ANTIDIABETIC AND TOXICOLOGICAL PROPERTIES OF SOME AFRICAN MEDICINAL PLANTS USED IN THE TREATMENT OF DIABETES AND ITS COMPLICATIONS

BY

OCHUKO LUCKY ERUKAINURE

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

SUPERVISOR: Prof. MS ISLAM, PhD

PREFACE

The information presented in this thesis is an original work by the candidate. Unless otherwise indicated in the chapters that some works have been done partly by the local or international researcher collaborators, this work was carried out in the Department of Biochemistry, School of Life Sciences, College of Agriculture, Engineering and Science, University of KwaZulu-Natal, Westville Campus, Durban, South Africa from April, 2016 to December, 2018 under the supervision of Prof. MS Islam and has not otherwise been submitted in any form for any degree or diploma to any other University in the world. Apart from research collaborators, all the assistance obtained from others has been duly acknowledged in the acknowledgement section.

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Candidate: Ochuko Lucky Erukainure

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Supervisor: Prof. MS Islam

DECLARATION 1 - PLAGIARISM

I, **Ochuko Lucky Erukainure**, declare that

1. The research reported in this thesis, except where otherwise indicated, is my original research.
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DECLARATION 2 - PUBLICATIONS

DETAILS OF CONTRIBUTION TO PUBLICATIONS that form part and/or include research presented in this thesis (include publications in preparation, submitted, *in press* and published and give details of the contributions of each author to the experimental work and writing of each publication).

Ochuko Lucky Erukainure carried out the works published in the following articles under the supervision of Prof. MS Islam (PhD), while the other authors contributed partly in the work. Prof. Islam has also done final editorial work before the submission to journals for publication.

PUBLICATIONS FROM THIS THESIS

1. **Erukainure OL**, Oyebode OA, Chukwuma CI, Matsabisa MG, Koorbanally NA, Islam MS (2018) Raffia Palm (*Raphia hookeri*) Wine Inhibits Glucose Diffusion; Improves Antioxidative Activities; and Modulates Dysregulated Pathways and Metabolites in Oxidative Pancreatic Injury. Journal of Food Biochemistry. (In press, accepted on October 25, 2018)

2. **Erukainure OL**, Oyebode OA, Ibeji CU, Koorbanally NA, Islam MS. (2018). *Vernonia Amygdalina* Del. Stimulated Glucose Uptake in Brain Tissues Enhances Antioxidative Activities; and Modulates Functional Chemistry and Dysregulated Metabolic Pathways. Metabolic Brain Disease (In press, accepted on December 4, 2018)

3. **Erukainure OL**, Chukwuma CI, Sanni O, Matsabisa MG, Islam MS (2018) Histochemistry, phenolic content, antioxidant, and anti- diabetic activities of Vernonia amygdalina leaf extract. Journal of Food Biochemistry. e12737. https://doi.org/10.1111/jfbc.12737

4. **Erukainure OL**, Sanni O, Islam MS (2018) *Clerodendrum volubile*: Phenolics and Applications to Health. In: Watson R, Preedy V, Zibadi S (Eds). Polyphenols: Mechanisms of Action in Human Health and Disease. 2nd Edn., Elsevier. DOI: 10.1016/B978-0-12-813006-3.00006-4

5. **Erukainure OL**, Mopuri R, Chukwuma CI, Koorbanally NA, Islam MS (2018) *Phaseolus lunatus* (Lima Beans) Abates Fe2+-induced hepatic redox imbalance; inhibits Intestinal

Glucose Absorption and Major Carbohydrate Catabolic Enzymes; and Modulates Muscle Glucose Uptake. Journal of Food Biochemistry. DOI: 10.1111/jfbc.12655

6. **Erukainure OL**, Narainpersad N, Singh M, Olakunle S, Islam MS (2018) Clerodendrum volubile inhibits key enzymes linked to type 2 diabetes but induces cytotoxicity in human embryonic kidney (HEK293) cells via exacerbated oxidative stress and proinflammation. Biomedicine and Pharmacotherapy. 106: 1144–1152

7. **Erukainure OL**, Oyebode OA, Sokhela MK, Koorbanally NA, Islam MS (2017) Caffeine – Rich Infusion from *Cola nitida* (Kola Nut) inhibits Major Carbohydrate Catabolic Enzymes; Abates Redox Imbalance; and Modulates Oxidative Dysregulated Metabolic Pathways and Metabolites in Fe^{2+} - induced Hepatic Toxicity. Biomedicine and Pharmacotherapy. DOI: 10.1016/j.biopha.2017.11.120

8. **Erukainure OL**, Mopuri R, Oyebode OA, Koorbanally NA, Islam MA (2017) *Dacryodes edulis* enhances antioxidant activities, suppresses DNA fragmentation in oxidative pancreatic and hepatic injuries; and inhibits carbohydrate digestive enzymes linked to type 2 diabetes. Biomedicine and Pharmacotherapy. 96: 37–47

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8. Sanni O, **Erukainure OL**, Chukwuma CI, Koorbanally NA, Ibeji CU, Islam MS. (2019). Azadirachta indica inhibits key enzyme linked to type 2 diabetes in vitro, abates oxidative hepatic injury and enhances muscle glucose uptake ex vivo. Biomedicine and Pharmacotherapy. 109: 734–743
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10. Oyebode OA, **Erukainure OL**, Chukwuma CI, Ibeji CU, Koorbanally NA, Islam S. (2018). *Boerhaavia diffusa* inhibits key enzymes linked to type 2 diabetes in vitro and in silico; and modulates abdominal glucose absorption and muscle glucose uptake *ex vivo*. Biomedicine and Pharmacotherapy. 106: 1116–1125

11. Oyebode OA, **Erukainure OL**, Koorbanally NA, Islam S. (2018). *Acalypha wilkesiana* 'Java white': Identification of some bioactive compounds by GC-MS and effects on key enzymes linked to type 2 diabetes. Acta Pharm. 68**:** 425–439
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14. **Erukainure OL**, Mesaik MA, Atolani O, Muhammad A, Chukwuma CI, Islam MS. (2017). Pectolinarigenin from the leaves of Clerodendrum volubile shows Potent Immunomodulatory Activity by Inhibiting T – Cell Proliferation and Modulating Respiratory Oxidative Burst in Phagocytes. Biomedicine and Pharmacotherapy 93: 529–535
15. **Erukainure OL**, Hafizur RM, Choudhary MI, Adhikari A, Mesaik AM, Atolani O, Banerjee P, Preissner R, Muhammad A, MS Islam. (2017). Anti-diabetic effect of the ethyl acetate fraction of Clerodendrum volubile: protocatechuic acid suppresses phagocytic oxidative burst and modulates inflammatory cytokines. Biomed Phar Biomedicine and Pharmacotherapy. 86: 307–315
16. **Erukainure OL**, Mesaik AM, Muhammad A, Chukwuma CI, Manhas N, Singh P, Aremu OS, Islam MS. (2016). Flowers of Clerodendrum volubile Exacerbate Immunomodulation by Suppressing Phagocytic Oxidative Burst and Modulation of COX-2 Activity. Biomedicine and Pharmacotherapy. DOI: 10.1016/j.biopha.2016.09.002

SUBMITTED PAPERS (UNDER REVIEW) FROM THIS THESIS

17. **Erukainure OL,** Chukwuma CI, Islam MS. (2018). Short-term Fermentation of Raffia Palm (*Raphia hookeri*) Wine may not affect its Qualitative Sugar Profile but Alters Its Functional Chemistry and Antidiabetic Properties. Food Bioscience (FBIO_2018_571)

18. **Erukainure OL**, Reddy R, Islam MS. (2018). Raffia Palm (*Raphia hookeri*) Wine Extenuates Redox Imbalance and Modulates Activities of Glycolytic and Cholinergic Enzymes in Hyperglycemia Induced Testicular Injury in Type 2 Diabetes Rats. Journal of Food Biochemistry (JFBC-10-18-0815)

19. **Erukainure OL**, Oyebode OA, Mansoor S, Koorbanally NA, Islam MS. Raffia (*Raphia hookeri*) Palm Wine Modulates Dysregulated Lipid Metabolic Pathways and Metabolites in Diabetic Testicular Toxicity. Biomedicine and Pharmacotherapy (BIOPHA_2018_5945)

20. **Erukainure OL**, Ijomone OM, Sanni O, Aschner M, Islam MS. Type 2 Diabetes Induced Oxidative Brain Injury involves Altered Cerebellar Neuronal Integrity and Elemental distribution, and Exacerbated Nrf2 Expression: Therapeutic Potential of Raffia Palm (Raphia hookeri) Wine. Metabolic Brain Disease (MEBR-D-18-00410)

21. **Erukainure OL**, Oyebode OA, Ijomone OM, Chukwuma CI, Koorbanally NA, Islam MS. Raffia Palm (Raphia hookeri) Wine Modulates Glucose Homeostasis by Enhancing Insulin Secretion; Inhibiting Redox Imbalance, Glycolytic and Cholinergic Enzymes Activities; and Downregulates Pancreatic Nrf2 Expression in Type 2 Diabetic Rats. Chemico-Biological Interactions (CHEMBIOINT_2018_1532)

PROSPECTIVE PUBLICATIONS FROM THIS THESIS

22. **Erukainure OL**, Islam MS. Phytol – Rich Extracts of *Vernonia Amygdalina* Stimulates Muscle Glucose Uptake, and Modulates Redox Activities and Functional Chemistry in Oxidative Hepatic Injury (In preparation)
23. **Erukainure OL**, Daramola OA, Koorbanally NA, Islam MS. Flowers of *Clerodendrum volubile* Modulates Redox Homeostasis and Suppresses DNA Fragmentation in Fe2+ - induced Oxidative Injury; and Inhibits Carbohydrates and Lipids Catabolic Enzymes linked to Type 2 Diabetes (In preparation)
24. **Erukainure OL**, Sanni O, Ijomone OM, Ibeji CU, Islam MS. *Cola nitida* Modulates Glucose Homeostasis in Type 2 Diabetes by Inducing Insulin Secretion; Abating Redox Imbalance and Dyslipidemia; Inhibiting Glycolytic and Cholinergic Enzymes Activities; and Downregulating Nrf2 Expression (In preparation)

25. **Erukainure OL**, Ijomone OM, Oyebode OA, Aschner M, Islam MS. Hyperglycemia-Induced Oxidative Brain Injury: Therapeutic Effects of *Cola nitida* Infusion against Redox Imbalance, Cerebellar Neuronal Insults, and Upregulated Nrf2 Expression in type 2 diabetes Rats (In preparation)
26. **Erukainure OL**, Ijomone OM, Chukwuma CI, Xiao X, Matsabisa MG, Islam MS. Antidiabetic Properties of the Butanol Fraction of Dacryodes edulis L. Ethanol Extract: Modulatory Effects on Redox Imbalance and Nrf2 Expression; and Inhibitory Effects on Glycolytic and Cholinergic Enzymes Activities in Type 2 Diabetic Rats (In preparation)

PRESENTATIONS

27. **Erukainure OL**, Oyebode OA, Koorbanally NA, Ibeji CU, Islam MS. *Vernonia Amygdalina* Enhances Brain Glucose Uptake, Ameliorates Redox Imbalance and Proinflammatory Markers: An ex vivo Study. 18th International Congress of Endocrinology / 53rd SEMDSA Congress (December, 2018; Prospective)

28. **Erukainure OL**, Islam MS. Type 2 Diabetes Alters Cerebellar Neuronal Integrity and Elemental distribution and upregulates Nrf2: Therapeutic Effect of Raffia Palm (Raphia hookeri) Wine. College of Agriculture, Engineering, and Sciences Research Day and Exhibition, University of KwaZulu-Natal, Durban, South Africa (October, 2018)

29. **Erukainure OL**, Islam MS. Enhanced glucose uptake in brain by *Vernonia amygdalina*: antioxidative and anti-proinflammatory effects. Nueroinflammation Symposium, Department of Human Physiology, University of KwaZulu-Natal, Durban, South Africa (August, 2018)

30. **Erukainure OL**, Islam MS. Enhanced glucose uptake in brain by *Vernonia amygdalina*: antioxidative and anti-proinflammatory effects. College of Agriculture, Engineering, and Sciences Research Day and Exhibition, University of KwaZulu-Natal, Durban, South Africa (October, 2017)

31. **Erukainure OL**, Mopuri R, Oyebode OA, Koorbanally NA, Islam MA. *Dacryodes edulis* enhances antioxidant activities, suppresses DNA fragmentation in oxidative pancreatic and hepatic injuries; and inhibits carbohydrate digestive enzymes linked to type 2 diabetes. 7th

General Assembly and National Conference of the Nigerian Young Academy, Effurun, Nigeria (August, 2017)

32. **Erukainure OL**, Islam MS. *Phaseolus lunatus* (Lima Beans) Abates Fe2+-induced hepatic redox imbalance; inhibits Intestinal Glucose Absorption and Major Carbohydrate Catabolic Enzymes; and Modulates Muscle Glucose Uptake. School of Life Sciences Research Day and Exhibition, University of KwaZulu-Natal, Durban, South Africa (April, 2017)

DEDICATION

To my parents, Mr. and Mrs. **Vincent O. Erukainure**

To my darling wife, **Isoken** and beautiful daughter, **Zoe**

And to the memory of late Mr. **Darlington Dafevwakpo,** a brother who always called me "Doctor"

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I will not fail to appreciate the moral supports and care of my mother in-law, Hajia S. Omoregie as well as my brother in-law, Mr. D.O. Omoregie towards my wife and daughter in my absentia in the course of this program.

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LIST OF ABBREVIATIONS

DM	Diabetes mellitus
T1D	Type 1 diabetes
IDDM	Insulin dependent diabetes mellitus
T2D	Type 2 diabetes
NIDDM	Non-insulin dependent diabetes mellitus
T3D	Type 3 diabetes
AD	Alzheimer disease
GDM	Gestational diabetes mellitus
MHC-HLA	Major histocompatibility complex - Human leukocyte antigen
IGT	Impaired glucose tolerance
SGLT2	Sodium/glucose co-transporter 2
GLP1	Glucagon-like peptide 1
ROS	Reactive oxygen species
CVD	Cardiovascular disease
GPx	Glutathione peroxidase
GLUT2	Glucose transporter 2
H_2O_2	Hydrogen peroxide
$O_2^{\cdot -}$	Superoxide anion
$OH^{\cdot}$	Hydroxyl radical
HOCl	Hypochlorous acid

HCl	Hydrochloric acid
$ONOO^-$	Peroxynitrate
TCA	Tricarboxylic acid
NADH	Reduced nicotinamide adenine dinucleotide
NAD+	Oxidized nicotinamide adenine dinucleotide
RAGE	Receptor for advanced glycation end products
AGEs	Advanced glycation end products
NF-κB	Nuclear factor kappa B
GFAT	Glutamine:fructose-6 phosphate amidotransferase
PKC	Protein kinase C
VEGF	Vascular endothelial growth factor
PPAR-γ	Peroxisome proliferator-activated receptor gamma
SGLT2	Sodium glucose co-transporters inhibitors
DPP	Dipeptidyl peptidase
DPPH	2,2'-diphenyl-1-picrylhydrazyl
FRAP `	Ferric reducing antioxidant power
DCM	Dichloromethane
MeOH	Methanol
BuOH	Butanol
$FeCl_3$	Iron (III) chloride
TBARS	Thiobarbituric acid reactive substances

MDA	Malondialdehyde
ACE-I	Angiotensin converting enzyme-1
RPW	Raffia palm wine
BFDE	Butanol fraction of *Dacryodes Edulis*

ABSTRACT

This study investigated the antioxidant, antidiabetic and toxicity properties of antidiabetic medicinal plants comprising of *Vernonia amygdalina*, *Cola nitida*, Raffia palm (*Raphia hookeri*) wine, *Phaseolus lunatus*, *Dacryodes edulis*, and *Clerodendrum volubile* using *in vitro, ex vivo, in silico* and *in vivo* models. The leaves of *V. amygdalina* and *D. edulis*, as well as *C. volubile* flower were sequentially extracted with solvents of increasing polarity to yield ethyl acetate, ethanol and aqueous extracts. *Cola nitida* and *V. amygdalina* were infused in hot water to yield infusion extracts. *Phaseolus lunatus* was subjected to aqueous extraction to yield aqueous extract, while Raffia palm wine was concentrated to yield the concentrate. The extracts and concentrate were screened for their *in vitro* and *ex vivo* antioxidant activities, as well as their inhibitory effect on α-glucosidase, α-amylase and pancreatic lipase activities, and their ability to stimulate muscle glucose uptake and inhibit intestinal glucose absorption *in vitro*. The ethanol extracts of *D. edulis*, *C. volubile and V. amygdalina* were subjected to GC-MS analysis, while the aqueous extract of *P. lunatus*, palm wine concentrate and the infusions were analyzed with LC-MS to elucidate the active compounds that may be responsible for their bioactivities. The ethanol extracts of *C. volubile* and *D. edulis* were further subjected to liquid-liquid fractionation to yield the hexane, dichloromethane, ethyl acetate, butanol and aqueous fractions. These fractions were also assayed for their antioxidant and antidiabetic properties *in vitro* and *ex vivo*. The dichloromethane, ethyl acetate and butanol fractions were subjected to GC-MS analysis to elucidate their active compounds. The identified compounds were molecularly docked with the test enzymes *in silico* to further validate their bioactivities. The antidiabetic properties of palm wine concentrate, *C. nitida* infusion, and *D. edulis* butanol fraction were investigated in a type 2 diabetes rat model. The *in vivo* study revealed a potent hypoglycemic activity, with concomitant amelioration of oxidative stress in the serum, pancreas, testes and brain. This was further substantiated by the downregulation of Nrf2 expressions in the pancreas and brain. These results further validate the use and safety of these plants in diabetes management.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.0 Introduction

Diabetes mellitus (DM) is the fastest growing global epidemic scourge affecting over 425 million people in 2017 (IDF 2018), which depicts a 2.4% rise in prevalence from 415 million in 2015 (IDF 2016). This is expected to rise by 48% to 629 million in 2045, with Africa predicted to have a 156% upsurge (**Figure 1.1**) (IDF 2018). This predicted upsurge in Africa is a major concern, owing to her poor health infrastructures, economic burden and rising incidences of other diseases like HIV/AIDS, malaria and tuberculosis. Increased urbanization owing to rural – urban drift, sedentary life styles with less physical exercise, and ageing population have been linked to the upsurge (Bos and Agyemang 2013; Erukainure et al. 2017a). The number of people living with diabetes in Africa was estimated to be 16 million in 2017 (IDF 2018), depicting as the lowest. However, difficulty in data collection coupled with lack of high quality data suggests that the number may be much higher.

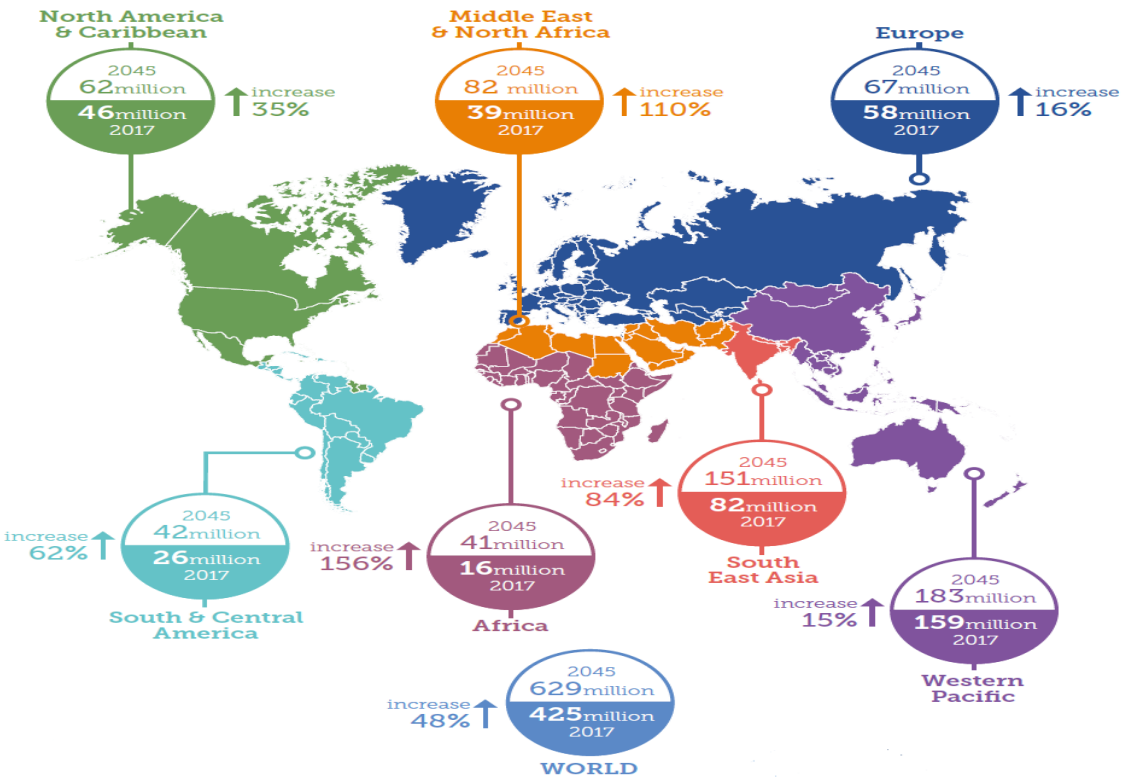

Figure 1. 1: Global epidemic picture of DM in 2017 and 2045. (adapted without permission from IDF Diabetes Atlas, 8th edition (I.D.F. 2018)).

Diabetes is ranked among the top 10 contributors to global mortality **(Figure 1.2)** (I.D.F. 2018), a major factor being 30-80% of diabetics are undiagnosed. Multiple complications arising from prolonged DM has also been implicated in diabetic mortality (Motala 2002).

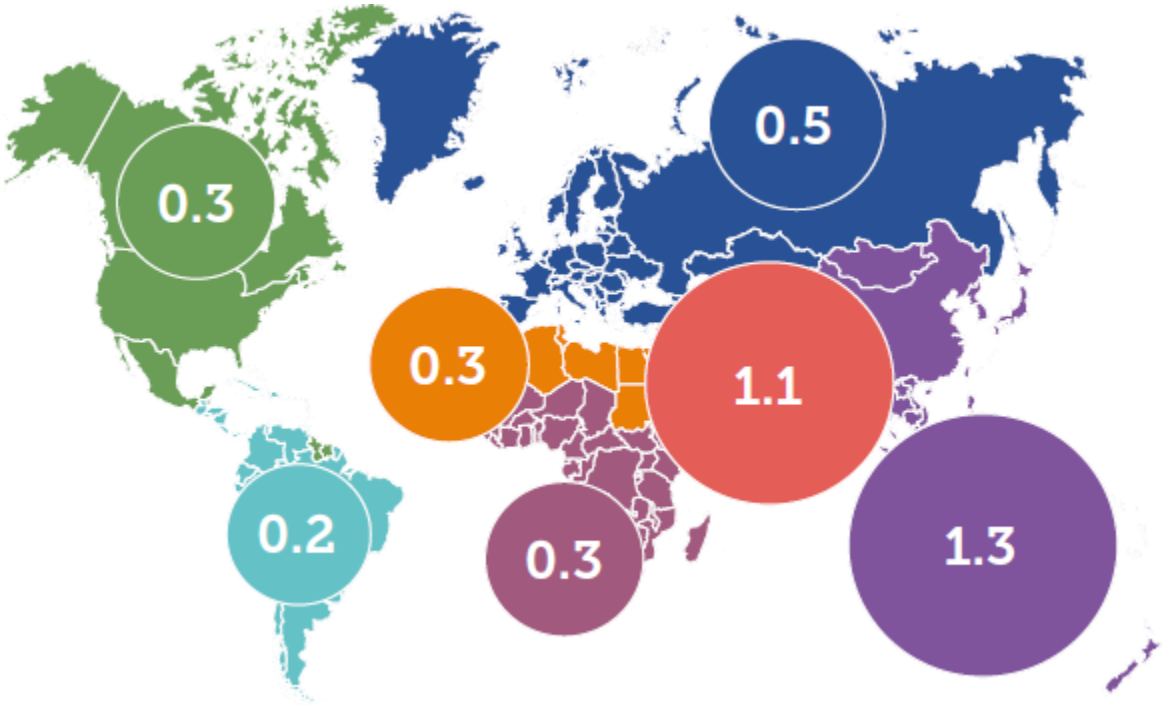

Figure 1.2: Estimated global mortality (20 – 75 years) due DM in 2017 (adapted without permission from IDF Diabetes Atlas, 8th edition (I.D.F. 2018)). *values are in millions

This has led to continuous increase in global healthcare expenditure. The global healthcare expenditure for DM was USD727 billion in 2017, which portrays an 8% rise from the 2015 cost (**Figure 1.3**) (I.D.F. 2018). This increase has been attributed to increased population growth, urbanization and lifestyle changes particularly in Sub-Saharan Africa and other developing countries (I.D.F. 2016).

