2). This could be linked to the active principles present and the differences observed could be due to variation and concentration of the phytochemicals present in each part.

Moreover, it is an established fact that α-amylase and α-glucosidase inhibitors from natural sources play a significant role in diabetic management and control. This is achieved via a decrease in postprandial hyperglycemia through inhibition of α-amylase and α-glucosidase actions (16). However, for effective control of postprandial hyperglycemia, moderate α-amylase inhibition and potent α-glucosidase inhibition provide better options for controlling the availability of dietary glucose for absorption in the intestinal tract (36). This is due to adverse effects associated with strong α-amylase inhibition such as abdominal distension, flatulence, bowel necrosis and diarrhea (37). In this study, various solvent extracts demonstrated mild α-amylase inhibition and potent α-glucosidase inhibition, indicating a potential role as an anti-diabetic agent. The inhibitory effects of E. caryophyllata bud reported by Adefegha and Oboh (16) correspond with the results of this study. Furthermore, various solvent extracts from the leaf and stem could be good substitutes for the bud as potential antidiabetic agents, as the bud is being used locally in most parts of the world.

Phytochemical analysis of the most active parts resulted in the identification of compounds with potential medicinal usage (20). For example, eugenol (1) has already been implicated with a wide array of therapeutic application such as antioxidative, antidiabetic and antimicrobial effects. Interestingly, eugenol (1) and caryophyllene (3) were present in all parts while others like 2-acetyl-4(2-propenyl) anisole (4) appear in the bud and leaf but not the stem. The availability of eugenol (1) in most parts of E. caryophyllata has already been reported (38-40). Additionally, long chain aliphatic acids (6-10), also detected in the bud could synergistically or independently contribute to the observed higher activities of this part compared to others. Furthermore, the hydroxyl group present in compounds 1 and 2 could directly or indirectly be the key feature that contributed to the higher antioxidative and antidiabetic effects depicted by the bud, leaf and stem extracts (Fig. 7). The low reduction potentials of phenolics, hydroxyls and other related compounds inactive and terminate the initiation and propagation of chain reactions associated with oxidative damage (41). In a similar way, phenolics and hydroxyls were reported to interfere with some surface amino acid side chains in both α-amylase and α-glucosidase structures (42). This causes some conformational changes on the enzyme structure, thereby decreasing their actions and causing reduction on blood glucose levels and subsequently reduced postprandial hyperglycemia.

CONCLUSIONS

In conclusion, various solvent extracts from the bud, leaf and stem of E. caryophyllata possessed antioxidative as well as antidiabetic effects in vitro while the root extracts showed very low or no significant effects in the same assays. The effects of the leaf extracts were comparable to that of the bud and could therefore serve as a good substitute for various culinary and medicinal potentials of the bud. Hence, it is recommended that bioassay-guided fractionation of the EtOH extracts could be done in order to fully investigate the in vivo antidiabetic and antioxidative effects of this extract.

Declaration of interest

There is no conflict of interest within this article.
Acknowledgments

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REFERENCES


Received: 17.09.2014
Appendix II

NMR SPECTRA
1H NMR spectrum of 6-paradol

Expanded 1H NMR spectrum of 6-paradol
13C NMR spectrum of 6-paradol

Expanded 13C NMR spectrum of 6-paradol
DEPT spectrum of 6-paradol

Expanded DEPT spectrum of 6-paradol
HSQC NMR spectrum of 6-paradol

HMBC NMR spectrum of 6-paradol
Expanded HMBC NMR spectrum of 6-paradol (1)

Expanded HMBC NMR spectrum of 6-paradol (2)
**1H NMR spectrum of 6-shagaol**

**Expanded 1H NMR spectrum of 6-shagaol (1)**
Expanded 1H NMR spectrum of 6-shagaol (2)

13C NMR spectrum of 6-shagaol
NM/15-AM/5(21-31) in C6D6

Expanded 13C NMR spectrum of 6-shagaol

DEPT spectrum of 6-shagaol
Expanded DEPT spectrum of 6-shagaol

COSY spectrum of 6-shagaol
NOESY spectrum of 6-shagaol

HSQC NMR spectrum of 6-shagaol
HMBC NMR spectrum of 6-shagaol

Expanded HMBC NMR spectrum of 6-shagaol (1)
Expanded HMBC NMR spectrum of 6-shagaol (2)

1H NMR spectrum of 6-gingerol
Expanded 1H NMR spectrum of 6-gingerol

13C NMR spectrum of 6-gingerol
1H NMR spectrum of oleanolic acid

Expanded 1H NMR spectrum of oleanolic acid
13C NMR spectrum of oleanolic acid

Expanded 13C NMR spectrum of oleanolic acid