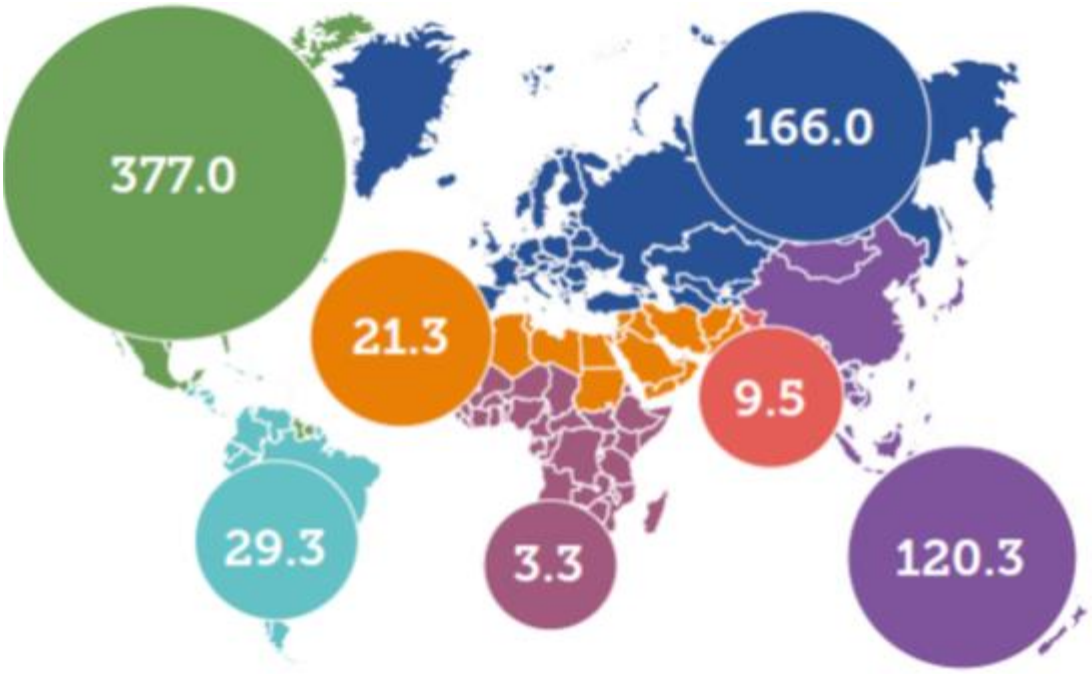

Figure 1.3: Global healthcare expenditure (in USD billions) on DM in 2017 (adapted without permission from IDF Diabetes Atlas, 8th edition (I.D.F. 2018))

Due to the cost associated with DM and the economic burden in developing, there have been increased search for novel and affordable alternative treatment with little or no side effects. This has led to increase paradigm shift to natural products, particularly from plant source (Erukainure et al. 2018). However, the safety and toxicity of these alternative treatments remains a huge concern. Hence this project, not only aims at studying the efficacies of selected antidiabetic medicinal plant but also the toxicity associated with their use for the treatment of diabetes, particularly type 2 diabetes (T2D).

1.1 Diabetes mellitus

Diabetes mellitus (DM) is a metabolic disorder which affects carbohydrate, fat, and protein metabolism owing to defects in insulin secretion and/or action (Erukainure et al. 2013; Vinayagam et al. 2016), which leads to chronic hyperglycemia (high blood glucose level) with multiple etiologies and pathogenesis (Association 2010; Vinayagam et al. 2016). It is often accompanied with glucotoxicity, lipotoxicity, increased inflammation/oxidative stress, and endoplasmic reticulum-induced stress leading to apoptotic and necrotic cell death of the pancreatic β-cells

(Jadhav et al. 2013; Ndisang et al. 2014). These also lead to the pathogenesis of related complications such as hepatopathy, retinopathy, nephropathy, neuropathy, and cardiovascular diseases (CVD) (Lin and Sun 2010; Papatheodorou et al. 2016; Tripathi and Srivastava 2006). These complications have been implicated in the morbidity and mortality of DM, affecting over 50% of diabetic patients (Islam 2011; Pari and Saravanan 2004).

Insulin is regarded as the main anabolic hormone in the body as it regulates the metabolism of carbohydrate, fatty acids, and protein (Voet and Voet 2011). It is a polypeptide hormone synthesized by the pancreatic β-cells of the islets of Langerhans, and involved mainly in the regulation of blood glucose level (Qaid and Abdelrahman 2016; Wilcox 2005). It lowers blood glucose level by facilitating its uptake from the blood into cells and skeletal muscles, thereby stimulating glycogenesis with concomitant inhibition of glycogenolysis. It decreases plasma fatty acid level by stimulating fatty acids uptake into the adipose and muscle tissues; decreasing the rate of lipolysis in adipose tissues as well as fatty acid oxidation in muscle and liver (Delarue and Magnan 2007; Dimitriadis et al. 2011). It also promotes cell division and growth via its mitogenic activities, thus also regarded as a growth hormone (Wilcox 2005). Therefore, its deficiency, reduced secretion and/or underutilization have tremendous metabolic fate which has been implicated in the pathogenesis of DM (Delarue and Magnan 2007; Dimitriadis et al. 2011; Lin and Sun 2010; Wilcox 2005).

1.2 Types of Diabetes

There have been much discussions, deliberations and revisions over the years on the classifications of DM owing to its etiologies (Baynest 2015; I.D.F. 2018; Ize-Ludlow and Sperling 2005). It is commonly classified into 2 types namely: type 1 diabetes (T1D) formerly known as Insulin Dependent Diabetes Mellitus (IDDM), and type 2 diabetes (T2D) formerly known as Non-Insulin Dependent Diabetes Mellitus (NIDDM). A third type of diabetes has been proposed as type 3 diabetes (T3D) or brain diabetes, which has been linked to Alzheimer disease (AD) (de la Monte and Wands 2008; Leszek et al. 2017; Shaw 2017; Suzanne 2014). Gestational diabetes mellitus (GDM) is also another common type of diabetes which occurs during pregnancy. A rare type of diabetes is diabetes insipidus, which impairs the synthesis, transportation and/or release of vasopressin (an anti-diuretic hormone) (Perkins et al. 2006; Saborio et al. 2000). Others include specific types due to other inducements such as monogenic diabetes syndromes, exocrine pancreas

diseases, endocrinopathies, and drug/chemical – induced diabetes (Association 2015; Kerner and Brückel 2014).

1.2.1 Type 1 Diabetes (T1D)

T1D accounts for 10% of global diabetic cases (Association (2001); Ozougwu et al. 2013). It is regarded as an autoimmune disease which selectively destroys the pancreatic β-cell, leading to insulin deficiency and ensuing hyperglycemia (Atkinson et al. 2014; Gregory et al. 2013; Ozougwu et al. 2013). It was initially thought to be a childhood and adolescent metabolic disorder but has been discovered to affect adults too. The classic trio or symptoms consisting of polydipsia, polyphagia, and polyuria are the most common form of its diagnosis (Atkinson 2012; Atkinson et al. 2014; Gregory et al. 2013).

Factors such as genetics, immunology, and environment have been implicated in the autoimmune destruction of the pancreatic β – cells (**Figure 1.4**). The autoimmune destruction of the pancreatic β-cell is considered an inflammatory process as it is mediated by inflammatory cytokines particularly β-cell autoantigens, macrophages, dendritic cells, B-lymphocytes, and T-lymphocytes (Pugliese 2016; Yoon and Jun 2005). Genetic predispositions have also been reported to induce autoimmune destruction of the pancreatic β-cells (Pirot et al. 2008). Several chromosomal loci have been identified in the development of T1D, with the human leukocyte antigen (HLA) locus reported as the most predisposing (Jahromi and Eisenbarth 2007; Pirot et al. 2008; Todd et al. 2007). Others include the cytotoxic T-lymphocyte antigen 4 (CTLA4), insulin, and phosphatase non-receptor type 22 (PTPN22) loci (Jahromi and Eisenbarth 2007; Kim and Polychronakos 2005; Pirot et al. 2008). These loci and others identified are located within the MHC-HLA class II region (Cucca et al. 2001; Hakonarson et al. 2007). The predisposing roles of MHC haplotypes and non-HLA loci in have also been reported (Atkinson and Eisenbarth 2001; Barrett et al. 2009; Fernando et al. 2008). These genes cause an aggravated inflammatory and immune response to stimuli, thereby increasing the risk of autoimmunity which may affect the β-cell function and presentation of antigens in individuals (Åkerblom et al. 2002; Pirot et al. 2008). Environmental factors have also been reported to play major roles in the pathogenesis of T1D (Åkerblom et al. 2002; Atkinson and Eisenbarth 2001; Pirot et al. 2008). These include the diet types particularly infant and adolescent diets, vitamin D deficiency, climate influences, viral infections, growth, toxins, ante-

and perinatal risk factors, vaccinations, and stress (Åkerblom et al. 2002; Atkinson and Eisenbarth 2001; Pirot et al. 2008). These environmental factors act as modifiers of genetic predisposition, which in turn induces the activation of cytokines involved in autoimmune destruction of the pancreatic β-cell (Jahromi and Eisenbarth 2007).

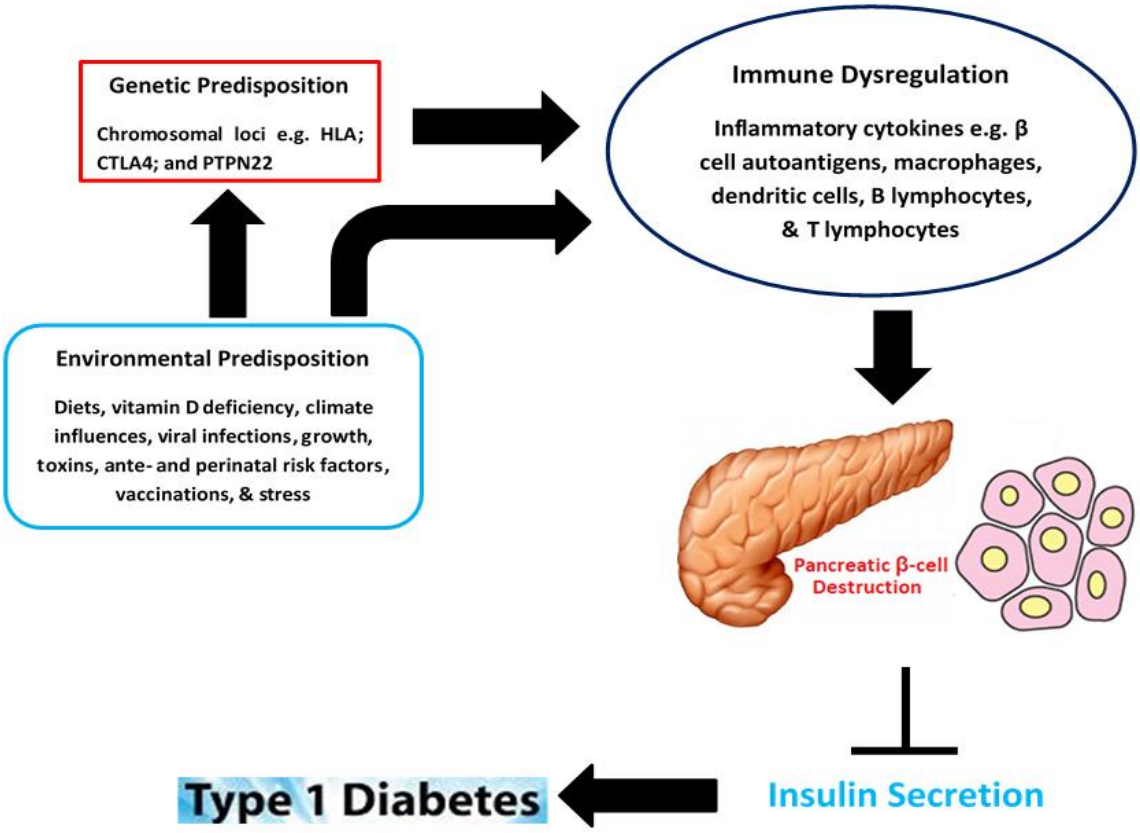

Figure 1.4: Pathophysiology of type 1 diabetes.

1.2.2 Type 2 Diabetes (T2D)

T2D for approximately 90% of all types of diabetes (I.D.F. 2016; I.D.F. 2018), making it the most prevalent and a major contributor to diabetic mortality and morbidity. It is a complex heterogenous metabolic disorder characterized by impaired insulin secretion and/or action leading to hyperglycemia (DeFronzo et al. 2015; Mohammed et al. 2015). This has been attributed to β-cell dysfunction, insulin resistance and chronic inflammation of the pancreatic tissues.

Interactions involving both genetic and environmental factors have been implicated in the pathogenesis of T2D (DeFronzo et al. 2015). The genetic factors are pancreatic β-cell dysfunction

and insulin resistance, while sedentary lifestyle, lack of physical activities, weight gain, oxidative stress and consumption of high calorie diets constitutes environmental factors (DeFronzo et al. 2015; Kahn et al. 2014). Decreased physical activity and obesity leads to decreased insulin sensitivity which when coupled to the genetic burden of insulin resistance, causes the pancreatic β-cells to enhance insulin secretion to offset the insulin inaction (Defronzo 1997; DeFronzo 2009). This activity helps in maintaining normal glucose tolerance. However, prolonged augmentation of insulin secretion will cause the pancreatic β-cell function to begin to decline (DeFronzo 2004; DeFronzo 2009). This is followed by increased fasting plasma glucose concentration, leading to insulin insensitivity and subsequently diabetes (DeFronzo 2009).

Hepatic glucose production, reduced insulin secretion, and impaired insulin sensitivity (the triumvirate) have been reported as the main defects in the onset of T2D – induced hyperglycemia, with pancreatic β-cell failure occurring much earlier (Figure 1.5) (DeFronzo et al. 2015; Lin and Sun 2010; Stumvoll et al. 2005).

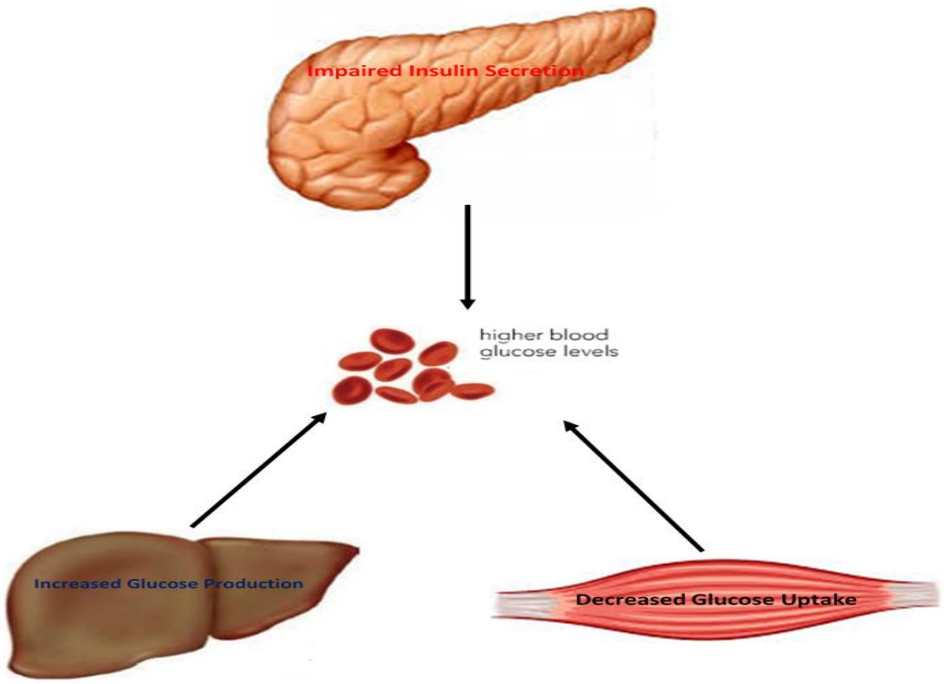

Figure 1.5: The triumvirate pathophysiology of T2D

A maximal and/or near-maximal insulin resistance have been documented for individuals at the upper tertile of impaired glucose tolerance, with over 80% of their pancreatic β-cell function being

lost (DeFronzo 2009). However, an "ominous octet" has been proposed to replace the triumvirate. They are the adipocytes (increased lipolysis), gastrointestinal tract (incretin deficiency/resistance), α-cell (hyperglucagonemia), kidney (accelerated glucose reabsorption), and brain (insulin resistance/neuro-dysfunction) in addition to the liver, muscle and pancreatic β-cell (**Figure 1.6**) (DeFronzo 2009; DeFronzo et al. 2015). Increased glucagon level and heightened hepatic glucagon sensitivity cause the liver to produce excess glucose (DeFronzo et al. 2015). There is an increased rate of lipolysis with concomitant increased plasma free fatty acid (FFA) levels owing to insulin resistance in the adipocytes, which further exacerbate insulin resistance in the liver and muscle, and contributing to pancreatic β-cell failure (DeFronzo et al. 2015; Gustafson et al. 2015). Hyperglycemia is being maintained by accelerated glucose reabsorption in the renal tissues by the sodium/glucose co-transporter 2 (SGLT2) as well as increased glucose spillage in the urine (DeFronzo 2009; DeFronzo et al. 2015; Meyer et al. 1998). Insulin resistance is also aggravated by the resistance to leptin, glucagon-like peptide 1 (GLP1), and amylin which are appetite-suppressive (Cummings and Overduin 2007; DeFronzo et al. 2015; Williams et al. 2006). This is further aggravated by low dopamine and serotonin levels in the brain (DeFronzo et al. 2015; Mooradian 1988).

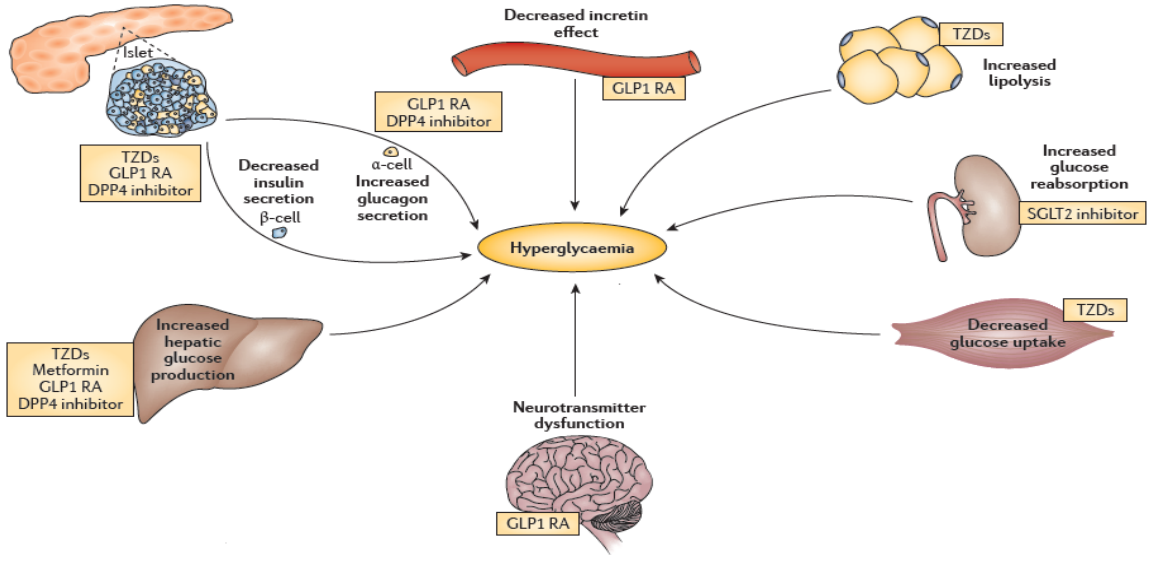

Figure 1.6: The ominous octet pathophysiology of T2D. (Adapted without permission from DeFrenzo et al. (2015))

1.3 Complications of T2D

T2D is often described as a multifactorial disease owing to the complications associated with it. Chronic hyperglycemia in T2D has been linked to the production of reactive oxygen species (ROS), which overwhelms the body's endogenous antioxidant system resulting to oxidative stress (Erukainure et al. 2017a). Oxidative stress has been implicated in the pathogenesis of T2D complications such as retinopathy, neuropathy, nephropathy, cardiovascular diseases (CVD), and reproductive toxicity.

Hyperglycemia has also been recognized to mediate the pathogenesis of T2D complications via activation of inflammatory cytokines which promotes inflammation (Aronson 2008; Guha et al. 2000). Thus, making hypoglycemia a major player in T2D complications (Aronson 2008).

1.3.1 Oxidative stress and T2D

High concentrations of ROS are generated in most tissues under chronic hyperglycemic condition in T2D, with the pancreatic β-cells being the most susceptible compared to any other organs (Brownlee 2001; Kajimoto and Kaneto 2004). This have been attributed to its high efficiency for glucose uptake on exposure to excess glucose because of high expression of GLUT2 (Kajimoto and Kaneto 2004). The expression levels of the antioxidant enzymes glutathione peroxidase (GPx) and catalase (CAT) have also been reported to be extremely low in pancreatic β-cells, thus making the cells highly susceptible to oxidative damage (Pi et al. 2010).

Glucose oxidation has been recognized as the main trigger of free radical productions in T2D. Glucose is oxidized in its enediol form to an enediol radical in the presence of transition metal, which leads to the production of reactive ketoaldehydes and superoxide anion ($O_2^{\cdot -}$) radicals (Maritim et al. 2003). Superoxide dismutase then converts the generated $O_2^{\cdot -}$ to hydrogen peroxide (H_2O_2). The continuous oxidation of glucose owing to hyperglycemia will lead to high concentration of $O_2^{\cdot -}$ and accumulation of H_2O_2 which overwhelms the cells' GPx and catalase activities, thereby leading to production of hydroxyl radicals ($OH^{\cdot}$). Myeloperoxidase can also convert H_2O_2 to hypochlorous acid (HOCl) in the presence of hydrochloric acid (HCl). Nitric oxide can also react with $O_2^{\cdot -}$ to give peroxynitrate ($ONOO^-$).

Enhanced glycolysis leading to the non-oxidative catabolism of glucose to lactate have also been implicated in hyperglycemia induced generation of free radicals in T2D (Chikezie et al. 2015). The continuous generation of lactate from pyruvate causes an increase in free cytosolic NADH/NAD+ ratio, which impairs the oxidation of NADH to NAD+ (Chikezie et al. 2015). Thus, increasing the cell levels of the electron donor NADH.

Additionally, acceleration of the tricarboxylic acid (TCA) cycle in hyperglycemia have been reported as another mechanism of glucose generation of free radicals (Araki and Nishikawa 2010; Brownlee 2001). Pyruvate from the glycolytic pathway is oxidized via the TCA cycle to generate NADH and FADH2 (Brownlee 2001). The increased production of these electron donors in hyperglycemia leads to the generation of a high mitochondrial membrane potential, which inhibits electron transport at complex II with concomitant reduction of oxygen to (O_2) to $O_2^{\cdot -}$ (Brownlee 2001; Du et al. 2001).

Hyperglycemia have also been shown to induce oxidative stress via activation of the polyol pathway; advanced glycation end-product (AGE) formation; the hexosamine pathway; and protein kinase C (PKC) isoforms (Aronson 2008; Brownlee 2001; Wiernsperger 2003)

a) Activation of the Polyol Pathway

This pathway involves the oxidation of sorbitol to fructose by sorbitol dehydrogenase, with concomitant reduction of NAD+ to NADH (**Figure 1.7**) (Bonnefont-Rousselot 2002; Brownlee 2001). In non-diabetics, this metabolic pathway for glucose oxidation is very minimal but increased in hyperglycemic state. This has been attributed to the high exposure of the cells to glucose which saturates the hexokinase, thereby channeling the excess glucose to the polyol pathway (Brownlee 2001; Kanwar et al. 2011). The channeled glucose is first reduced by aldose reductase to sorbitol, which is then oxidized by sorbitol dehydrogenase to fructose. The continuous channeling of glucose to this pathway in hyperglycemia leads to accumulation of sorbitol in the cells and consequent production of fructose. The former causes osmotic stress, while the latter have been reported for its high glycating activity (Obrosova 2005). Furthermore, aldose reductase oxidizes NADH to NAD+ which decreases the cells ability to regenerate reduced glutathione (GSH). The continuous generation of the electron donor (NADH), production of fructose, and

concomitant depleted GSH levels indicates a potential of redox imbalance in the cells (Brownlee 2005).

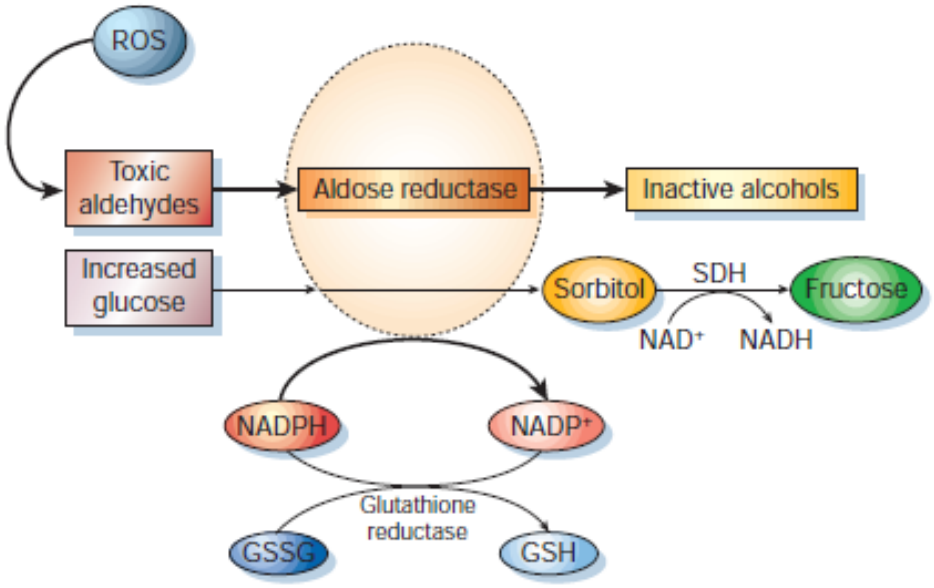

Figure 1.7: The polyol pathway (adapted without permission from Brownlee (2001))

b) Increased Formation of Advanced Glycation End-Product (AGE)

Increased formation of advanced glycation end products (AGEs) has been recognized as one of the pathological mechanism of hyperglycemia – induced oxidative injury (Aronson 2008; Vistoli et al. 2013). It is a non-enzymatic glycosylation of proteins and/or lipids owing to prolonged exposure to glucose as seen in hyperglycemic states (Goldin et al. 2006). The presence of AGEs alters enzymatic activities, cellular functions, disrupts molecular conformation, as well as affect receptor recognition (Aronson 2008; Goldin et al. 2006). Receptor for advanced glycation end products (RAGE) has been reported to be present in most cells (Goldin et al. 2006). The binding of AGEs with these receptors are responsible for AGEs pathological contribution to diabetic complications. This binding activates RAGE, leading to a series of pathological events particularly: 1) upregulation of nuclear factor kappa B (NF-κB) and its target genes leading to inflammation; 2) activation of monocytes, and inhibition monocyte migration across the membrane; 3) increased transportation of macromolecule across the endothelial membrane; and 4)

blockage of endothelial NO activity, leading to the production of ROS (oxidative stress) (Aronson 2008; Brownlee 2001; Brownlee 2005; Goldin et al. 2006; Vistoli et al. 2013)

c) Activation of the Hexosamine Pathway

In hyperglycemic state, excess glucose can be channeled into the hexosamine pathway using fructose-6-phospahte as the substrate. It involves the conversion of fructose-6-phosphate to glucosamine-6-phosphate by glutamine:fructose-6 phosphate amidotransferase (GFAT), and then the final product uridine diphosphate (UDP) *N*-acetyl glucosamine (Aronson 2008; Brownlee 2005). UDP *N*-acetyl glucosamines are involved in O-glycosylation by adding N-acetylglucosamine (GlcNAc) to a protein's serine and threonine residues (Aronson 2008). Phosphorylation of these residues are critical steps in the regulation of most enzymes, thus implying that O-glycosylation could alter normal enzyme activities leading to detrimental consequences (Vistoli et al. 2013). Modification of transcription factors and other nuclear and cytoplasmic proteins by O-linked GlcNAc have been reported, leading to complications such as cardiomyocyte dysfunction, increased carotid artery plaques and abnormal express of the glomerular cell gene (Aronson 2008; Brownlee 2001; Brownlee 2005; Goldin et al. 2006).

d) Activation of Protein Kinase C (PKC) Isoforms

Hyperglycemia have also been reported to mediate the pathogenesis of T2D via activation of protein kinase C (PKC) and its isoforms (Aronson 2008; Brownlee 2001; Brownlee 2005). Protein kinase Cs are enzymes involved in the regulation of protein functions by phosphorylation of their serine and threonine amino acid residues (Aronson 2008; Brownlee 2005). Hyperglycemia influences PKC activities by increasing the synthesis of diacylglycerol from glyceraldehyde-3-phosphate and dihydroxy-acetone phosphate (Aronson 2008). Diacylglycerol has been shown to be an important co-factor for the activation of PKC isoforms: α, β, and γ which are widely distributed in cells (Brownlee 2001; Brownlee 2005). Activation of PKC leads to the activation of NADPH oxidase which has been implicated in the generation of ROS in blood vessels (Inoguchi et al. 2000). PKC has been shown to be involved in the activation of NF-kB by $O_2^{\cdot -}$ (Ogata et al. 2000). Yakubu et al. (Yakubu et al. 2004) also reported that vasculopathy in hyperglycemia is due to the suppression of NO production and NO-dependent relaxation by PKC-coupled generated ROS.

These factors are considered the potential unifying mechanisms **(Figure 1.8)** by which hyperglycemia mediates oxidative cellular dysfunction which have are implicated in the pathogenesis of in T2D complications. These complications are broadly divided into two namely: microvascular and macrovascular complications.

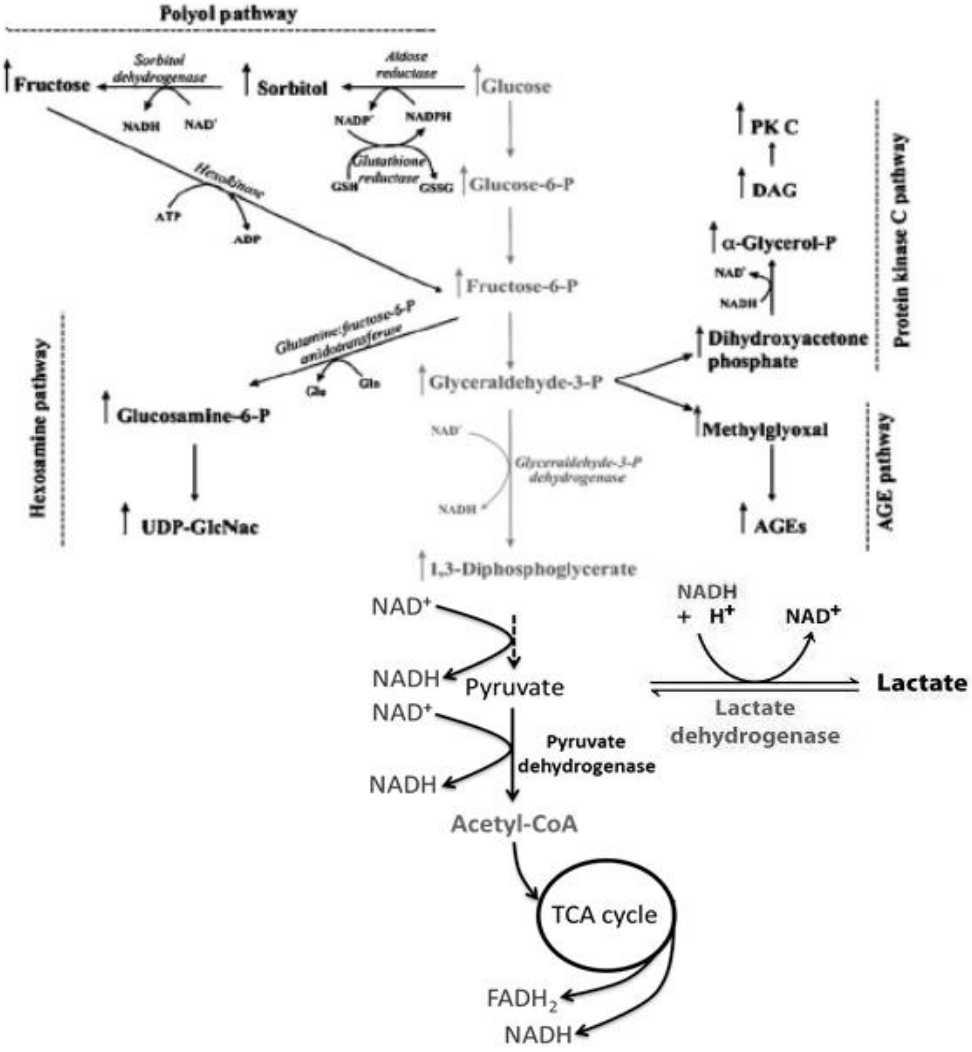

Figure 1.8: Potential unified mechanism of hypoglycemia induced oxidative cellular dysfunction in T2D (adapted without permission (Luo et al. 2016; Rolo and Palmeira 2006))

1.3.2 Microvascular complications

Microvascular complications are diseases associated with small blood vessels often developed in diabetes. The most notables of this complications are retinopathy, nephropathy and neuropathy.

a) Diabetic retinopathy

Diabetic retinopathy is one of the microvascular complications and main cause of blindness in prolonged diabetics. It is characterized by vascular lesions in the retinal blood vessels, leading to the formation of microaneurysms, haemorrhages and amplified leakage (Ahmed et al. 2010; Tripathi and Srivastava 2006). Thereby, resulting to retinal edema and severe exudation of lipid (Ahmed et al. 2010). In proliferative retinopathy, there is development of abnormal blood vessels and fibrous tissues in the retina with subsequent retinal detachment and visual impairment (Ahmed et al. 2010; Forrester and Knott 1997). Hyperglycemia activation of the polyol pathway causes accumulation of sorbitol in the lens, thereby leading to cataract (Ahmed et al. 2010; Fong et al. 2004).

b) Diabetic nephropathy

Diabetic nephropathy has been recognized as the leading cause of end-stage renal failure in diabetics, affecting type 2 diabetics more susceptible (Fowler 2008). It is characterized by glomerular hemodynamic abnormalities which causes glomerular hyper-filtration, leading to glomerular damage (microalbuminuria) (Ighodaro and Adeosun 2017). Prolong and untreated microalbuminuria often progress to overt proteinuria and end-stage renal failure (Fowler 2008; Ighodaro and Adeosun 2017). Increased thickening of the glomerular basement owing to synthesis and catabolism of the glomerular basement membrane macromolecules has been implicated in the pathogenesis of microalbuminuria (Tripathi and Srivastava 2006). Increased levels of VEGF have also been recognized as a causative factor in increasing glomerulus permeability (Ritz and Orth 1999).

c) Diabetic neuropathy

Diabetic neuropathy is a group of diseases that involves the somatic and autonomic nervous system due to prolonged hyperglycemia. It is often grouped into polyneuropathy, mono-neuropathy, and/or autonomic neuropathy, with polyneuropathy being the most common (Ahmed et al. 2010; Ighodaro and Adeosun 2017). Polyneuropathy is characterized by loss of peripheral sensations, leading to non-healing ulcers (wounds) and subsequently, non-traumatic amputation (Tripathi and Srivastava 2006). This has been attributed to decreased microfilaments, thickened axons, monocytes adhesion, and narrow capillary (Tripathi and Srivastava 2006), which may result from

hyperglycemia-mediated injury of the nerve parenchyma and/or neuronal ischemia (Chen and Reaven 1997).

1.3.3 Macrovascular complications

These are complications mostly in T2D associated with large blood vessels. Atherosclerosis leading to the narrowing of arterial walls have been recognized as the underlying factor for the pathogenesis of macrovascular diseases (Fowler 2008; Ighodaro and Adeosun 2017). Thus, making CVDs a major causative factor of mortality in T2D, and account for the highest healthcare expenditures (Fowler 2008). This include congestive heart failure, peripheral vascular disease, myocardial infarction, and coronary artery disease (Tripathi and Srivastava 2006). The occurrence of T2D in metabolic syndrome settings, also makes individuals with T2D susceptible to CVD (Fowler 2008).

Chronic inflammation and oxidative injury to the peripheral arterial wall or coronary vascular system have been implicated in the triggering of atherosclerosis (Fowler 2008; Ighodaro and Adeosun 2017). This is due to the accumulation of oxidized lipids in the endothelial walls of the arteries, causing the narrowing down of the arteries lumen. This is followed by infiltration of monocytes across the arterial wall, differentiating into macrophages which form foam cells on accumulation with oxidized lipids (Fowler 2008). The formed foam cells further stimulate the proliferation of macrophages, attracting T-lymphocytes. T-lymphocytes then induce proliferation of the arterial walls' smooth muscle, attracting collagen accumulation which leads to acute vascular infarction.

1.4 Management of T2D

Individuals with T2D are commonly recommended to modify their diets and lifestyle, particularly for long term management. However, pharmacological/therapeutic interventions are often introduced together with these modification for cases of chronic hyperglycemia and/or later stages of T2D. These interventions often involve the use of therapeutic drugs in regulating the high blood glucose level in chronic hyperglycemic state. These drugs are sub-divided into six major classes namely: α-glucosidase inhibitors, biguanides, sulfonylureas, thiazolidinediones, sodium glucose co-transporters (SGLT2) inhibitors, and dipeptidyl peptidase (DPP) IV inhibitors. **Figure 1.9** shows the different classes and their target organ of action.

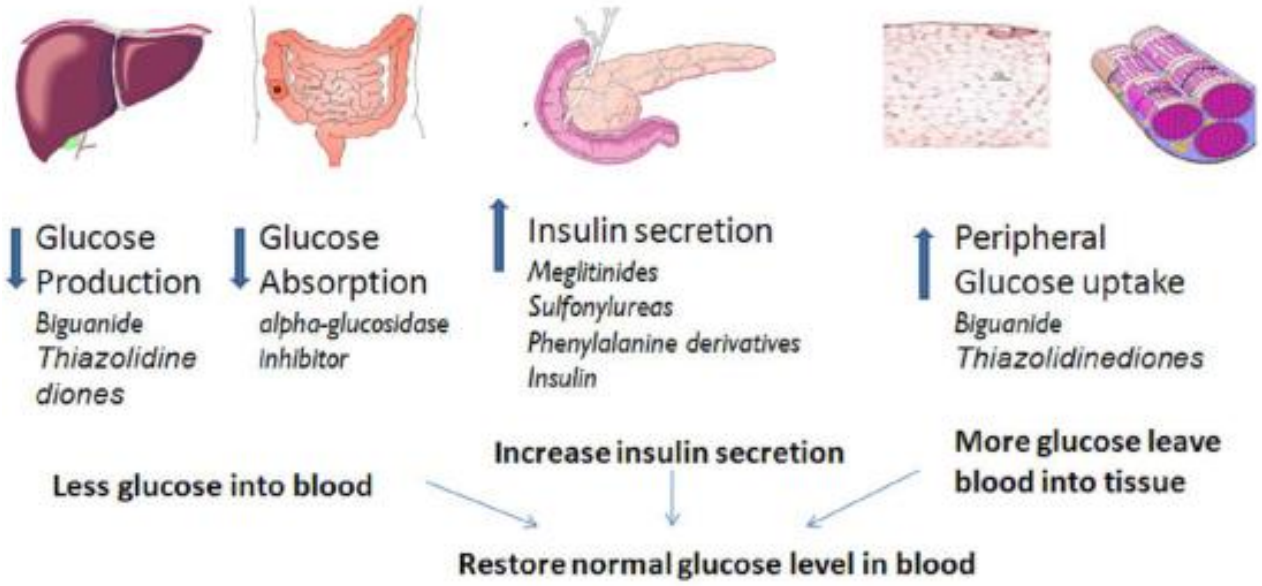

Figure 1.9: Classes of antidiabetic drugs, their mechanism and action sites (adapted without permission (Hui et al. 2009))

1.4.1 α-glucosidase inhibitors

This class constitutes drugs that control blood glucose level by the competitive inhibition of intestinal α-glucosidases activities. Alpha-glucosidases are enzymes involved in the breakdown of dietary oligosaccharide to monosaccharides in the small intestine, leading to postprandial rise in blood glucose level. Their inhibition will therefore delay the digestion of dietary carbohydrate, thus reducing the level of glucose released into the blood stream.

Examples of commonly used α – glucosidase inhibitors are acarbose, miglitol, and voglibose, with α – glucosidase being the most popular drug. However, side effects such as gastrointestinal disturbances, bloating, and flatulence have been associated with acarbose (Yee and Fong 1996).

1.4.2 Biguanides

This class of therapeutic drugs lowers blood glucose level by reducing hepatic glucose output, while enhancing insulin-stimulated glucose uptake and utilization in skeletal muscles and peripheral tissues (Campbell 2007; Tripathi and Srivastava 2006). The most common drug in this class is metformin. Its ability to increase insulin mediated phosphorylation of insulin-receptor and

tyrosine-kinase A, improves insulin actions in tissues that are less sensitive to insulin (Tripathi and Srivastava 2006). Metformin has been shown to upregulate GLUT-4 and GLUT-1 transporters in skeletal muscles and adipocytes (Klip and Leiter 1990). It increases glucose metabolism in the splanchnic bed (Bösenberg and van Zyl 2008). It has also been reported to suppress fatty acid oxidation, while reducing triglyceride levels (Bailey and Turner 1996). It is readily absorbed and eliminated in the urine (Bösenberg and van Zyl 2008). However, metformin medication has been associated with diarrhea, nausea, abdominal spasm, lactic acidosis, and metallic aftertaste (Bösenberg and van Zyl 2008).

1.4.3 Sulfonylureas

This class of antidiabetic drugs lowers blood glucose level by increasing pancreatic β-cell secretion of insulin. They bind directly to the β-cells via the transmembrane sulphonylurea receptor (SUR-1), leading to the closure of ATP-sensitive K^+ channels (Bösenberg and van Zyl 2008; Tripathi and Srivastava 2006). This reduces potassium efflux, triggering depolarization of the membrane and opening of the voltage-dependent $Ca2^+$-channels which causes an influx of calcium (Bösenberg and van Zyl 2008). This in turn stimulates insulin release from pancreatic secretory granules. Glibenclamide is the most popular drug in this class, others are gliclazide, glipizide, and glimepiride. However, the use of these drugs can lead to hypoglycemia owing to their ability to directly induce insulin secretion (Chiniwala and Jabbour 2011).

1.4.4 Thiazolidinediones

Also known as insulin sensitizer, this class of diabetic drugs improves insulin sensitivity via binding to the PPAR-γ receptor which is abundantly expressed in adipocytes (Bösenberg and van Zyl 2008). peroxisome proliferator-activated receptor gamma is a transcription factor which when activated insulin-dependent suppression of hepatic glucose output, and regulations lipid metabolism (Tripathi and Srivastava 2006). It is activated when bound to thiazolidinediones (TZDs).

Common drugs in this class are troglitazone, pioglitazone, and rosiglitazone. Their sides effects include oedema, weight gain, and hypoglycemia. Pioglitazone and rosiglitazone are restricted in Canada owing to increased risks of bladder cancer and non-fatal heart attack respectively (Bösenberg and van Zyl 2008).

1.4.5 SGLT2 inhibitors

This class of antidiabetic drugs reduce blood glucose level by inhibiting SGLT2, leading to the blockage of glucose reabsorption in the proximal renal tissues (Chaudhury et al. 2017). These drugs are often considered safe for chronic T2D owing to their ability to reduce blood glucose level independent of insulin, thus reducing the work pressure on the pancreatic β-cells. They also cause mild weight loss and reduce blood pressure. However, they have been associated with genital and urinary infections which could lead to urosepsis, pyelonephritis, and genital mycosis (Chaudhury et al. 2017).

Dapagliflozin and canagliflozin are presently the only commercial available SGLT2-inhibitors. However, the former is only marketed in Europe and unapproved by the FDA (Osadebe et al. 2015).

1.4.6 Dipeptidyl peptidase (DPP) IV inhibitors

This class consists of antidiabetic drugs that lower blood glucose level by inhibiting dipeptidyl peptidase IV (DPP-4), which is an antagonist of incretin hormones particularly the glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) (Osadebe et al. 2015; Pratley and Salsali 2007). These hormones are gastrointestinal and are released in response to meal. They increase β-cell secretion of insulin, while suppressing the release of glucagon (Osadebe et al. 2015). By preventing the inactivation of these hormones, DDP IV inhibitors thus reduces postprandial blood glucose rise.

Examples of antidiabetic drugs in this class are sitagliptin, vidagliptin, saxagliptin, alogliptin, and linagliptin (Osadebe et al. 2015). Their side effects include pharyngitis, headache and acute pancreatitis

1.5 Medicinal plants in the treatment and management of T2D

Over the years, medicinal plants have been part of the world's healthcare system, where they are employed in the folkloric treatment, management and prevention of human diseases. It is being estimated that 80% of the third world population rely on the use of herbs in traditional medicine as their primary source of healthcare (Ekor 2014), which is an indication of the importance of these plants even in modern times. There has also been an increased surge in the patronage and usage of

complementary and alternative medicines (CAMs) in developed countries (Anquez-Traxler 2011; Ekor 2014), with herbs being a major part. Studies on the medicinal efficacies of these plants have led to the development of novel drugs.

Several studies have documented the use of medicinal plants in the management of T2D and treatment of its complications (Erukainure et al. 2018; Ezuruike and Prieto 2014). This has been in practice from time immemorial, with the choice of plants and method of administration often influenced by both trado-and geo-cultural factors. Their accessibility and affordability have made the folkloric use of these plants an important form of healthcare in most developing countries with the burden of poor health infrastructures and political instability (W.H.O. 2013). There has also been an increased paradigm shift from the use of synthesized drugs to medicinal plants (Erukainure et al. 2017b), with some diabetics often relying on combined therapies of herbs and prescription drugs (Ezuruike and Prieto 2016). The antidiabetic efficacies of some of these plants have been attributed to a lot of factors, particularly the phytochemical constituents. These phytochemicals have been documented for their antidiabetic and antioxidative activities (Sabu and Kuttan 2002), with the polyphenols playing a major role (Bahadoran et al. 2013).

1.5.1 Toxicity of medicinal plants in the treatment of T2D

Despite the efficacy, health benefits, and wide acceptance of medicinal plants, there are however concerns about their safety and toxicity. These plants consist of a cocktail of phytochemicals of which a few may be therapeutically effective (Angelova et al. 2008). These phytochemicals may also elicit herbal – drug, herbal – food or herbal – herbal interactions with detrimental consequences (Ezuruike and Prieto 2014; Shan et al. 2007; Yüce et al. 2006). There are also concerns on their standardization, characterization, dosing and preparation (Shan et al. 2007), thereby making self-medication of herbal preparations a major issue to health practitioners. The method of extraction from different plant parts are also issues of concerns as they elicit different biological activities, some of which could be toxic due to the type of phytochemicals extracted (Ezuruike and Prieto 2014; Liu 2004).

Studies on some antidiabetic plants showed specific organ toxicity, particularly nephrotoxic and hepatotoxic, with concomitant effects on some liver and kidney function enzymes activities (Yüce et al. 2006). Some of these plants have been reported for their cytotoxic activities (Hilmi et al.

2014; Kadan et al. 2013). *In vitro* studies have also revealed that some of these plants can modulate P-glycoprotein and some cytochrome P450 enzymes, while some can alter the activities of phase 2 metabolic enzymes (Ezuruike and Prieto 2014). Thereby leading to potential alteration of the pharmacokinetics of co-administered drugs, such as glibenclamide which is a substrate of P-glycoprotein (Ezuruike and Prieto 2014).

Therefore, it is important to study the efficacies of medicinal plants used in the treatment of T2D and its complications, as well as the toxicity associated with their use.

1.6 Aims and objectives

The aim of this study was to investigate the antidiabetic and toxicological effects of selected anti-diabetic African medicinal plants namely: *Cola nitida, Clerodendrum volubile, Dacryodes edulis, Phaseolus lunatus, Vernonia amygdalina,* and *Raffia palm* (*Raphia hookeri*) *wine* using different *in vitro, ex vivo,* and *in vivo* models. The research objectives included the following:

1. **Chemical properties**
    - Investigating the chemical properties of the selected medicinal plants using GC-MS, LC-MS, HPLC, FTIR, and NMR.
2. ***In vitro* study**
    - Investigating the antioxidant, and enzyme inhibitory effects of the selected medicinal plants
    - Investigating the cytotoxic effect and mechanism of toxicity of the selected medicinal plants on human embryonic kidney cells (HEK 293) and 3T3-L1 adipocytes.
3. ***Ex vivo* study**
    - Investigating the antioxidative effect of the selected medicinal plants in oxidative pancreatic and hepatic tissues
    - Investigating the inhibitory effect of the selected medicinal plants on glucose absorption in isolated small intestines of rats
    - Investigating the effect of the medicinal plants on glucose uptake in rats' muscle and brain

- Investigating the effect of the medicinal plants on oxidative dysfunctional metabolic pathways using combined metabolomics

4. *In silico* studies

- Predicting the ADME properties of identified compounds of the most active medicinal plants
- Predicting the toxicity class of identified compounds of the most active medicinal plants
- Molecular docking of identified compounds of the most active medicinal plants with relevant target protein

5. *In vivo* studies

Three of the plants with the best *in vitro* and *ex vivo* activities were selected for this part of the study and were investigated for:

- antidiabetic, antioxidative, and antilipidemic effects in type 2 diabetic rats
- modulatory effects on cholinergic purinergic enzymes in type 2 diabetic rats
- modulatory effects on neuronal, testicular, hepatic and pancreatic microarchitecture of type 2 diabetic rats
- modulatory effects on dysfunctional metabolic pathways in type 2 diabetic rats
- molecular mechanism behind glucose uptake in muscle and brain of type 2 diabetic rats
- molecular mechanism of toxicity in type 2 diabetic rats

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CHAPTER 2

MATERIALS AND METHODS

2.1 Plant Materials

2.1.1 Collection of Plant Materials

The seeds of *Cola nitida*, and the leaves and flowers of *Clerodendrum volubile* were collected from Ifon, Ondo State Nigeria. Raffia palm (*Raphia hookeri*) wine, and the leaves of *Dacryodes edulis* and *Vernonia amygdalina* were collected from Benin city, Edo State, Nigeria. The seeds of *Phaseolus lunatus* were collected from Irrua, Edo State, Nigeria. All samples, except *Dacryodes edulis* were identified, authenticated and deposited at the herbarium of the department of Botany, University of Benin, Benin city, Edo State, Nigeria. They were assigned the voucher numbers: UBH_{C284} (*C. volubile*), UBH_{C323} (*C. nitida*), UBH_{P284} (*P. lunatus*) and UBH_{V342} (*V. amygdalina*). *Dacryodes edulis* were identified, authenticated and deposited at the herbarium of the department of Botany, University of Lagos, Lagos, Nigeria. It was assigned voucher numbers, LUH: 6964.

All samples except raffia palm wine were air dried, blended, and stored in airtight bags for subsequent analysis.

2.1.2 Preparation of Crude Extracts

One hundred gram each of the blended samples of *D. edulis*, *V. amygdalina*, and *C. volubile* (flowers) were defatted using n-hexane, and then sequentially extracted with solvents of increasing polarity to yield ethyl acetate, ethanol and aqueous extracts (Erukainure et al. 2017b). All extracts except aqueous were concentrated *in vacou* using a rotatory evaporator (Buchi RII; Flawil, Switzerland) and stored in glass vials at 4^{o}C until further analysis. The aqueous extracts were concentrated with a water bath at 50°C, and subsequently stores in glass vials at 4^{o}C until further analysis.

One hundred gram of the blended sample of *C. volubile* leaves was subjected to methanol (MeOH) extraction. The resulting extract was concentrated *in vacou* using a rotatory evaporator (Buchi RII; Flawil, Switzerland) and stored in glass vials at 4^{o}C until further analysis.

One hundred gram of the blended seeds of *P. lunatus* were defatted with n-hexane, and then subjected to aqueous extraction with distilled water. The extract was concentrated with a water bath at 50°C and stored in glass vials at 4^{o}C until further analysis.

Fresh raffia palm wine tapped from *Raphia hookeri* was first filtered with prewashed cotton to remove any debris present. It was subsequently divided into 3 batches of 300 mL each. The first batch was concentrated at 50°C and labeled as unfermented. The other batches were subjected to open air fermentation for 24 and 48 h, respectively. They were concentrated at 50°C with a water bath and stored in glass vials at 4°C until further analysis.

2.1.3 Preparation of hot infusions

Ten grams each of the blended samples of *C. volubile* (leaves), *C. nitida*, and *P. americana* were infused in boiling distilled water and allowed stand overnight (Erukainure et al. 2017c). The extracts were filtered and concentrated in a water bath at 50^{o}C, then stored in glass vials at 4°C until further analysis.

2.1.4 Fractionation of crude extracts

The ethanolic crude extracts of *D. edulis*, and *C. volubile* (flowers) as well as the MeOH extract of *C. volubile* leaves were further subjected to liquid – liquid fractionation using a separating funnel. Ten grams of each extract was dissolved in distilled water and then fractionated with solvents of gradient polarity in the order of hexane, dichloromethane (DCM), ethyl acetate, and butanol (BuOH). The aqueous residue was considered as aqueous fraction and was concentrated at 50°C with a water bath and stored in glass vials at 4°C until further analysis. The other fractions were concentrated *in vacou* using a rotatory evaporator (Buchi RII) (Erukainure et al. 2018; Erukainure et al. 2017a)

2.1.5 Preparation of stock solution

A 1 mg/mL stock solution was prepared from each extract and fraction in distilled water. Different concentrations consisting of 15, 30, 60, 120 and 240 mg/mL were prepared from the stock solution for subsequent *in vitro* and *ex vivo* activities (Erukainure et al. 2017c).

2.2 Total phenolic content

The Folin–Ciocalteu reagent assay was used in determining the total phenolic content of each extract and fraction (Liu and Yao 2007).

Twenty microliters of each extract and fraction (240 µg/mL) was incubated with 100 µL of 10% diluted Folin ciocalteau reagent and 80 µL of 0.7 M Na2CO3 for 30 minutes at room temperature. Absorbance was measured at 765 nm and results expressed as milligrams of gallic acid equivalents (GAE) per gram of dry weight.

2.3 *In vitro* antioxidant assays

The 2,2'-diphenyl-1-picrylhydrazyl (DPPH) scavenging (Braca et al. 2002) and ferric reducing antioxidant power (FRAP) assays (Benzie and Strain 1996) were used in the determination of the in vitro antioxidant activities of the plant extracts and fractions. Ascorbic acid was used as standard for both activities.

2.3.1 DPPH scavenging activity

One hundred µL of each sample and standard concentrations (15 – 240 mg/mL) was incubated with 50 µL of 0.3 mM DPPH solution (in methanol) in the dark for 30 mins at room temperature. The absorbance was then read at 517 nm against blank without sample or standard.

2.3.2 FRAP activity

One hundred µL of each sample and standard concentrations was mixed with 100 µL sodium phosphate buffer (0.2 M, pH 6.6) and 100 µL of 1% potassium ferricyanide and then incubated at 50°C for 30 mins. The mixture was acidified with 100 µL of 10% trichloroacetic acid (TCA), 100 µL of distilled water and 200 µL of 0.1% iron (III) chloride ($FeCl_3$) were then added. Absorbance was read at 700 nm. Results were expressed as a percentage of the absorbance of the samples to that of gallic acid as shown below:

$$\text{Ferric reducing antioxidant power (\%)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of gallic acid}} \text{x } 100$$

2.4 Enzyme inhibitory activities

The antidiabetic and anti-obesogenic potentials of the extracts and fractions were ascertained via their ability to inhibit key enzymes linked to type 2 diabetes (α-glucosidase and α-amylase) and obesity (pancreatic lipase) *in vitro* (Kim et al. 2010; Oboh and Ademosun 2011; Shai et al. 2010)

2.4.1 α-glucosidase Inhibition

The ability of the extracts and fractions to inhibit α-glucosidase *in vitro* was carried out according to the method described by Oboh and Ademosun (2011), with slight modifications. Fifty microliter of each sample concentrations were incubated with equal volume of α-glucosidase (1.0 U/mL) in phosphate buffer (100 mM, pH 6.8) at 37°C for 15 mins. Thereafter, 100 µL of 5 mM *p*-nitrophenyl-α-D-glucopyranoside (pNPG) solution in phosphate buffer (100 mM, pH 6.8) was added to the reaction mixture, which was further incubated for 20 minutes at 37°C. The absorbance of the liberated p-nitrophenol was measured at 405 nm and the inhibitory activity expressed as a percentage of the control lacking inhibitors as shown in the formula below:

$$\% \text{ inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \text{x } 100$$

Acarbose was used as the standard drug.

2.4.2 α-amylase inhibition

The ability of the extract to inhibit α-amylase was investigated *in vitro* according to the method described by Shai et al. (2010), with slight modification. Fifty microliter of each sample concentrations or Acarbose was incubated with equal volumes of porcine pancreatic amylase (2 U/mL) in phosphate buffer (100 mM, pH 6.8) for 10 minutes at 37°C. A 50 µL of 1% starch solution in phosphate buffer (100 mM, pH 6.8) was added to the reaction mixture and incubated at 37°C for 10 mins. 100 µL of the dinitrosalicylate (DNS) color reagent was then added to the mixture and boiled for 10 minutes. Absorbance was then read at 540 nm and the inhibitory activity was expressed as percentage of the control without inhibitors as shown below:

$$\% \text{ inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \text{x } 100$$

2.4.3 Pancreatic lipase inhibition

The ability of the extracts and fractions to inhibit pancreatic lipase activity *in vitro* was determined by the method of Kim et al. (2010), with slight modifications. Fifty microliters of each sample or orlistat (standard drug) concentrations was added to 84.5 µL of Tris buffer (100 mM Tris–HC1 and 5 mM $CaCl_2$, pH 7.0), 20 µL of porcine pancreatic lipase (2.5 mg/mL in 10 mM MOPS (morpholine propane sulphonic acid) and 1 mM EDTA, pH 6.8) was then added and incubated at 37°C for 15 mins. 5 µL of 10mM p-NPB (p-nitrophenyl butyrate in dimethyl formamide) was added to the reaction mixture and further incubated 30 min at 37°C. Absorbance was then read at 405 nm and the inhibitory activity was expressed as percentage of the control without inhibitors as shown below:

$$\% \text{ inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \text{ x } 100$$

2.5 *In vitro* glucose uptake, absorption and diffusion

2.5.1 Glucose uptake/ transport by yeast cells

The effect of the extracts and fractions on glucose uptake/ transport by yeast cells were carried out according to previous established protocol (Cirillo 1962; Nirupama et al. 2014). Different concentration of the extracts and fractions were dissolved in 1 mL of distilled water containing 25 mM glucose. The resulting solution was incubated for 10 mins at 37°C. Thereafter, 100 μ L of 1% yeast suspension was added, vortexed and incubated for 60 mins at 37°C. Glucose concentration of the solution was determined with dinitro salicylic acid method (DNS), and calculated by the formula:

$$\% \, Uptake = \frac{Absorbance \; (control) - Absorbance \; (sample)}{Absorbance \; (control)}$$

2.5.3 Glucose diffusion

The ability of the extracts and fractions to inhibit glucose diffusion was carried out using a previous established method which monitors the movement of glucose across a dialysis bag (Gallagher et al. 2003; Nirupama et al. 2014). One milliliter of 50 µg/mL of the extracts in phosphate buffer

(100 mM, pH 6.8) was mixed with 0.22 M glucose and 0.15 M sodium chloride (NaCl) in a dialysis bag. The bag was sealed at both ends and placed in 45 mL of NaCl on an orbital shaker at 37°C. Glucose movement across the bag was monitored by estimating its concentration in the external solution at regular time intervals using the formula:

$$\% \text{ inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \text{x } 100$$

2.6 *Ex vivo* antioxidative and anti-proinflammatory activities

Tissues of liver and pancreas harvested from normal male albino rats (180 g) were homogenized with phosphate buffer (10 mM, pH6.8) on ice, then centrifuged at 15000 rpm for 15 min at 4°C. The supernatant was collected and stored at −20°C.

2.6.1 Induction and treatment of oxidative stress *ex vivo*

Equal volumes of each sample concentration and tissues (pancreatic or hepatic) homogenates were incubated with 30% volume of pro-oxidant (0.1 mM $FeSO_4$) at 37°C in 5% CO_2 for 30 mins. A reaction containing no extract or fraction (untreated) served as negative control. (Erukainure et al. 2017b; Erukainure et al. 2017c). The incubated samples were analyzed for oxidative and proinflammatory biomarkers as described below.

2.6.2 Oxidative stress biomarkers

1. Determination of reduced glutathione (GSH) levels

Reduced glutathione (GSH) levels were determined by the Ellman's method (Ellman 1959), which is based on the oxidation of GSH by 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) to form the yellow derivative 5′-thio-2-nitrobenzoic acid (TNB).

The standards were prepared from GSH stock in increasing concentrations: 0.025, 0.05, 0.1, 0.2 and 0.4 mM. Their absorbance was read at 415 nm and plotted against the concentration to get the standard curve (**Figure 2.1**)

The samples were deproteinize with an equal volume of 10% TCA and then centrifuged at 3500 rpm for 5 mins at 25ºC. Thereafter, 200 µL of the supernatant was pipetted into a 96 well plate. A 50 µL of Ellman reagent was then added and allowed to stand for 5 mins. Absorbance was read at

415 nm. The GSH level was then extrapolated from the standard curve of plotted GSH concentrations.

2. Determination of superoxide dismutase (SOD) activities

Superoxide dismutase activity was determined using previously established method based on the principle that H_2O_2 from SOD catalyzed dismutation of $O_2^{\cdot -}$ oxidizes 6-hydroxydopamine (6-HD) to produce a coloured product (Gee and Davison 1989).

A 170 µL of 0.1 mM diethylenetriaminepentaacetic acid (DETAPAC) was added to and 15 µL of the samples in a 96-well plate. Thereafter, 15 µL of 1.6 mM 6-HD was added and mixed by gently tapping all four sides of plates. Absorbance of the resulting mixture was measured at 492 nm for 5 mins at 1 min interval. The enzyme activity was calculated by the formula:

$$\text{Activity} = 1000 \times [(A_1 - A_b)/\varepsilon 490\ ] \times 0.5\ \text{nmol/min/µg protein}$$

$\varepsilon 490$ = Molar absorptivity at 490nm = 1.742/mM/cm

A_1 and A_b = Reaction rate for sample and blank respectively

3. Determination of catalase activities

The catalase activity of the sample was determined using previously established protocol which is based on the measurement of decreased absorbance of test samples owing to H_2O_2 decomposition (Aebi 1984). A 340 µL of 50 mM sodium phosphate buffer (pH 7.0) was mixed with 10 µL of the samples, then 150 µL of 2 M H_2O_2 was added to the mixture. Absorbance was read at 240 nm for 3 min at 1 min interval.

4. Determination of lipid peroxidation levels

Lipid peroxidation levels of the samples were determined by measuring thiobarbituric acid reactive substances (TBARS), expressed as malondialdehyde (MDA) equivalent (Chowdhury and Soulsby 2002; Janero 1990). One hundred microliters of the samples were mixed with an equal volume of 8.1% SDS solution, 375 µL of 20% acetic acid, 1 mL of 0.25% thiobarbituric acid (TBA), and 425 µL of distilled water. The reaction mixture was heated at 95°C for 1 h in a water bath. Thereafter, 200 µL of the heated mixture was pipetted into 96-well plate and absorbance read at 532 nm. A standard curve was generated from the absorbance of standard MDA, from which the TBARS concentration was extrapolated.

5. Determination of non- protein thiol group (NPSH) levels

The NPSH levels of the samples were determined using Ellman's reagent according to previous established protocol (Adefegha et al. 2017; Habig et al. 1974). Two hundred microliters of the samples were deproteinized with 10% triton, then vortexed and allowed to stand for 10 mins. Thereafter, 200 µL of 20% TCA was added, vortexed and centrifuged for 10 mins at 4000 rpm at 4°C. 50 µL of Ellman's reagent was then added to 100 µL of the supernatant and incubated for 1 h at room temperature. Absorbance was read at 412 nm. The thiol groups (SH) levels was extrapolated from a standard curve of cysteine.

2.6.3 Anti-proinflammatory activity

1. Determination of NO levels

The NO levels of the samples were determined using the Griess method (Sun et al. 2003). One hundred microliters of the samples or distilled water (blank) and was incubated with an equal volume of Griess reagent for 30 mins at 25°C in the dark. Absorbance was read at 548 nm and the result was calculated using the formula:

$$\textbf{Nitric oxide conc.} = (\text{Absorbance of sample} - \text{Absorbance of blank}) \text{ x } 0.1305$$

2. Determination of myeloperoxidase activities

The myeloperoxidase (MPO) activities of the tissue samples were determined using previous established method with slight modifications (Ajayi et al. 2015; Granell et al. 2003). One hundred microliters of the samples or distilled water (blank) were incubated with 100 µL of 5 mM KCl and 25 µL of 2 M H_2O_2 for 10 mins. Thereafter, 50 µL of 1.25% ammonium molybdate was added to reaction mixture and allowed to stand for 5 mins. Absorbance was read at 405.

2.6.4 Purinergic enzymes inhibitory activities

1. Determination of ATPase activities

The ATPase activities of the tissue samples were determined according to previous established protocol (Adewoye et al. 2000; Erukainure et al. 2017b). Two hundred microliters of the samples were incubated with 200 µL of 5 mM KCl, 1300 µL of 0.1 M Tris-HCl buffer, and 40 µL of 50

mM ATP at 37°C in a shaker for 30 mins. Thereafter, 1 mL of distilled water and 1.25% ammonium molybdate were added respectively to stop the reaction. 1 mL of freshly prepared 9% ascorbic acid was then added to the reaction mixture and allowed to stand for 30 mins. Absorbance was read at 660 nm and ATPase activity was calculated as the amount of inorganic phosphate (Pi) released/min/mg protein.

2. Determination of E-NTPDase activities

The E-NTPDase activities of the tissue samples were determined according to a previously established protocol with slight modifications (Ademiluyi et al. 2016; Schetinger et al. 2007). Twenty microliters of the samples were incubated with 200 µL of the reaction buffer (1.5 mM $CaCl_2$, 5 mM KCl, 0.1 mM EDTA, 10 mM glucose, 225 mM sucrose and 45 mM Tris-HCl) for 10 mins at 37°C. Thereafter, 20 µL of 50 mM ATP was added to the reaction mixture and further incubated for 20 mins at 37°C in a shaker. The reaction was stopped with 200 µL of 10% TCA and incubated with 200 µL 1.25% ammonium molybdate and freshly prepared 9% ascorbic acid for 10 mins in ice. Absorbance was read at 600 nm and E-NTPDase activity was calculated as the amount of inorganic phosphate (Pi) released/min/mg protein.

2.6.5 Determination of glucose-6-phosphatase activities

The glucose-6-phosphatase activities of the tissue samples were determined according to previously established protocol (Mahato et al. 2011), with slight modification (Erukainure et al. 2017b). Two hundred microliters of tissues were incubated with 100 µL of 0.25 M glucose, 200 µL of 5 mM KCl, 1300 µL of 0.1 M Tris-HCl buffer, and 40 µL of 50 mM ATP at 37°C in a shaker for 30 mins. Thereafter, 1 mL of distilled water and 1.25% ammonium molybdate were added respectively to stop the reaction. 1 mL of freshly prepared 9% ascorbic acid was then added to the reaction mixture and allowed to stand for 30 mins. Absorbance was read at 660 nm and ATPase activity was calculated as the amount of inorganic phosphate (Pi) released/min/mg protein.

2.6.6 Quantitation of DNA fragmentation

Induction and treatment of tissues *ex vivo* were carried out as described in **section 2.5.1** except for 4 h incubation. The percentage DNA fragmentation of the tissue samples were quantitated with diphenylamine (DPA) according to previous established protocol (Erukainure et al. 2017b;

Erukainure et al. 2017c; Muhammad et al. 2016; Sellins and Cohen 1987). The samples were centrifuged at 200g for 10 mins at 4°C. The supernatant was collected in new tubes and labelled S, while the tubes with the substrates (pellets) were labelled B. Thereafter, 100 µL of TTE solution was added to tube B, vigorously vortexed for 1 min and centrifuged at 20,000g at 4°C for 10 mins. The supernatant was collected into new tubes and labelled T. 100 µL of TTE solution was added again to the pellets in tube B. 100µl of 25% TCA was added to all tubes and vigorously vortexed for 1 min. They were incubated overnight at 4°C to allow precipitation of the DNA. The precipitated DNA was then recovered by pelleting for 10 min at 20,000g at 4°C. The supernatants were carefully discarded by aspiration. To hydrolyze the DNA, 160 µL of 5% TCA was added to tubes B and T, then heated at 90°C for 15 mins in a water bath. Subsequently, 320µl of freshly prepared DPA solution was added to all tubes, vortexed and incubated at 4°C for 4 h. 200 µL of the reaction solutions were pipetted into 96 well plate and absorbance read at 600 nm. Quantitation was calculated via the formula:

$$\% \text{ Fragmented DNA} = \frac{\text{Absorbance (T)}}{\text{Absorbance (T)} + \text{ Absorbance (B)}} \text{ x } 100$$

2.7 *Ex vivo* glucose absorption and uptake

Intestines, psoas muscles and brain were harvested from normal male albino rats (180 – 200 g). They were placed in ice and used immediately for the experiments. All animals used were maintained under the guidelines and approval of the animal ethics committee of the University of KwaZulu-Natal, Durban, South Africa (protocol approval number: AREC/020/017D).

2.7.1 Determination of glucose absorption in isolated rat jejunum

The effect of the extracts and fractions on intestinal glucose absorption was determined according to previously established protocol which measures the glucose level in a reaction solution containing the extracts and 5 cm isolated rat jejunum (Chukwuma and Islam 2015). The jejunal segments of the freshly harvested intestines were removed, flushed with Krebs buffer (118 mM NaCl, 5 mM KCl, 1.328 mM $CaCl_2 \cdot 2H_2O$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$ and 25 mM $NaHCO_3$) and subsequently divided into 5 cm segments. The villi of each segments were exposed and incubated with different concentrations of the extracts and fractions in 8 mL of Kreb's buffer containing 11.1 mM glucose under a 5% CO_2, 95% oxygen and 37°C conditions for 2 h. Tissues

incubated in Krebs buffer without the extract served as the normal control. Glucose with Krebs buffer without tissue or extracts served as the blank. Glucose concentration were measured before and after incubation with an Automated Chemistry Analyzer (Labmax Plenno, Labtest Inc., Costa Brava, Brazil). Intestinal glucose absorption was calculated with the formula:

$$\text{Glucose absorption / cm of rat jejunum} = \frac{\text{GC2} - \text{GC1}}{\text{Length of jejunum used in cm}}$$

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

2.7.2 Determination of glucose uptake in isolated rat psoas muscles

The effect of the extracts and fractions in isolated rat psoas muscles were determined according to the method of Chukwuma and Islam (2015). Different concentrations of the extracts and fractions were each incubated with 0.5 g of the isolated muscle in 8 mL of Krebs buffer containing 11.1 mM glucose under a 5%CO_2, 95% oxygen and 37°C conditions for 2 h in a CO_2 incubator (Lasec, South Africa). Tissues incubated in Krebs buffer without the extract served as the normal control. Glucose with Krebs buffer without tissue or extracts served as the blank. Metformin was used as the standard drug. Glucose concentration were measured before and after incubation with an Automated Chemistry Analyzer (Labmax Plenno, Labtest Inc., Costa Brava, Brazil). Muscle glucose absorption was calculated with the formula:

$$Glucose\ uptake\ per\ gm\ of\ rat\ psoas\ muscle = \frac{GC1 - GC2}{Weight\ of\ muscle\ tissue\ (g)}$$

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

2.7.3 Determination of glucose uptake in rat brain

The effect of the extracts and fractions in rat brain were determined according to the method of Chukwuma and Islam (2015). Different concentrations of the extracts and fractions were each incubated with 0.6 g of the harvested brain in 8 mL of Krebs buffer containing 11.1 mM glucose under a 5%CO_2, 95% oxygen and 37°C conditions for 2 h in a CO_2 incubator (Lasec, South Africa). Tissues incubated in Krebs buffer without the extract served as the normal control. Glucose with Krebs buffer without tissue or extracts served as the blank. Metformin was used as the standard drug. Glucose concentration were measured before and after incubation with an Automated

Chemistry Analyzer (Labmax Plenno, Labtest Inc., Costa Brava, Brazil). Brain glucose uptake was calculated with the formula:

$$Glucose\ uptake\ per\ gm\ of\ rat\ psoas\ muscle = \frac{GC1 - GC2}{Weight\ of\ muscle\ tissue\ (g)}$$

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

2.7.3 Determination of antioxidative, anti-proinflammatory and purinergic enzymes activities in brain

After incubation, the muscle and the brain were homogenized in sodium phosphate buffer (10 mM, pH 6.8) on ice, then centrifuged at 15000 rpm for 15 min at 4°C. The supernatant was collected and stored at −20°C. They were subsequently analyzed for antioxidative stress, anti-proinflammatory, and purinergic enzymes activities as described in **sections 2.6.2 – 2.6.4.**

2.8 Metabolite profiling and pathway analysis

Equal volumes of 240 µg/mL of active extracts or fractions and tissue homogenates were incubated with 15% pro-oxidant (0.1 mM $FeSO_4$) overnight in 5% CO_2 at 37^{o}C.

2.8.1 Metabolic Profiling

The target metabolites were extracted from the incubated samples using a previously described method (Chan et al. 2013) with slight modifications (Erukainure et al. 2017c). A 200 µL of the treated samples were first deproteinized with 800 µL of 20% methanol/ethanol, before incubating in ice for 20 mins. Thereafter, they were centrifuged at 20,000g at 4^{o}C for 10 mins. The supernatant containing the metabolites was lyophilized overnight at 45°C.

The lyophilized samples were scanned at a spectral range of 300 – 4000 cm^{-1} on a FT infrared spectrophotometer. The chemical functional groups were determined by comparing the peak heights to an IR spectroscopy correlation table (Erukainure et al. 2017c).

A 200 µL of 0.1% formic acid was used in reconstituting the samples, which was then subjected to mass spectrometry via LC-MS (Shimadzu LCMS-2020 Single Quadrupole) (Erukainure et al.

2017c). Identification of the metabolites was done by direct search of mass spectral data against the Human Metabolome Database (Wishart et al. 2012).

2.8.2 Pathway Analysis

The MetaboAnalyst 3.0 (http://www.metaboanalyst.ca/) and FunRich (www.funrich.org) servers were used in analyzing pathways of significantly altered metabolites (Pathan et al. 2015; Xia and Wishart 2016).

2.9 Cytotoxic activity

The cytotoxicity of the most active fractions was investigated in human embryonic kidney cells (HEK293) and 3T3-L1 adipocytes by the 3-(4, 5-dimethyl thiazole-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) colormetric assay.

2.9.1 Cell culture

The cells were grown in Eagles Minimum Essential Medium (EMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 1% (v/v) L-glutamine, 100 U penicillin and 100 µg/mL streptomycin at 37^{o}C in a humidified atmosphere with 5% CO_2.

2.9.2 MTT assay

The cytotoxic effect of the fractions on HEK293 and 3T3-L1 adipocytes cells was evaluated using the standard MTT colorimetric assay (Mosmann 1983). One hundred microliters of different concentrations of the fractions were incubated with equal volume of HEK293 cells (1 x 10^4 cells/mL) for 48 h at 37°C in a humidified atmosphere of 5% CO_2. The medium was replaced with a fresh one and incubated with 200 µL of MTT (5 mg/mL in PBS) for 4 h at 37°C. The medium and MTT were removed from the wells and replaced with 200 µL of DMSO to dissolve the formazan salt. Absorbance was read at 570 nm and the percentage inhibition was calculated using the formula:

% inhibition = (mean of absorbance of sample/mean of absorbance of control) x 100

2.9.3 Determination of antioxidative, anti-proinflammation, and purinergic enzymes activities

HEK293 cells were seeded into a 24-well plate at a density of 3.5 - 4.5 x 10^5 cells/well and incubated for 24 h. The cells were then incubated with the fractions for 48 h in a humidified atmosphere with 5% CO_2 at 37°C. A sample-free cells served as the positive control. The medium was removed and attached cells were detached gently scraping after rinsing with PBS. Detached cells were stored with PBS in 2 mL Eppendorf tubes at -20°C until further analysis. The cells were assayed for antioxidative, anti-proinflammatory and purinergic enzymes activities as described in **Sections 2.6**

2.9.4 Apoptosis Assay

The apoptotic effect of the fractions on the cells was determined by the acridine orange and ethidium bromide (AO/EB) dual staining method (Maiyoa et al. 2016). Cells were seeded and treated as described in **Section 2.9.3**. After incubation and rinsing with PBS, a 10 µL dual stain (AO: 100 µg/mL, EB: 100 µg/mL in PBS) was added and allowed to stand for 5 min at 25°C. Thereafter, they were viewed for morphological changes under an Olympus inverted fluorescent microscope fitted with a CC12 fluorescent camera, at X200 magnification. Apoptotic index was calculated by the formula:

$$\text{Apoptotic index} = \frac{\text{number of apoptotic cells}}{\text{number of total cells counted}}$$

2.10 Determination of chemical profiles of active extracts and fractions

The active extracts and fractions were subjected to chemical profiling to identify the phytochemical constituents that may be responsible for the observed biological activities.

2.10.1 Gas Chromatography-Mass Spectroscopy (GC-MS) profiling

The extracts and fractions were analyzed with GC-MS using an Agilent technologies 6890 series GC coupled with (an Agilent) 5973 Mass Selective Detector and driven by Agilent Chemstation software. The operating parameters were:

Column: HP-5MS capillary column (30 m × 0.25 mm ID, 0.25 μm film thickness, 5% phenylmethylsiloxane); **Carrier gas**: ultra-pure helium; **Flow rate**: 1.0 mL min^{-1} and a linear velocity of 37 cm sec^{-1}; **Injector temperature**: was set at 250°C. Initial oven temperature: 60°C, programmed to 280°C at the rate of 10°C min^{-1}. **Injection**: 1 μL made in split mode at a split ratio of 20:1; **Electron ionization mode**: 70 eV; **Electron multiplier voltage**: at 1859 V; **Ion source temperature**: 230°C; **Quadrupole temperature**: 150°C; **Solvent delay**: 4 min; **Scan range**: 50-70 amu.

Compounds were identified by directly comparing the retention times and mass spectral data with those in the NIST library.

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

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

Photodiode Array (PDA) sampling frequency: 1.5625 Hz; **Operating mode**: low pressure gradient; **Pump A**: LC-2030 Pump; **Mobile Phase A and B**: water and methanol respectively; **Flow rate:** 0.200 ml/min; **Start and End wavelengths**: 190 and 800 nm respectively; **Cell Temp**.: 40 °C; **Start and End time**: 0.00 and 4.00 mins respectively; **Acquisition mode**: Scan; **Scan Speed**: 1667 u/s; **Polarity**: Positive; **Event Time**: 1.00 s; **Detector Voltage**: +1.00 kV; **Threshold**: 0; **Start and End m/z**: 50.00 and 1700.00 respectively.

Compounds were identified by direct comparing mass spectral data with those in the NIST library online and Food Database [FooDB Version 1.0] (FoodDB, 2017).

2.10.3 High Pressure Liquid Chromatography (HPLC) analysis

The reverse phase HPLC with an Agilent 1100 Series HPLC-diode-array detector (DAD) was used in the profiling of the extracts and fractions. A C18 column (4.6 ×150 mm) packed with 5-μm diameter particles was utilized for this method. The operating parameters were:

Mobile phase A and B: methanol and 100% water respectively; **Initial and final temperature of left and right columns**: 27.3 °C and 28.5 °C respectively; Left and right columns pressure:

112.9 and 121.3 bar respectively**; Detector Lamp Burn Times setups**: DAD 1, UV Lamp: (Current On-Time) 3.89 h, (Accumulated On-Time) 4041.3 h; DAD 1, Visible Lamp: 3.53 3781.6 h.

Identification and quantification of compounds were done by comparing the retention time and UV absorption spectra with those of the studied standards.

2.10.4 Fourier Transform Infrared Spectroscopy (FTIR) Analysis

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

2.10.5 Proton NMR spectroscopy analysis

Thirteen mg each of the *C. nitida* infusion, unfermented and fermented Raffia palm wine samples were first dissolved in 0.8 mL D_2O, then filtered through prewashed cotton before subjecting to ^{1}H-NMR spectroscopy (400 MHz). Chemical shifts were reported in δ (ppm) values. Compounds were identified by direct search and comparison of chemical shift data against the Food Database [FooDB Version 1.0] (FoodDB, 2017).

2.10.6 Amino acid profiling of *P. lunatus*

The amino acid profile of the aqueous extract of *P. lunatus* was determined according to previous established protocol by loading the extract into a Technicon sequential Multi-Sample Amino Acid Analyzer (TSM) and operated according to the manufacturer's manual (Benitez 1989).

2.11 *In silico* predictions

2.11.1 *In silico* prediction of ADME and oral toxicity of identified compounds

The ADME (absorption, distribution, metabolism, and excretion) properties and oral toxicity of the identified compounds of the extracts and fractions were predicted *in silico* using the SwissADME (Daina et al. 2017) and ProTOX (Drwal et al. 2014) online servers.

2.11.2 Molecular docking

Molecular docking was carried out to determine the binding affinities of D-glucose and identified compounds with to ATPase, cholinesterase, catalase, myeloperoxidase, glucose transporter 4 (GLUT4), and α-glucosidase. Their 3D crystal structures were retrieved from protein data bank, with PDB access codes- ATPase: 4HYT (Laursen et al. 2013), cholinesterase: 1TBU (Yang et al. 2013), catalase: 1EVE (Kryger et al. 1999), myeloperoxidase: 1DNW(Blair-Johnson et al. 2001), GLUT4, and α-glucosidase: 3TON (Ren et al. 2011). The protein resolutions were 3.4 Å, 2.2 Å, 2.5 Å and 1.9 Å for to ATPase, cholinesterase, catalase, and myeloperoxidase respectively. The structure of D-glucose and compounds were retrieved from PUBMED and optimized using Gaussian 09 (Frisch et al. 2009). The grid box size was determined with AutoDock tools (Sanner 1999) 1.5.4 for the potential binding sites for all the proteins except α-glucosidase. The optimum binding sites of the ligands were determined by the Lamarckian genetic algorithm method (Yang et al. 2013). While AutoDock Tools graphical user interface was used in computing the Gasteiger charges (Morris et al. 2009).

For α-glucosidase, the active site was identified using the CASTp server (Liang et al. 1998). The Genetic Optimization of Ligand Docking (GOLD) software was used in docking the compounds with the active sites of the protein. The docking parameters were set as described in previous studies (Erukainure et al. 2017b).

2.12 *In vivo* antidiabetic activity in a type 2 diabetic model of rats

Based on the results from the *in vitro* and *ex vivo* studies, 3 plants were selected for the *in vivo* studies in type 2 model diabetic rats. The selected plants were: *C. nitida* infusion, BuOH fraction of *D. edulis* ethanol extract, and the aqueous extract of *P. lunatus*. The extracts and fractions were prepared as described in **section 2.1** but in kilograms to get larger yields.

2.12.1 Experimental animals

Male albino rats of Sprague-Dawley strain weighing about 180–200 g were obtained from the Biomedical Research Unit (BRU), University of KwaZulu-Natal, Durban, South Africa. They were acclimatized for 1 week on pelletized chows, with water given *ad libitum*. Standard laboratory conditions of natural photo period of 12-h light-dark cycle were maintained. All animals used were maintained under the guidelines and approval of the animal ethics committee of the

University of KwaZulu-Natal, Durban, South Africa (protocol approval number: AREC/020/017D).

2.12.2 Animal grouping and induction of type 2 diabetes

The rats were divided into 6 groups consisting of 5 in each normal and 7 in each diabetic group as follows:

Normal Control (NC): Normal rats (non-diabetic and not treated)

Diabetic Control (DC): Diabetic and untreated control

Diabetic + low dose (DLD): Diabetic rats administered low dose (150 mg/kg bw) of extract or fraction

Diabetic + high dose (DHD): Diabetic rats administered high dose (300 mg/kg bw) of extract or fraction

Diabetic + standard drug metformin (DSM): Diabetic rats administered 200 mg/kg bw metformin.

Normal Toxicological Group (NTXC): Non-diabetic rats administered high dose (300 mg/kg bodyweight [bw]) of extract or fraction

This grouping was applied to the 3 extracts selected for *in vivo* studies.

Insulin resistance was induced in the diabetic groups by supplying them with 10% fructose *ad libitum* for 2 weeks. They were then fasted overnight and injected (i.p) with streptozotocin (40 mg/kg BW) dissolved in citrate buffer (pH 4.5). The normal groups were injected (i.p) with citrate buffer. A week later, the non-fasting blood glucose (NFBG) levels of all the rats were checked with a glucometer. Rats with blood glucose level >200 mg/dL were considered diabetic and used for the studies.

2.12.3 Intervention trial

Diabetic rats in groups DLD and DHD were orally administered with low and high doses of the respective extract or fraction using a gastric gavage needle. Group NTX was administered high

dose of the respective extract or fraction. While groups NC and DC were administered with distilled water only. Daily food and water intake were recorded, while NFBG level was measured each weekly throughout the intervention trial. Treatment lasted for 6 weeks, except for rats treated with raffia palm wine which was 5 weeks

2.12.4 Oral glucose tolerance test (OGTT)

Oral glucose tolerance test (OGTT) was conducted in the last week of the intervention period to estimate the ability of the rats to tolerate glucose. The rats were orally administered glucose solution (2 g/kg bw) after overnight fasting. Thereafter, their blood glucose level was measured at 0 (before administration of glucose), 30, 60, 90 and 120 mins after glucose ingestion using a portable glucometer (Glucoplus Inc., Quebec, Canada).

2.12.5 Collection of blood and organs

At the end of the experiment, the rats were humanely sacrificed by euthanizing with halothane anesthesia. Blood was collected via cardiac puncture and centrifuged at 3000 rpm for 20 mins to obtain the serum, which was collected in Eppendorf tubes and stored at -20°C until further studies. The pancreas, liver, muscle, and brain were collected, washed in 0.9% NaCl, weighed and stored at -20°C until further studies. Small portion of the pancreas was cut and stored in neutral buffered formalin and 2.5% glutaraldehyde (in 0.1 M phosphate buffer; pH 7.2) for histopathological and microscopy analysis respectively.

2.12.6 Analytical methods

Serum insulin concentration was determined using an ultrasensitive rat insulin ELISA kit (Mercodia, Uppsala, Sweden) in multi-plate ELISA reader (Biorad-680, BIORAD Ltd., Japan). Serum lipid profile (total cholesterol, triglycerides, and HDL-cholesterol), calcium ion, fructosamine, urea, and creatinine, aspartate and alanine aminotransferases (AST and ALT), and alkaline phosphatase (ALP) were analyzed with an Automated Chemistry Analyzer (Labmax Plenno, Labtest Co. Ltd., Lagoa Santa, Brazil) with commercial assay kits according to manufacturer's manual.

The low-density lipoprotein (LDL) cholesterol level was calculated with formula (Friedewald et al. 1972)

$$\text{LDL} - \text{cholesterol} = \text{Total cholesterol} - \frac{\text{Triglyceride}}{2.22} + \text{HDL} - \text{cholesterol}$$

Insulin resistance and β-cell function were computed using the homeostatic assessment models, HOMA-IR and HOMA-β respectively as shown below:

$$\text{HOMA} - \text{IR} = \frac{\text{Serum insulin (U/L) x Blood glucose level (mmol/L)}}{22.5}$$

$$\text{HOMA} - \beta \text{ cell function} = \frac{20 \text{ x Serum insulin (U/L)}}{\text{Blood glucose level (mmol/L)} - 3.5}$$

2.12.8 Determination of oxidative stress and proinflammation biomarkers, purinergic and cholinergic enzyme activities

The collected organs (0.5 g) were first chopped with scissors in ice, then homogenized in in 4 mL of homogenization buffer (50 mM sodium phosphate buffer with triton X-100, pH 7.5). The homogenized samples were centrifuged at 15,000 rpm for 10 mins at 4°C. The supernatants were then collected in 2 mL Eppendorf tubes and stored at -20°C until further analysis.

The tissue homogenates and serum were analyzed for oxidative stress and proinflammation biomarkers (**section 2.6.2 – 2.6.3**), and purinergic enzymes activities (**section 2.6.4**).

Acetylcholinesterase activity was determined by the Ellman's method (Ellman et al. 1961). This was carried out by mixing 20 µL of the tissue with 10 µL of 3.3 mM Ellman's reagent (pH 7.0) and incubating with 50 µL of 0.1 M phosphate buffer (pH 8) for 20 min at 25 °C. 10 µL of 0.05 M acetylcholine iodide was then added to the reaction mixture and read absorbance read at 412 nm at 3 min intervals.

2.12.9 Determination of carbohydrate metabolic enzymes activities

The serum and pancreatic homogenate were analyzed for α-amylase activities as describes in **section 2.4.2**. However, the serum and the tissue were used as the enzyme source and no extract or standard drug was utilized.

The glucose 6 phosphatase and glycogen phosphorylase activities were determined in the liver homogenate as described in **section 2.6.5**. For glycogen phosphorylase activity, glycogen was used as the substrate source (Balogun and Ashafa 2017; Cornblath et al. 1963).

Fructose-1,6-bisphosphatase activity was determined in the liver homogenate using a previously established protocol (Balogun and Ashafa 2017; Gancedo and Gancedo 1971), with slight modification. One hundred microliters of the tissue homogenate were mixed with 1200 µL of Tris–HCl buffer (0.1 M, pH 7.0), 100 µL of fructose (0.05 M), 250 µL 0.1 M $MgCl_2$, 100 µL 0.1 M KCl, and 250 µL 1 mM EDTA. The reaction mixture was incubated at 37°C for 15 mins. The reaction was stopped with 1 mL of 10% TCA. The mixture was then centrifuged for 10 mins at 3000 rpm at 4°C. 100 µL of the supernatant was pipetted into a 96-well plate and incubated with 50 µL of 1.25% ammonium molybdate and freshly prepared 9% ascorbic acid for 20 mins at normal room temperature for colour development. Absorbance was read at 680 nm with a microplate reader and the activity calculated as the amount of inorganic phosphate (Pi) released/min/mg protein.

2.12.10 Extraction of metabolites and metabolomics

The metabolites of the collected tissues were extracted as described in **section 2.8**. Metabolic profiling was conducted via GC-MS analysis on an Agilent technologies 6890 Series GC coupled with (an Agilent) 5973 Mass Selective detector and driven by Agilent chemstation software. The metabolites were separated using a HP-5MS capillary column. Injections of 1 µL of the samples were made in splitless mode, with ultra-pure helium used as the carrier gas at a constant flow rate of 60 mL h^{-1}. The initial oven temperature was set at 60 °C for 2 min before increasing to 285°C at the rate of 5 °C min^{-1} with a hold time of 3 min. The temperatures of the ion source and quadrupole were set at 230 and 150°C respectively, with electron ionization mode and electron multiplier voltage operated at 70 eV and 1859 V.

The pathways were analyzed as described in **section 2.8**

2.12.12 Histopathological and microscopic analysis

Histological analysis of the brain (hypothalamus) and pancreatic tissues were carried out according to standard protocol for paraffin embedding. The respective tissues were sectioned to 4 µm in

slides. They were then deparaffinize with *p*-xylene and rehydrated in decreasing ethanol concentration gradient (100%, 80%, 70%, 50%). They were rinsed with tap water. The slides were stained with hematoxylin and nisili for pancreas and brain respectively for 5 mins, then rinsed with tap water. They were thereafter stained with eosin. The slides were mounted in DPX and cover-slipped. Leica slide scanner (SCN 4000, Leicabiosystems Germany) was used in viewing the images.

Microscopic analysis of the brain (hypothalamus and pancreatic tissues were carried out with a scanning electron microscope (SEM; Zeiss Ultra Plus). The fixed tissues were subjected to buffer wash to remove glutaraldehyde. This was done thrice at 5 mins intervals. Thereafter, they were post-fixed with 0.5% osmium tetroxide for 2 h. The tissues were washed with distilled water thrice at 5 mins interval, before subjecting to dehydration using ethanol of increasing concentrations: 25% (twice at 5 mins interval), 50% (twice at 5 mins interval), 75% (twice at 5 mins interval) and 100% (twice at 10 mins interval). The tissue samples were dried in a critical-point-dryer apparatus, gold coated, and observed at an accelerating voltage of 20–25 k with a SEM (Zeiss Ultra Plus).

2.12.13 Immunohistochemistry

Thin sections of 5 µm thickness were obtained from routine paraffin embedded pancreatic tissues prepared during histology. After deparaffinization, sections were subjected to heat-mediated antigen retrieval in citrate-based solution, pH 6.0. Endogenous peroxidase blocking was performed in 0.3 % hydrogen peroxide. Sections were then incubated overnight at 4 ^{0}C in primary rabbit antibodies: caspase-3 (Cell Signaling, MA, USA; #9662) and Nrf2 (Abcam, MA, USA; #ab31163) at 1:200 and 1:100 dilutions respectively. Secondary incubation was performed in ImmPRESS™ HRP Anti-Rabbit IgG (Peroxidase) Polymer Reagent, made in horse (Vector® #MP-7401). DAB Peroxidase (HRP) Substrate Kit (Vector® #SK-4100) was used for colour development, and sections were counter-stained in Harris hematoxylin. Control sections were similarly processed without primary antibody incubation. There was no specific immunoreactivity on the control sections. Immunoreactivity was quantified using the ImmunoRatio plugin on Image J software (NIH, USA) which separates and quantify percentage of DAB (positive immunoreactivity) by digital colour deconvolution (Tuominen et al. 2010).

2.13 Statistics

The one-way analysis of variance (ANOVA) was used in establishing the statistical significance and set at $p < 0.05$. Data were presented as mean ± SEM/SD. Statistical analyses were carried out using IBM SPSS for Windows, version 23.0 (SPSS Inc., Chicago, IL, USA).

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CHAPTER 3

THE ANTI-OXIDATIVE AND ANTI-DIABETIC ACTIVITIES OF RAFFIA PALM (*RAPHIA HOOKERI*) WINE *IN VITRO, EX VIVO,* AND *IN VIVO*

3.1 Raffia palm (*Raphia hookeri*) wine

3.1.2 Background

Palm wine is a traditional beverage with a milky flocculent appearance obtained from the sap of palm tree (Eze and Ogan 1988; Nwaiwu et al. 2016). It is consumed fresh (unfermented) and fermented. Its consumption cuts across West Africa, India and Indonesia. In Nigeria, its common name includes palmy, oguro, tombo, nkwu ocha, and nkwu enu. It is utilized in most socio-cultural activities in Nigeria, where it is consumed at most traditional events (Nwaiwu and Itumoh 2017) and employed in folkloric medicine. The high content of the yeast, *Saccharomyces cerevisiae* makes the palm wine susceptible to fermentation, leading to decreased sweetness with concomitant increase in alcohol content.

3.1.3 Ethnopharmacological uses

Children suffering from measles are often sprinkled with palm wine as part of the treatment process. It is often used as a macerating medium (unfermented or fermented) for some medicinal plants (Focho et al. 2009). Fresh (unfermented) palm wine are often given to breastfeeding mothers to boost milk production.

3.1.4 Biological activities

There are scanty scientific reports on the biological activities of palm wine. Few studies on its biological activities reveal palm wine as a good scavenger of free radicals (Oboh and Okhai 2012), which portrays its antioxidant activity.

3.1.5 Nutritional properties

Raffia palm wine is a rich dietary source of ascorbic acid and the B-vitamins complex, particularly thiamine (Bassir 1962; Eze and Ogan 1988; Tuley 1965). Obahiagbon and Osagie (2007) reported the presence of potassium, magnesium, calcium, sodium, phosphorus, and nitrogen. It has also

been reported to be rich in sucrose, glucose, xylose, raffinose and lactose (Ezeagu et al. 2003; Obahiagbon and Osagie 2007), which are responsible for its sweetness.

3.1.6 Phytochemistry

The volatile compounds of palm wine have been reported to consist of ethyl acetate, ethanol, trimethyldioxolane, propionic acid, propyl acetate, methyl cinnamate, butyl acetate, isopentyl alcohol, hexanoic acid, ethyl hexanoate, styrene, acetoin, ethyl lactate, methylpropylacetate, ethyl octanoate, acetic acid, methyl-2-methyl propanoate, ethyl dodecanoate, dimethylhydrazine, 3-methyl-6,7-benzylisoquinoline, tetraacetyl-D-xylonic nitrile, oleic acid, 2-phenylethanol, 2-phenylethanol, 4-hydroxy-2,5-dimethyl-3(2H)-furanone, 3-methylpentanoic acid, 2-ethyl-4-hydroxy-5-dimethyl-3(2H)-furanone, ethylcinnamate, 3-hydroxy-4,5-dimethyl-2(5H)-furanone, diethylsuccinate, c-dodecalactone, phenylacetic acid, vanillind, 4-Methoxy-2-methylphenol, and 2-/3-Methylbutanol (Lasekan et al. 2007; Nwaiwu et al. 2016; Uzochukwu et al. 1997). Uzochukwu et al. (1997) also reported the presence of 3 terpenes namely: a-pinene, 3-carene and Isocumene. Other acids identified are benzoic, phenyl acetic, and phenyl propionic (Uzochukwu et al. 1997).

3.1.7 Aims and objective

This study aims at investigating the anti-oxidative and antidiabetic properties of unfermented and fermented raffia palm wine by determining their *in vitro* free radical scavenging and enzyme inhibitory activities, glucose absorption and uptake *in vitro* and *ex vivo*, antioxidative effect and modulation of dysregulated metabolic pathways in oxidative pancreatic injury *ex vivo*. The antidiabetic and antioxidant protective effects in type 2 diabetes was also determined *in vivo*.

3.2 Fermentation does not Affect Sugar Quality of Raffia Palm (*Raphia hookeri*) Wine but Alters Its Functional Chemistry and Antidiabetic Properties

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Preface: This article investigated the *in vitro* antioxidant and enzyme inhibitory activities, as well as the *ex vivo* glucose absorption and uptake effects of raffia palm wine. It has been submitted to Food Bioscience for publication.

3.2.1 Abstract

The effect of short term fermentation on the sugar quality, functional chemistry, antioxidant and antidiabetic properties of raffia palm (*Raphia hookeri*) wine was investigated. Palm wine samples were subjected to open air fermentation for 24 and 48 hours, respectively. The samples showed significant ($p<0.05$) antioxidant activities with little or no differences between the unfermented and fermented. There was a dose-dependent inhibitory effect on α-glucosidase, α-amylase and intestinal glucose absorption with increasing fermentation time. All samples except the 48 h fermented caused an increased muscle glucose uptake dose-dependently. ^{1}H-NMR spectroscopy revealed the presence of allose, cellobiose, d-tagatose, fructose, galactitol, gluconic acid, rhamnose, sucrose, xylose and β-N-acetylglucosamine. FTIR spectroscopy revealed the presence of alcohols, phenols, 1° and 2° amines, aromatics functional groups in all samples. Fermentation led to the addition of the functional group, bend alkenes. These results demonstrate the antioxidative and antidiabetic properties of unfermented and fermented raffia palm wine.

Keywords: Antioxidant; Enzymes inhibition; Palm wine; and Type 2 Diabetes

3.2.2 Introduction

Diabetes mellitus (DM) has been recognized as one of the world's growing epidemic, and a major contributor of morbidity and mortality in Africa (Motala 2002). It is a metabolic disease affecting carbohydrate, protein and lipid metabolism, leading to increased blood glucose level (Saltiel and Kahn 2001). Inability of the pancreatic beta-cell to secrete insulin (type 1 diabetes) or inability for the body to utilized secreted insulin (type 2 diabetes[T2D]) have been implicated as the main culprit. T2D has been recognized as the most prevalent of diabetes, constituting more than 90% (IDF 2015). It is characterized by hyperglycemia, which has been implicated in the morbidity and mortality of T2D. Chronic hyperglycemia has been shown to alter the body's redox balance due to increased production of reactive oxygen species (ROS) (Maritim et al. 2003; Tiwari et al. 2013). Alteration of the body's redox balance in favour of ROS production over the body's antioxidant system, will lead to oxidative stress (Erukainure et al. 2017). Oxidative stress is an important contributor to the pathogenic micro- and macro-vascular complications of T2D (Maritim et al. 2003; Tiwari et al. 2013). Treatment of T2D with antioxidants have been demonstrated in several studies to reduce these complications have (Fiordaliso et al. 2004; Scott and King 2004; Ziegler et al. 2011).

The role of medicinal plants in the treatment and management of T2D and its complications are well documented (Abo et al. 2008; Ezuruike and Prieto 2014; Mohammed et al. 2014). This has been attributed to their phytochemical and nutritional properties (Harbilas et al. 2009; Patel et al. 2012). Some of these plants are often utilized singly or in combinations immersed in fresh (unfermented) or fermented palm wine.

However, there have been concerns on the safety and efficacy of the use of palm wine in combination with medicinal herbs particularly in the treatment of T2D, owing to its sweetness (unfermented) and alcoholic content (fermented). Thus, we investigated the effect of short term fermentation on the sugar quality, functional chemistry, antioxidant and anti-diabetic properties of raffia palm (*Raphia hookeri*) wine.

3.2.2 Materials and Methods

Kindly refer to Chapter 2; sub sections 2.1 – 2.5; 2.7; 2.10; and 2.13

3.2.3 Results and Discussion

The safety and efficacy of the use of palm wine in combination with antidiabetic medicinal herbs remains a major question to most health practitioners. This has been attributed to its sweetness and ease of fermentation. In this study, we reported for the first time the antidiabetic potential of raffia palm wine and the influential role of fermentation on its biological activity.

Total Phenolic Content

All samples had relative low total phenolic content as depicted in **Figure 3.2.1**. Fermentation for 24 h led to significant ($p<0.05$) reduction in the total phenolic content, this was however increased after 48 h fermentation. The role of polyphenolics in the medicinal properties of plants is well documented (Erukainure et al. 2017). They are secondary metabolites with reported antioxidant activities (Adedayo et al. 2010).

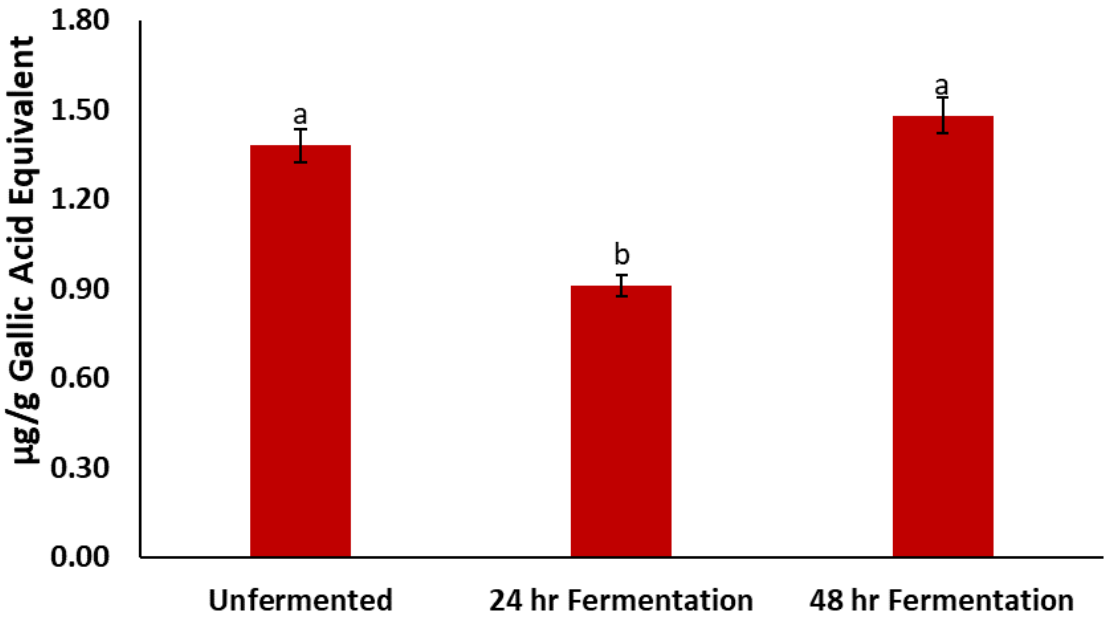
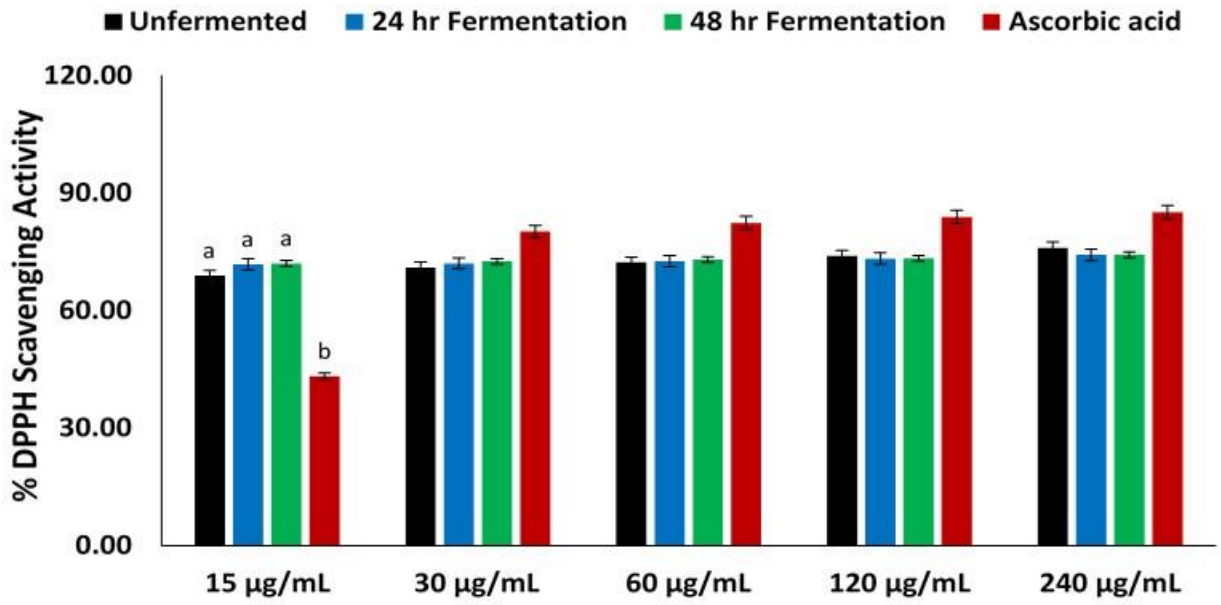


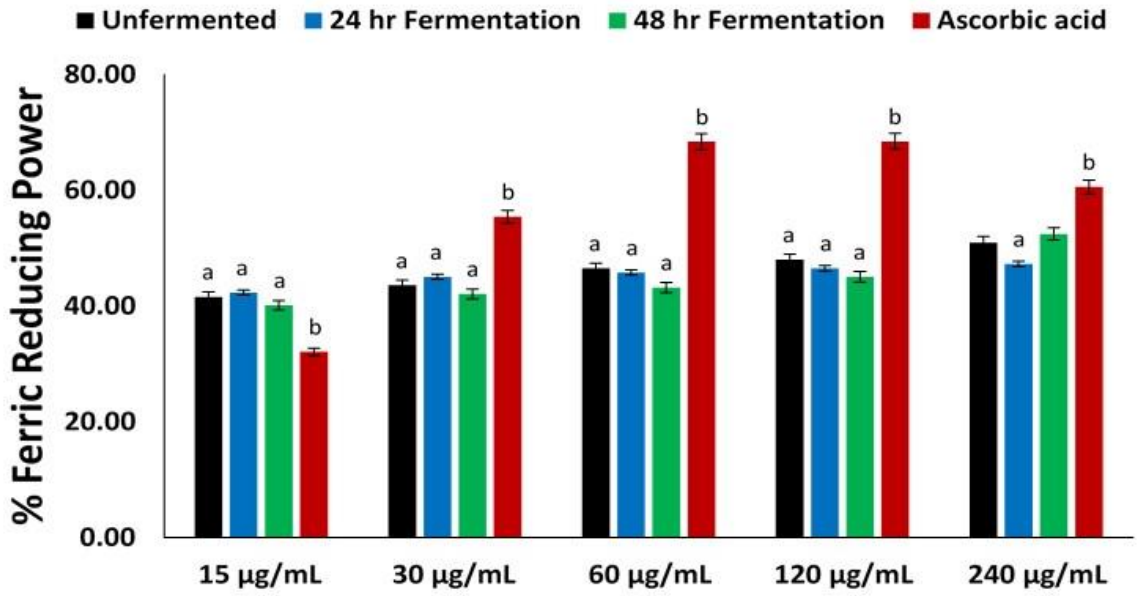
Figure 3.2.1: Total phenolic content of unfermented and fermented raffia palm wine. Data = mean ± SD; n = 3. [ab]Values with different letters above the bars for a given extract are significantly ($p < 0.05$) different from each other.

In Vitro Antioxidant Activity

There was with little or no significance difference between the antioxidant activities of the unfermented and fermented palm wine as shown **in Figures 3.1.2A and B**. Their IC_{50} values of 1.00 (unfermented), 2.00 (24 h fermented) and 1.99 (48 h fermented) µg/mL for DPPH scavenging activity portrays a potent quenching activity, even higher than ascorbic acid (8.31 µg/mL) (**Table 3.2.1**). Their IC_{50} values for FRAP activities, though higher than ascorbic also reveals a strong antioxidant potential. The role of antioxidants in the amelioration of complications implicated in type 2 diabetes is well documented (Rahimi et al. 2005). Thus, suggesting a protective role of palm wine against hyperglycemia induced oxidative stress. The antioxidant activities corresponds to previous reports on the DPPH scavenging properties of raffia palm wine (Oboh and Okhai 2012) and can be attributed to the total phenolic content (**Figure 3.2.1**).



(A)



(B)

Figure 3.2.2: (A) DPPH scavenging and (B) FRAP activities of unfermented and fermented raffia palm wine. Data = mean ± SD; n = 3. [ab]Values with different letters above the bars for a given concentration are significantly ($p < 0.05$) different from each other.

Table 3.2.1: IC_{50} values of biological activities of unfermented and fermented Raffia palm wine

Activities	Unfermented (µg/mL)	24 h Fermented (µg/mL)	48 h Fermented (µg/mL)	Ascorbic Acid (µg/mL)	Acarbose (µg/mL)
DPPH	1.00	2.00	1.99	8.31	–
FRAP	186.20	977.23	229.08	29.98	–
Alpha glucosidase	6.76	1.13	15.84	–	62.42
Alpha amylase	537.03	109.64	29.51	–	0.04
Glucose absorption	37.15	1.41	0.47	–	–
Glucose uptake	11.48	7.07	102.32	–	–

Carbohydrate Digesting Enzyme Inhibitory Activity

All samples showed significant inhibition of α-glucosidase as shown in **Figure 3.2.3A**. There was no dependent increased activity by the unfermented palm wine. However, the fermented samples showed a dose dependent inhibitory effect, with the 48 h fermented showing significantly ($p<0.05$) higher activity with increasing concentrations. Their IC_{50} values of 6.76, 1.13, 15.84 µg/mL for unfermented, 24 h and 48 h fermented respectively depicts a potent α-glucosidase inhibitory activity compared to the standard drug, acarbose (62.42 µg/mL) with the 24 h fermented showing the best activity (**Table 3.2.1**).

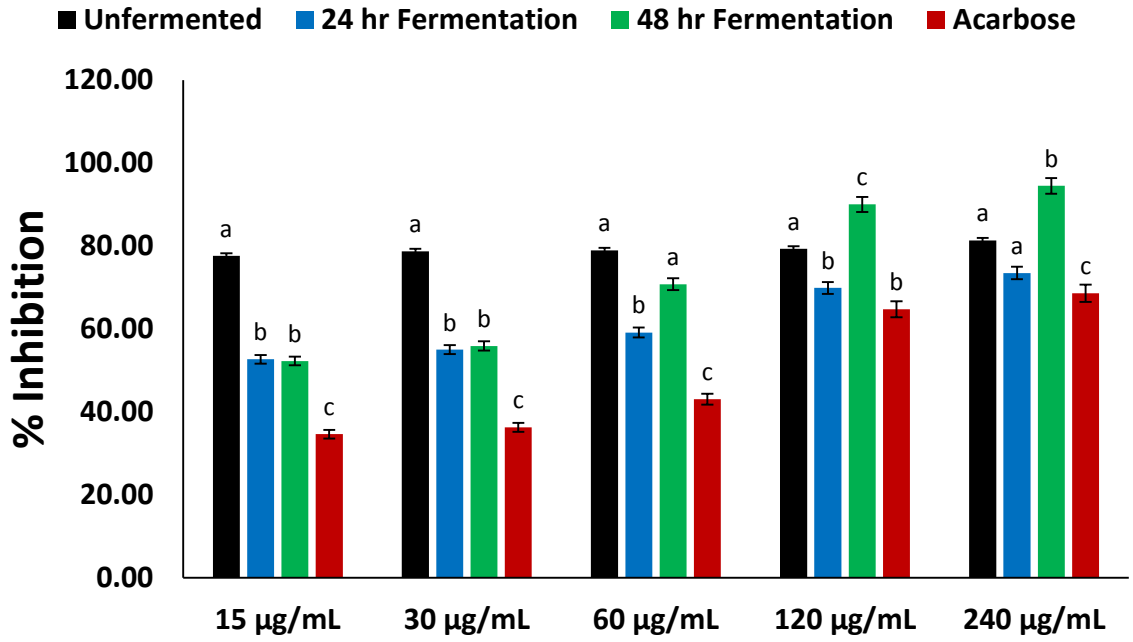


Figure 3.2.3A: α-glucosidase inhibitory activities of unfermented and fermented raffia palm wine. Data = mean ± SD; n = 3. [abc]Values with different letters above the bars for a given concentration are significantly ($p < 0.05$) different from each other.

The inhibitory effect of the palm wines on α-amylase activity was significantly lower than the standard drug, Acarbose as depicted in **Figure 3.2.3B**. All samples showed a dose – dependent inhibitory effect, which also increases with increasing time of fermentation. The low IC_{50} value for the 48 h fermented palm wine (29.51 µg/mL), insinuates the best activity (**Table 3.2.1**). The inhibition of these enzymes has been linked to the antidiabetic properties of most drugs and

medicinal plants (Bischoff 1995; Oboh et al. 2014). These enzymes involved in the breakdown of dietary carbohydrate to glucose. Therefore, their inhibition will limit the amount of glucose available to the blood stream. The inhibitory results from this study, thus demonstrates the antidiabetic properties of raffia palm wine. Fermentation has been shown to improve the antidiabetic properties of foods (Kumar et al. 2005; Kwon et al. 2010), which corroborates with the observed increased inhibitory activity of the fermented palm wines.

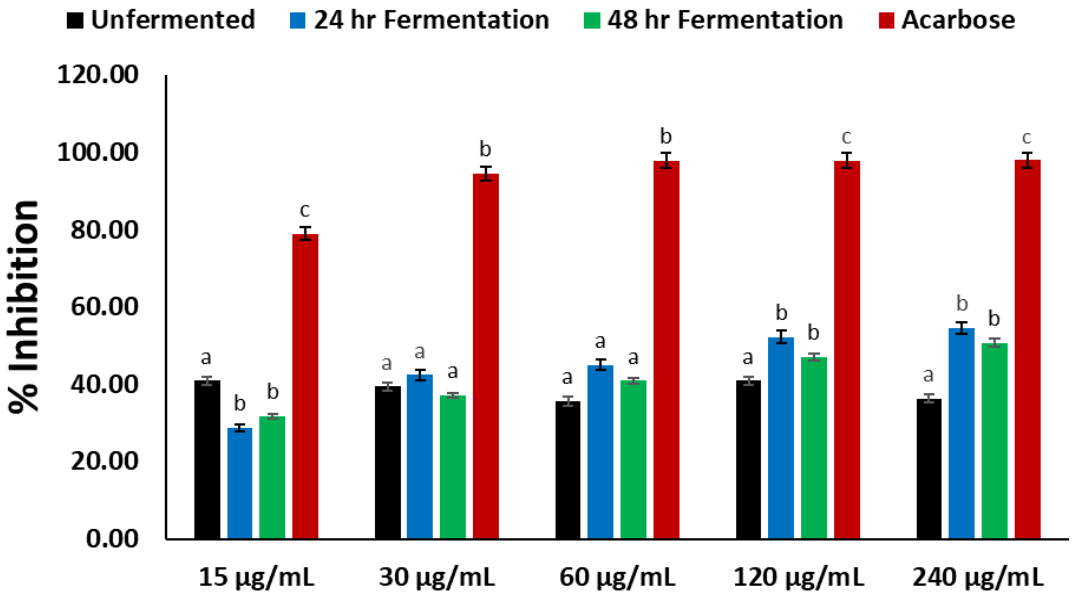


Figure 3.2.3B: α-amylase inhibitory activities of unfermented and fermented raffia palm wine. Data = mean ± SD; n = 3. [abc]Values with different letters above the bars for a given concentration are significantly ($p < 0.05$) different from each other.

Measurement of Glucose Absorption in Isolated Rat Jejunum

Incubation of isolated rat jejunum with unfermented and fermented palm wine led to significant ($p<0.05$) inhibition of intestinal glucose absorption as shown in **Figure 3.2.4**. All samples showed a dose – dependent activity, which increased with increasing time of fermentation. This is evident by the decreasing IC_{50} values of the palm wines as the fermentation time increased, with the 48 h fermented having a value of 0.47 µg/mL. This result further portrays the ability of raffia palm wine to reduce blood glucose level by inhibiting intestinal glucose absorption. Glucose arising from the α-glucosidase breakdown of dietary carbohydrate is rapidly absorbed in the small intestine,

particularly at the proximal region (jejunal and duodenal regions) (Kellett and Helliwell 2000; Meddah et al. 2009). Thereby leading to elevated blood glucose level. This also corroborates with its (palm wine) inhibitory effect on α-glucosidase activities (**Figure 3.2.3A**).

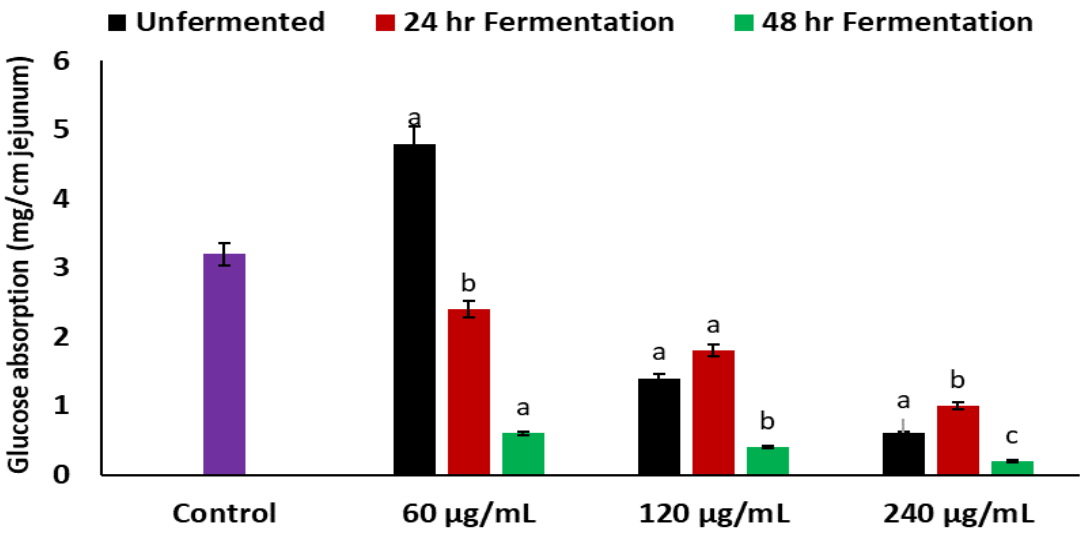


Figure 3.2.4: Effects of fermentation on glucose absorption in isolated rat jejunum by raffia palm wine. Data = mean ± SD; n = 3. [abc]Values with different letters above the bars for a given cocentration are significantly ($p < 0.05$) different from each other.

Measurement of Glucose uptake in Isolated Rat Psoas Muscles

There was a significant ($p<0.05$) increased glucose uptake in muscle tissues incubated with unfermented and 24 h fermented palm wines respectively, with the latter showing the best activity as depicted in **Figure 3.2.5** and **Table 3.2.1**. The activities were dose dependent with increasing concentration. The 48 h showed little or no significant activity. Skeletal muscles play a major role in carbohydrate metabolism and glucose homeostasis (Sinacore and Gulve 1993). These has been attributed to the high concentration of GLUT-4 which aids glucose uptake (Satoh 2014). Stimulation of muscle glucose uptake has been shown to be a major therapy for maintaining normal glycaemia in T2D (Pereira et al. 2017). Thus, the increased glucose uptake by the unfermented and 24 h fermented raffia palm wines indicates an anti-hyperglycemic potential.

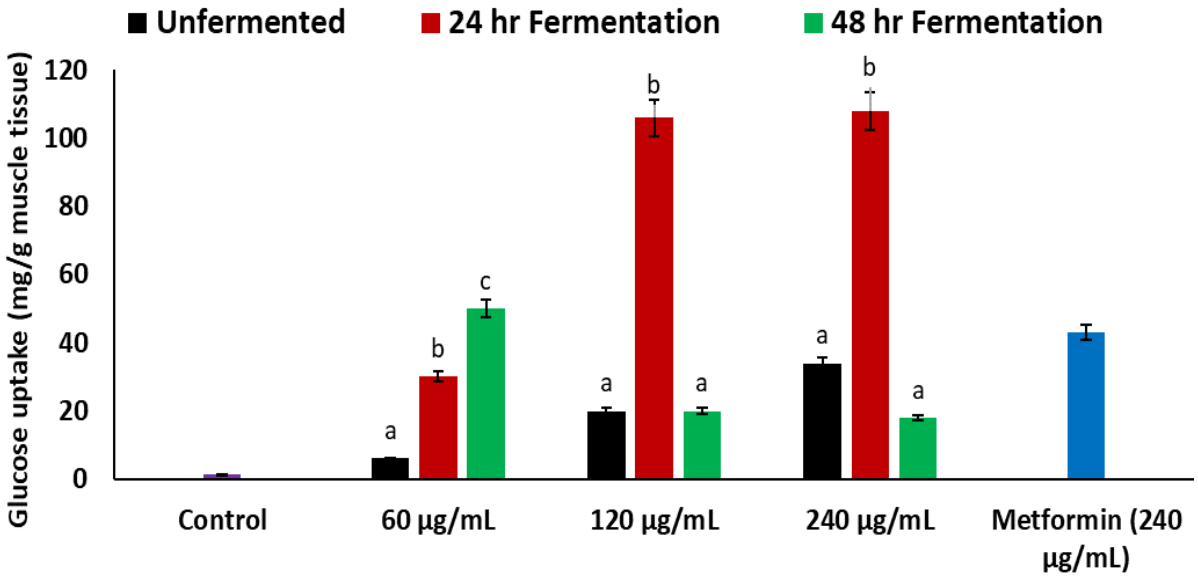


Figure 3.2.5: Effects of fermentation on glucose uptake in isolated rat psoas muscle by raffia palm wine. Data = mean ± SD; n = 3. [abc]Values with different letters above the bars for a given concentration are significantly ($p < 0.05$) different from each other.

Proton NMR Spectroscopy Analysis

^{1}H-NMR spectroscopy of the palm wine samples revealed that fermentation had no effect on the sugar contents. The identified sugars were: allose**,** cellobiose, d-tagatose, fructose, galactitol, gluconic acid**,** rhamnose, sucrose, xylose, and β-N-acetylglucosamine as depicted in **Figure 3.2.6**. The identified sugars corroborate previous studies on the sugar content of raffia palm wine. Eze and Ogan (1988); Obahiagbon and Osagie (2007) reported the presence of sucrose, fructose, glucose and raffinose in raffia palm wine, with sucrose being the major sugar. The presence of maltose (Ezeagu et al. 2003), l-arabinose, d-xylose, l-rhamnose, and cellobiose (Faparusi 1981) have also been reported. The little or no change in sugar quality of the fermented palm wine however contradicts previous reports by (Van Pee and Swings 1971); (Faparusi 1969); and Obahiagbon & Osagie (2007). They reported reduced sugar levels owing to fermentation to yield alcohol, carbon dioxide and acetic acid. In this present study, the fermentation time was short and the identified sugars were not quantified. The observed antidiabetic properties of the studied palm wines (**Figure 3.2.3** – **Figure 3.2.5**) can also be attributed to the synergetic effect of identified

sugars, particularly d-tagatose and rhamnose (Espinosa and Fogelfeld 2010; Lee et al. 2008). This also corroborates with reported antidiabetic properties of natural sweeteners (Chukwuma and Islam 2015; Chukwuma and Islam 2017).

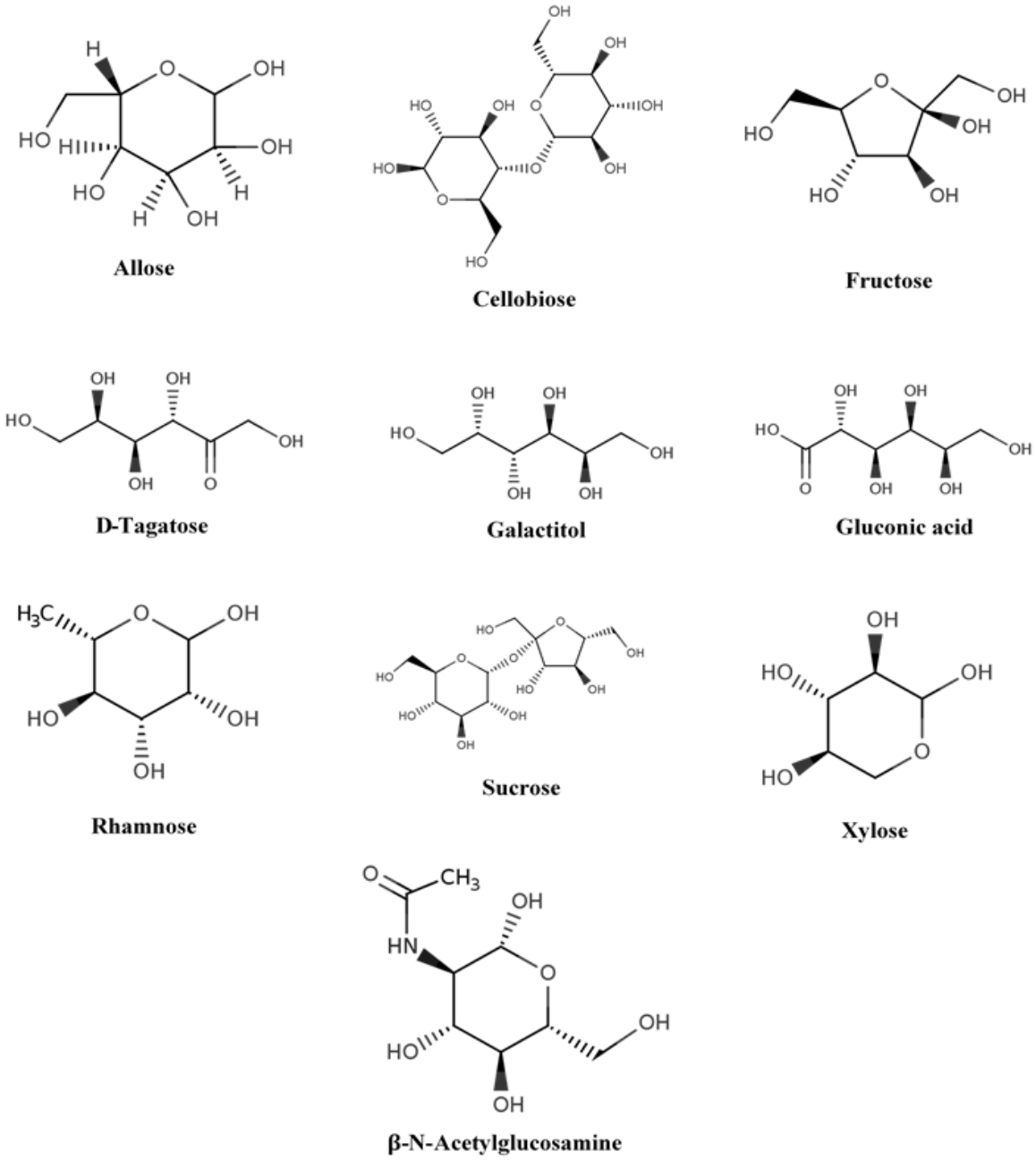


Figure 3.2.6: Identified sugars in unfermented and fermented raffia palm wine

Fourier Transform Infrared Spectroscopy (FTIR) Analysis

FTIR spectroscopy of the palm wine samples revealed the presence of O–H stretch, H–bonded, N–H stretch, C–H stretch, N–H bend and C–C stretch (in–ring) bonds depicting alcohols, phenols, 1° and 2° amines, aromatics functional groups in all samples as shown in **Figure 3.2.7** and **Table 2**. These chemical bonds portray an electron – deficient moiety, indicating susceptibility to accepting an electron (Erukainure et al. 2017; Harrold 2013). Thus, demonstrating an antioxidant potential which corroborates the observed antioxidative activity (**Figure 3.2.2**). Fermentation led to the addition of the functional group, bend alkenes. 48 h fermentation led to the addition of carbonyls (general), carboxylic acids, aldehydes, esters, saturated aliphatic and α, β–unsaturated esters functional groups as indicated by the presence of C=O stretch.

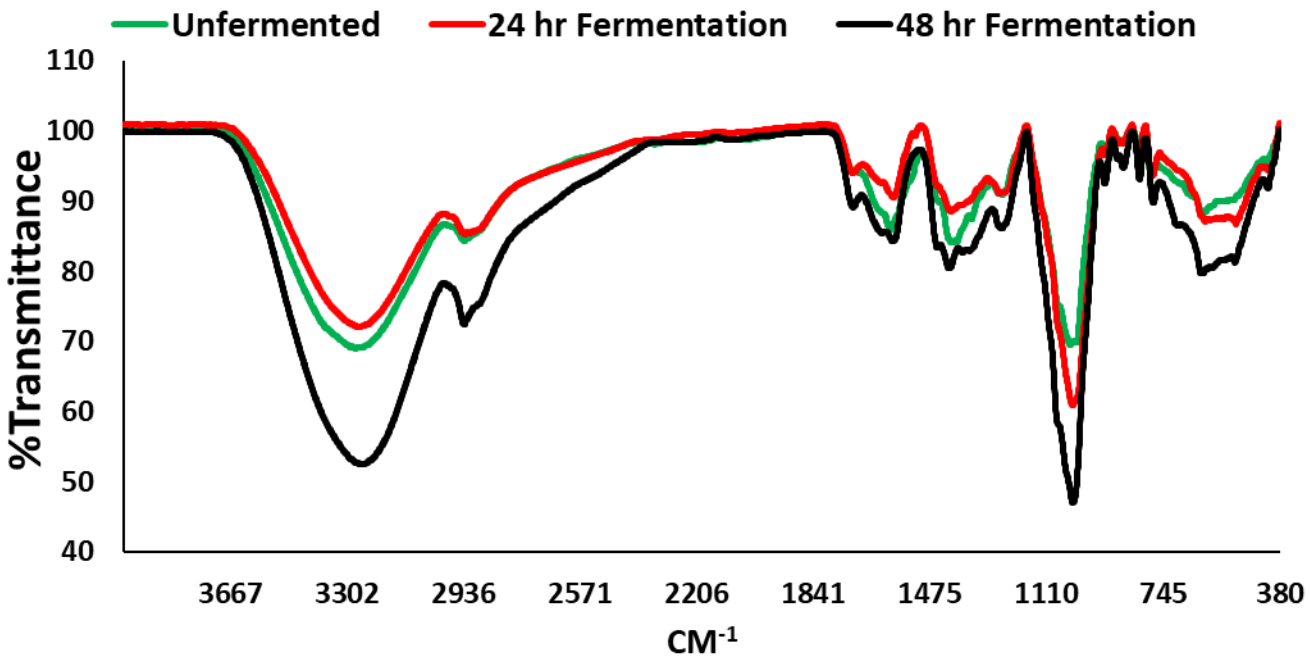


Figure 3.2.7: FTIR spectroscopy of unfermented and fermented palm wine.

Table 3.2.2: Quantitative analysis of FTIR spectroscopy of unfermented and fermented raffia palm wine

Unfermented (cm^{-1})	**24 hr Fermented (cm^{-1})**	**48 hr Fermented (cm^{-1})**	**Functional Group**	**Chemical Bonds**
3274.90	3260.89	3259.08	alcohols, phenols	O–H stretch, H–bonded
			1°, 2° amines, amides	N–H stretch
2933.71	2933.25	2935.19	Alkanes	C–H stretch
–	–	1716.05	carbonyls (general)	C=O stretch
			carboxylic acids	
			aldehydes, esters, saturated aliphatic	
			α, β–unsaturated esters	
1595.23	1591.31	1588.29	1° amines	N–H bend
			Aromatics	C–C stretch (in–ring)
1401.09	1408.01	1416.88	Aromatics	C–C stretch (in–ring)
1250.30	1255.68	1250.13	alcohols, carboxylic acids, esters, ethers	C–O stretch
1037.19	1029.66	1037.05	aliphatic amines	C–N stretch
926.27	–	927.53	carboxylic acids	O–H bend
			alkenes	=C–H bend
867.53	–	869.87	Aromatics	C–H “oop”
–	818.05	818.50	1°, 2° amines	N–H wag
–	779.54	776.18	bend alkenes	=C–H

619.97	–	626.86	bend alkynes	–C≡C–H: C–H

3.2.4 Conclusion

These results demonstrate the antioxidative and antidiabetic properties of unfermented and fermented raffia palm wine. These can be attributed to the synergetic effect of the identified sugars and functional groups. The increased enzyme inhibitory activity with increasing fermentation time indicates a fermenting effect. These results also validate its use as a major ingredient in some folkloric antidiabetic medicine. Further studies are however required to validate its anti-hyperglycemic activity *in vivo*

3.3 Raffia Palm (*Raphia hookeri*) Wine inhibits Glucose Diffusion; Exacerbates Antioxidative Activities; and Modulates Dysregulated Pathways and Metabolites in Oxidative Pancreatic Injury

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Preface: This article investigated the effect of raffia palm wine on glucose uptake and diffusion in yeast cells and across dialysis membrane respectively. Its antioxidative and anti-proinflammatory effects on oxidative pancreatic injury and metabolic pathways involved were also investigated *ex vivo*. It has been accepted for publication in Journal of Food Biochemistry (JFBC-08-18-0641).

3.3.1 Abstract

The antidiabetic, antioxidative and anti-proinflammatory effects of raffia palm (*Raphia hookeri*) wine was investigated using *in vitro* and *ex vivo* models. Unfermented, 24, and 48 hours fermented raffia palm wine samples were respectively investigated for their ability to promote glucose uptake and inhibit glucose diffusion. Their effect on reduced glutathione (GSH), malondialdehyde (MDA) and nitric oxide (NO) levels, as well as superoxide dismutase (SOD), catalase, myeloperoxidase, and ATPase activities were investigated in Fe^{2+} induced oxidative pancreatic tissues. The samples significantly ($p<0.05$) promoted glucose uptake in yeast cells and inhibited glucose diffusion across dialysis membrane. Except for GSH level, all studied antioxidant and proinflammatory biomarkers were significantly reversed after treatment with the palm wine samples. This may be

attributed to the LC-MS identified compounds which were mainly polyphenols and their glycosides, vitamins and amino acids. Investigation of the oxidative pancreatic metabolites revealed depleted metabolites with concomitant generation of citric acid cycle metabolites owing to activation of energetic metabolic pathways involved in superoxide generation. These were depleted in the treated tissues with the generation of adenosine metabolic intermediates, sugar and inositol phosphorylation intermediates, as well as generation of enzyme co-factors, selenium and vitamin metabolites owing to concomitant activation of vitamins, lipid, steroids, inositol and its phosphates, and sulfate/sulfite metabolic pathways. These results suggest the antidiabetic and antioxidative effects of unfermented and fermented raffia palm wine. These activities can be attributed to the inhibition of metabolic pathways involved in free radical generation, and activation of pathways involved in antioxidant activities.

Keywords: Antioxidative stress; Antidiabetes; *Ex vivo*; Metabolomics; and Raffia palm wine

3.3.2 Introduction

Plants have been employed from time immemorial in the treatment and management of several ailments and diseases including diabetes and its complications, making them a major contributor of the global health system (Iwu and Gbodossou 2000; Mpinga et al. 2013). These plants are major constituents of phytochemicals, which are responsible for their medicinal properties (Erukainure et al. 2017). These plants either utilized singly or macerated in other liquid mediums (Focho et al. 2009). Amongst such mediums is the raffia palm (*Raphia hookeri*) wine.

Diabetes mellitus (DM) has been recognized as a global epidemic affecting over 425 million people globally in 2017, with Africa accounting for 3.76% (IDF 2018). A 48% increase in prevalence have been predicted for 2045 which amounts to 629 million people that will living with diabetics globally (IDF 2018). Diabetes is a metabolic disease arising from inability of the pancreatic tissue to secret insulin (type 1 diabetes) and/or inability of the body to utilize the secreted insulin owing to insufficiency (type 2 diabetes). Thereby affecting carbohydrate, lipid and protein metabolism. This leads to an uncontrolled increased blood glucose level termed, hyperglycemia. Chronic hyperglycemia arising from insulin resistance and pancreatic β-cell dysfunction has been recognized as the hallmark of type 2 diabetes (T2D), which is the predominant of all diabetes types (Kahn et al. 2014). Hyperglycemia triggers the production of

free radicals, which when surpasses the body's endogenous antioxidant defense system causes oxidative stress (Maritim et al. 2003b). Oxidative stress has been implicated in the pathogenesis of complications associated with T2D (Erukainure et al. 2015; Maritim et al. 2003b) and often characterized by increased membrane lipid peroxidation and DNA insults owing to increased levels of superoxide ($O2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\cdot}OH$).

Despite the wide use of raffia palm wine in folkloric medicine, there is still a dearth in the scientific reports of its acclaimed medicinal properties and mechanism of actions. Thus, this study aims to report the antidiabetic and antioxidative properties of raffia palm wine by investigating its inhibitory activity on glucose diffusion and uptake, as well as the potential metabolic pathways involved in its antioxidative activities in Fe^{2+} - induced oxidative stress in pancreatic tissues.

3.3.3 Materials and Methods

Kindly refer to chapter 2; sub sections: 2.1.1 – 2.1.2; 2.5 – 2.6.2.4; 2.6.3 – 2.6.4.1; 2.8; 2.10.2; 2.11.1; and 2.13

3.3.4 Results

Incubation of the palm wine samples with yeast cells in the presence of glucose, led to significant ($p<0.05$) increase in glucose uptake in a dose dependent manner as depicted in **Figure 3.3.1A**. The 24 h fermented showed the best activity (IC_{50} = 158.80 µg/mL) as shown in **Table 3.3.1**, followed by the unfermented and 48 h fermented respectively.

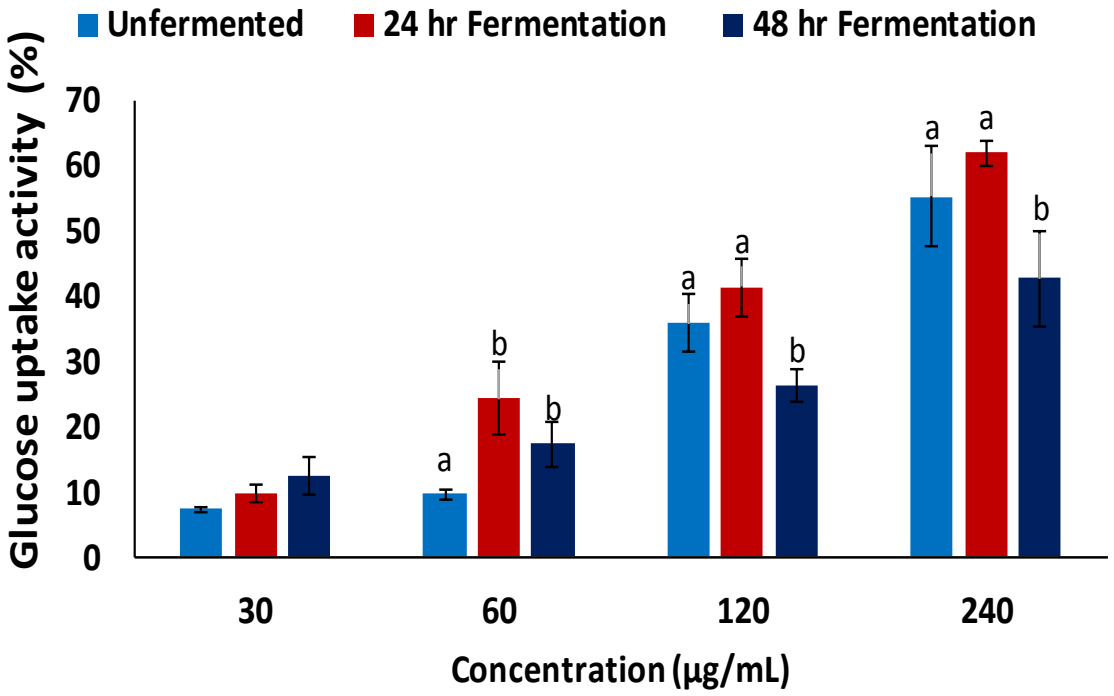

Figure 3.3.1A: Effect of raffia palm wine on glucose uptake/ transport in yeast cells. Values = mean ± SD; n = 3. [abc]Values with different letters over the bars for a given concentration are significantly different from each other ($p < 0.05$, Tukey's HSD-multiple range post-hoc test, IBM SPSS for Windows).

The palm wine samples significantly ($p<0.05$) inhibited the movement of glucose across the dialysis bag, indicating an inhibitory effect on glucose diffusion as shown in **Figure 3.3.2B**. The effect was dose-dependent. Based on the IC_{50} values (**Table 3.3.1**), the 48 h fermented sample had the best activity, followed by the 24 h fermented, while the unfermented was the least. Thus, indicating an increasing activity with increased fermentation time.

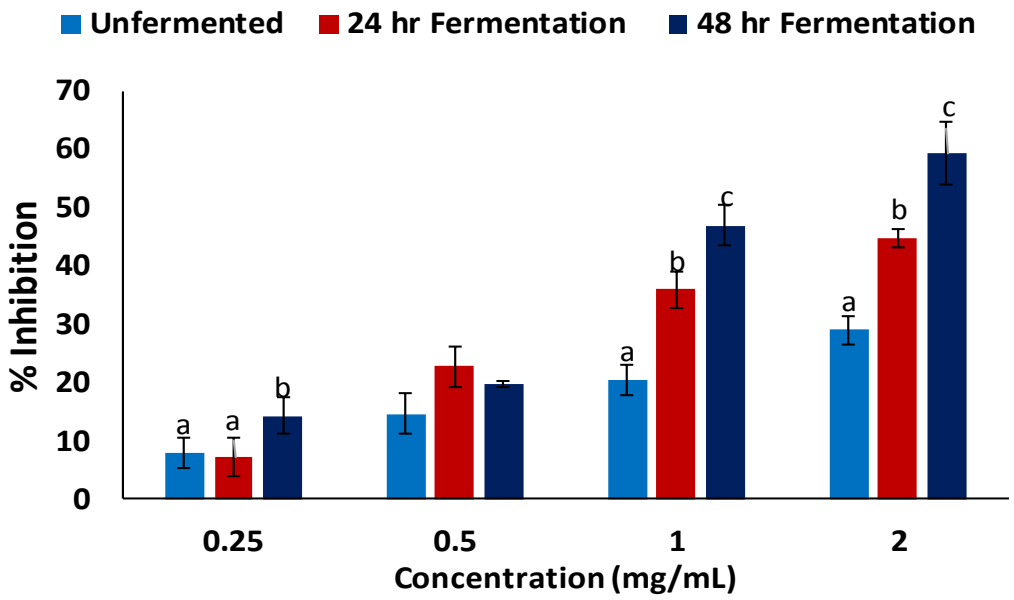

Figure 3.3.1B: Effect of raffia palm wine on glucose diffusion. Values = mean ± SD; n = 3. [abc]Values with different letters over the bars for a given concentration are significantly different from each other ($p < 0.05$, Tukey's HSD-multiple range post-hoc test, IBM SPSS for Windows).

Table 3.3.1: IC_{50} values of studied biological activities

Activities	Unfermented Palm wine	24 h fermented palm wine	48 h fermented palm wine	Gallic acid
Glucose Diffusion (mg/mL)	3.03	2.16	1.91	ND
Glucose uptake (µg/mL)	216.53	158.80	486.64	ND
GSH (µg/mL)	<1000	<1000	<1000	<1000
SOD (µg/mL)	0.95	0.08	2.74	<1000
Catalase (µg/mL)	0.08	0.01	0.17	<1000
Lipid Peroxidation (µg/mL)	0.01	<1000	15.61	26.74
NO (µg/mL)	208.86	338.81	438.99	38.94
Myeloperoxidase (µg/mL)	209.52	142.43	544.2	362.81
ATPase	0.61	5.99	<1000	0.01

ND: not determined.

Incubation of pancreatic tissues with $FeSO_4$ led to significant ($p<0.05$) reduction of GSH level as depicted in **Figure 3.3.2A**. Incubation with the palm wine samples showed little or no effect on the GSH level.

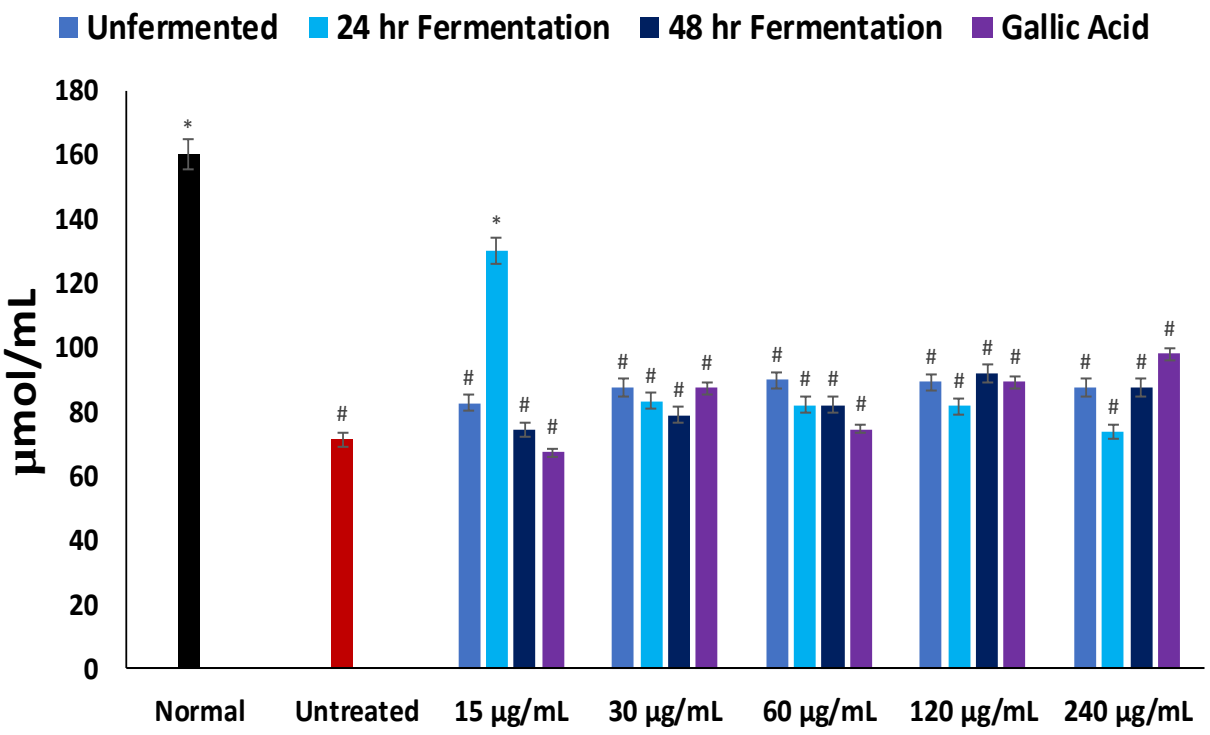

Figure 3.3.2A: Effect of raffia palm wine on GSH level in oxidative pancreatic injury. Values = mean ± SD; n = 3. *Significantly different from untreated sample and #Significantly different from normal sample ($p < 0.05$, Tukey's HSD-multiple range post-hoc test, IBM SPSS for Windows).

There was a significant ($p<0.05$) decrease in SOD and catalase activities on incubation of pancreatic tissues with $FeSO_4$ as shown in **Figure 3.3.2B** and **Figure 3.3.2C**. These were significantly ($p<0.05$) revised to near normal on incubation with the palm wine samples, indicating potent antioxidative potentials as portrayed by their low IC_{50} values (**Table 3.3.1**).

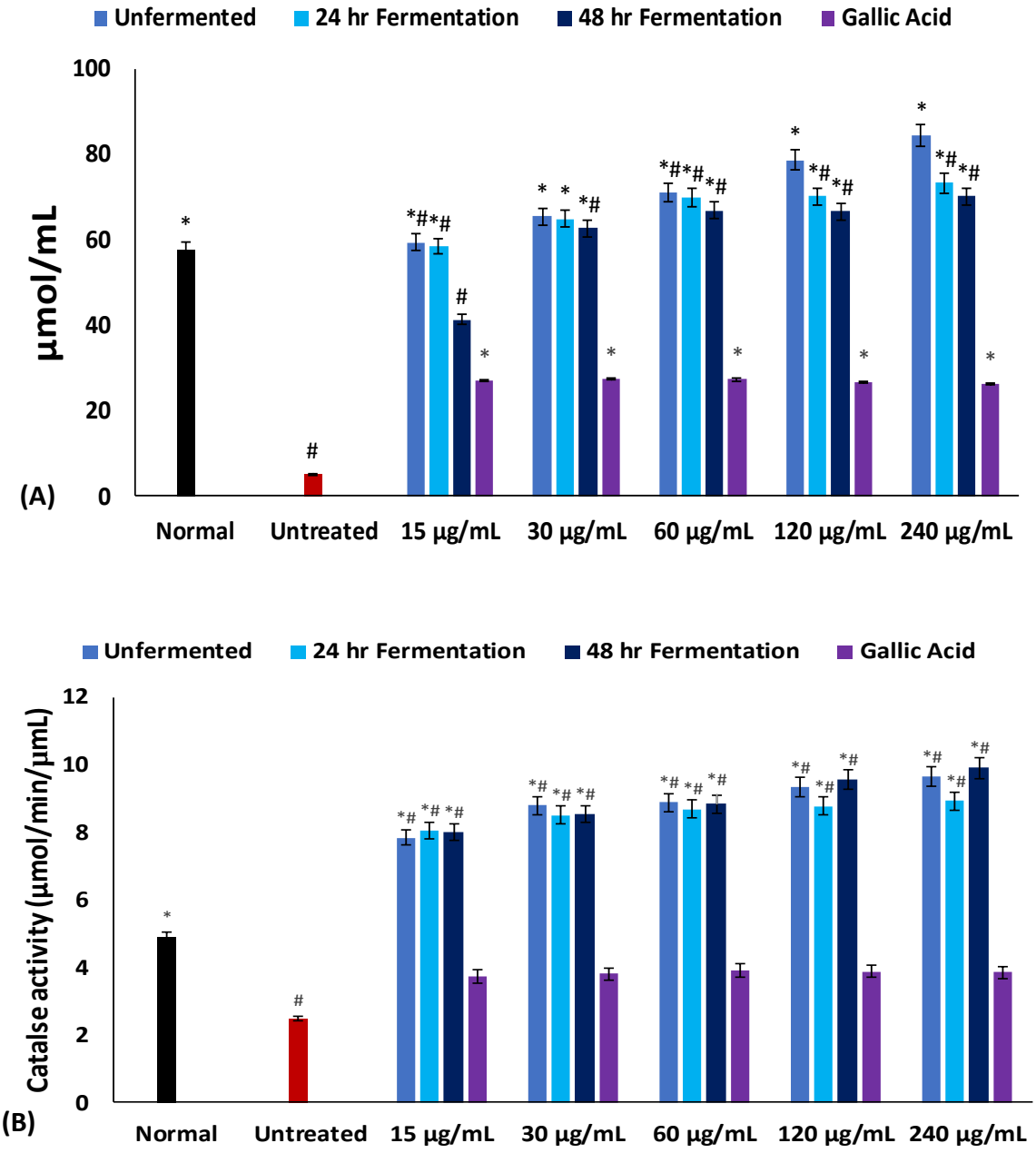

Figure 3.3.2: Effect of raffia palm wine on (B) SOD and (C) catalase activities in oxidative pancreatic injury. Values = mean ± SD; n = 3. *Significantly different from untreated sample and #Significantly different from normal sample ($p < 0.05$, Tukey's HSD-multiple range post-hoc test, IBM SPSS for Windows).

The significant ($p<0.05$) increased MDA levels on incubation with $FeSO_4$, indicates a peroxidative effect. This was reduced by the palm wine samples dose dependently (**Figure 3.3.2D**), with the unfermented showing the best activity (IC_{50} = 0.01 µg/mL).

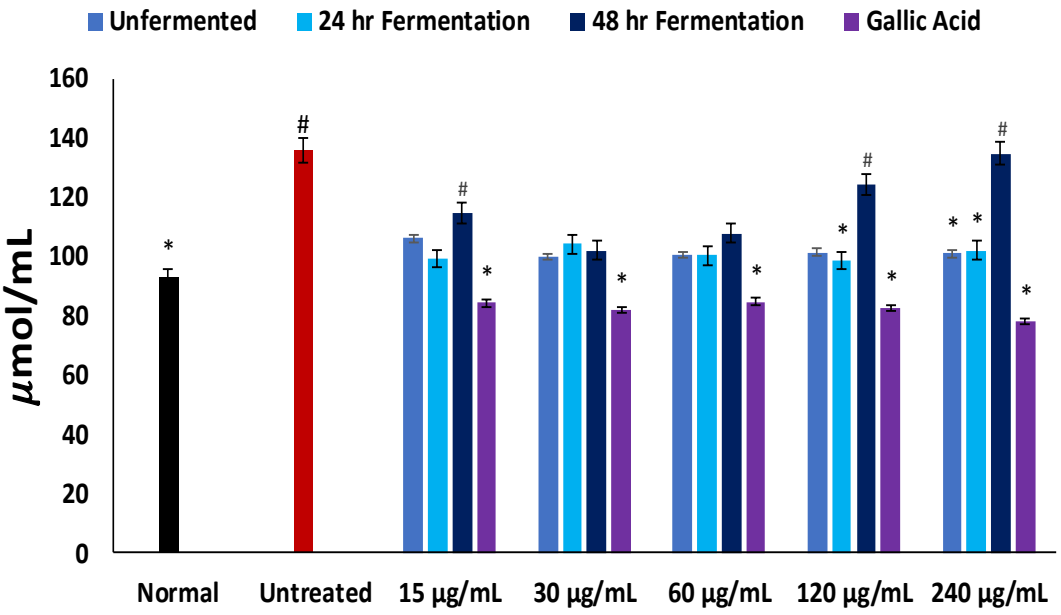

Figure 3.3.2.D: Effect of raffia palm wine on MDA level in oxidative pancreatic injury. Values = mean ± SD; n = 3. *Significantly different from untreated sample and #Significantly different from normal sample ($p < 0.05$, Tukey's HSD-multiple range post-hoc test, IBM SPSS for Windows).

Incubation of pancreatic tissues with $FeSO_4$ led to significant ($p<0.05$) increased levels of NO as depicted in **Figure 3.3.3A**. The palm wine samples showed a dose dependent reduction activity, with the unfermented showing the best activity (IC_{50} = 208.86 µg/mL) (**Table 3.3.1**).

The palm wine samples showed significant ($p<0.05$) inhibitory effect on myeloperoxidase activities in pancreatic tissues incubated with $FeSO_4$, indicating an anti-myeloperoxidase effect as shown in **Figure 3.3.3B**. The effect was dose – dependent for the unfermented and 24 h fermented palm wines, with the unfermented showing the best activities showing the best activity (IC_{50} = 142.43 µg/mL). While, the 48 h fermented palm wine showed a dose dependent decrease.

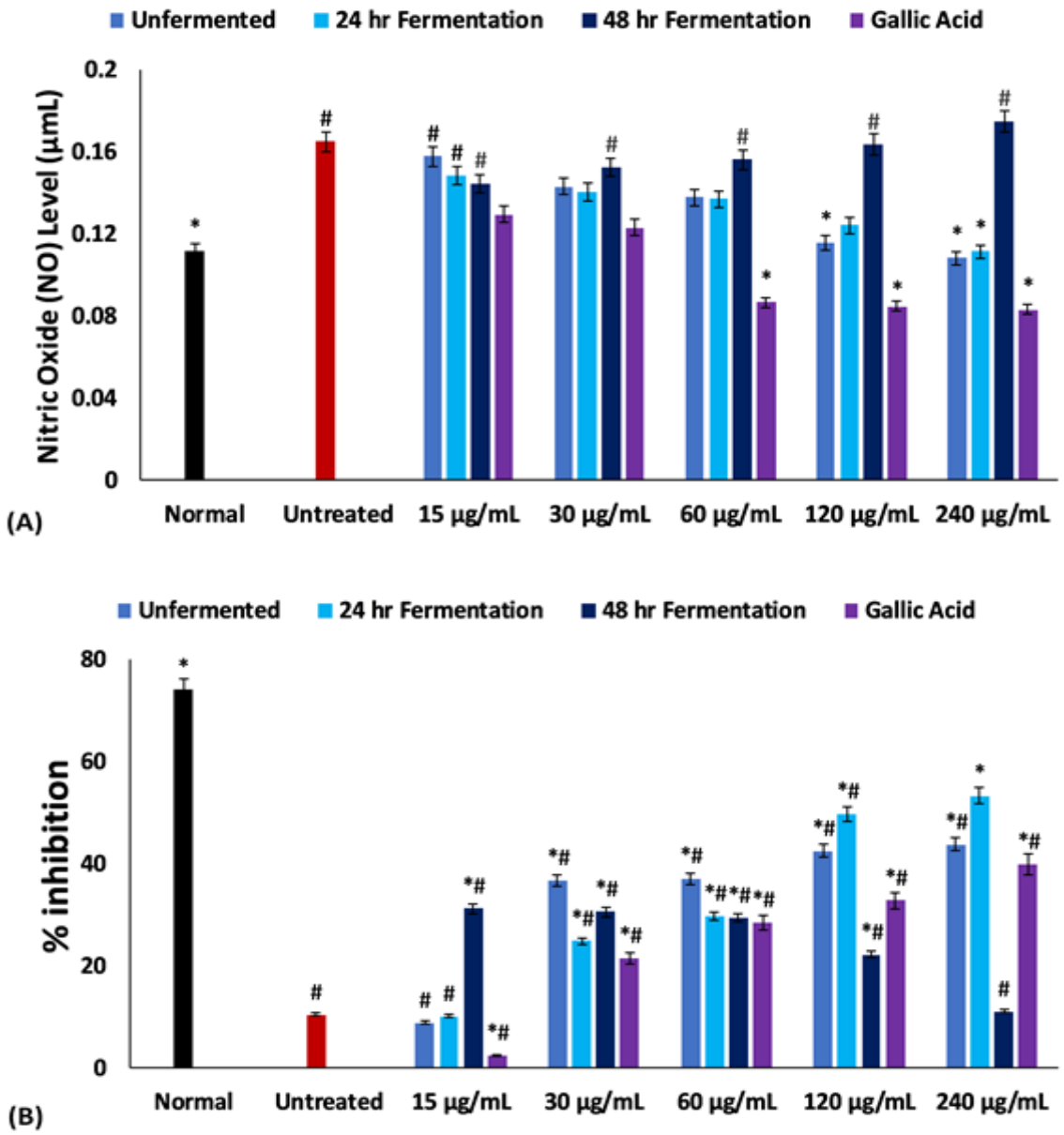

Figure 3.3.3: Effect of raffia palm wine on (A) nitric oxide level and (B) myeloperoxidase activity in oxidative pancreatic injury. Values = mean ± SD; n = 3. *Significantly different from untreated sample and #Significantly different from normal sample ($p < 0.05$, Tukey's HSD-multiple range post-hoc test, IBM SPSS for Windows).

The ATPase activity of pancreatic tissues incubated with the palm wine samples was significantly ($p<0.05$) reduced compared to the increased activity on incubation with $FeSO_4$ only as depicted in

Figure 3.3.4. The inhibitory effect was dose-dependent, with the unfermented palm wine showing the best activity (IC_{50} = 0.61 µg/mL).

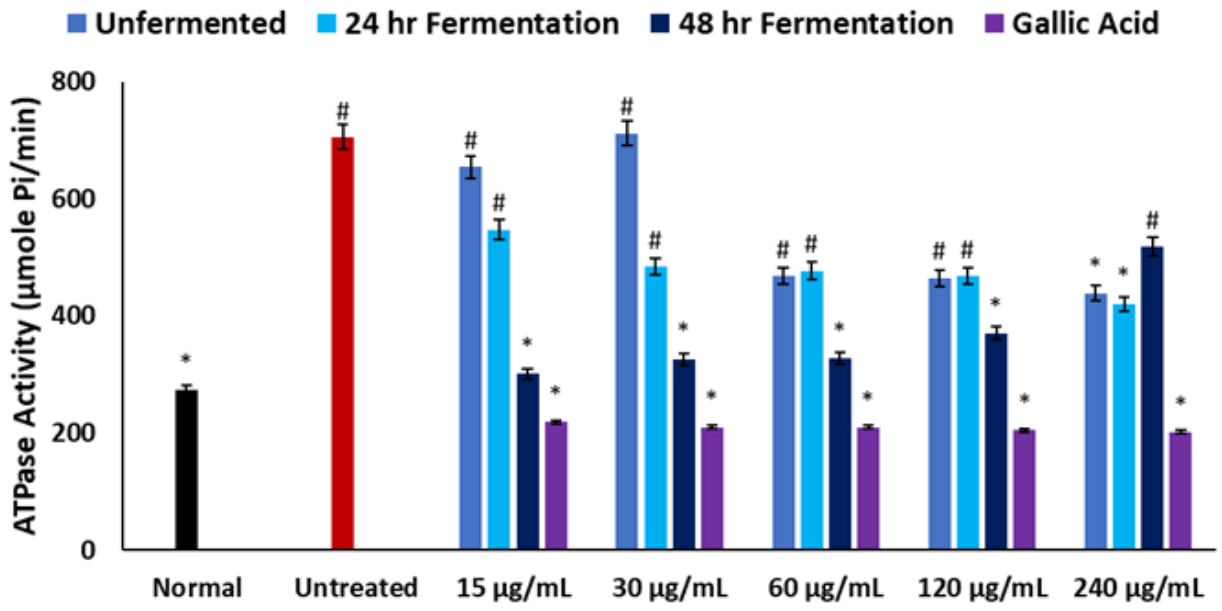

Figure 3.3.4: Effect of raffia palm wine on ATPase activity in oxidative pancreatic injury. Values = mean ± SD; n = 3. *Significantly different from untreated sample and #Significantly different from normal sample ($p < 0.05$, Tukey's HSD-multiple range post-hoc test, IBM SPSS for Windows)

LC-MS analysis of the palm wine samples revealed the presence of gallic acid, (±)-cystine, l-proline, l-sscorbic acid, l-phenylalanine and UDP-β-l-arabinofuranose in all three samples as revealed in **Figure 3.3.5 and Table 3.3.2**. Aside compounds common to all samples, 19 compounds were identified in the unfermented, while 7 and 3 compounds were identified in the 24 and 48 hrs fermented respectively. Of the 7 additional compounds identified in the 24 h fermented sample, 5 were common to those identified in the unfermented. Phenolics were noted to be the predominant compounds in all samples.

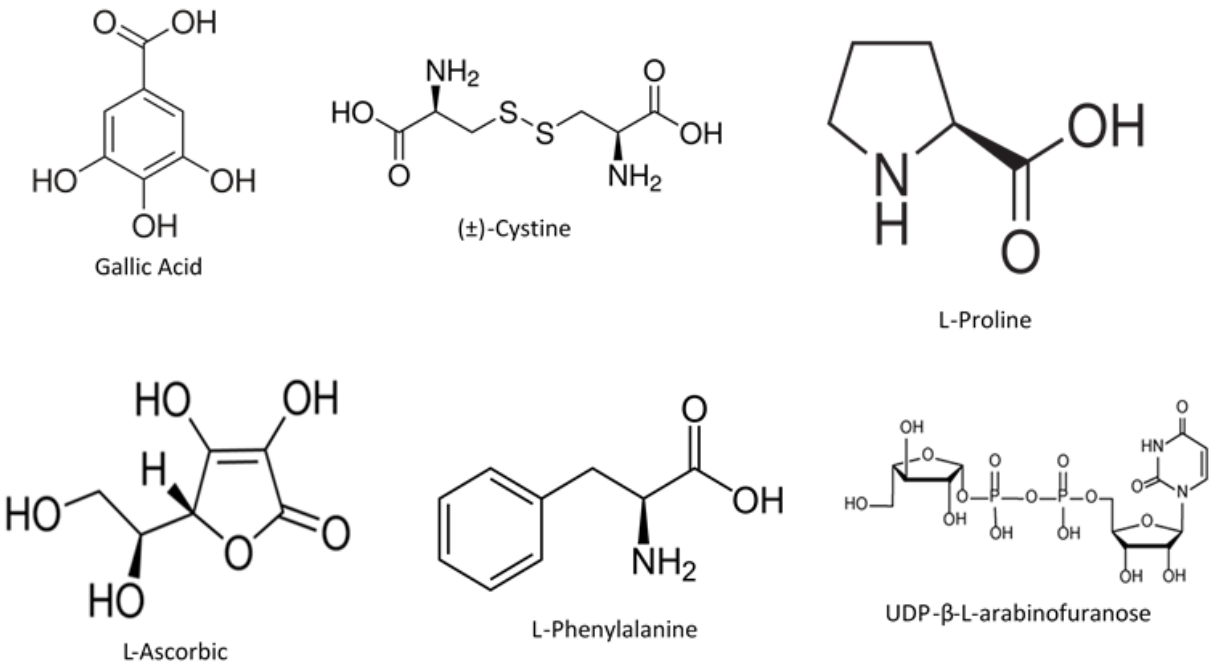

Figure 3.3.5: LC-MS identified compounds common in unfermented and fermented raffia palm wines

Oral toxicity prediction of the identified compounds showed all the compounds to fall on the class range from 4 – 6, except (±)-cystine and α-D-glucose 6-phosphate which had a toxicity class of 3 as depicted in **Table 3.3.2**.

Table 3.3.2: LC-MS identified compounds of unfermented and fermented raffia palm wine and their predicted toxicity

Peak No.	Compounds	Unfermented palm wine	24 h fermented palm wine	48 h fermented palm wine	Predicted LD_{50} (mg/kg)	Predicted toxicity class
105	Gallic acid	X	x	x	2000	4
115	Coumarin-4-carboxylic acid	X	x	-	1691	4
121	Hydroxycaffeic acid	X	-		2980	5
122	Daucic acid	X	-		1238	4
135	D-Lactic acid	x	x	-	75	3
143	(±)-Cystine	x	x	x	156	3
152	α-D-glucose 6-phosphate	x	x	-	1500	4
172	Luteic acid	x	-		3200	5
173	Tartaric acid	x	-		1600	4
192	L-Proline	x	x	x	2078	5
219	Caffeic acid	x			2980	5
221	L-Ascorbic acid	x	x	x	3367	5
242	L-Phenylalanine	x	x	x	2400	5
244	Oxalic acid	x	-		660	4
262	Selenomethyl selenocysteine	x	x	-	660	4
290	UDP-β-L-arabinofuranose	x	x	x	16877	6
293	7,4'-Dihydroxyflavone	x	x	-	2500	5
306	1-Methylnicotinamide iodide	x	-	-	2500	5
349	2,7,4'-trihydroxyisoflavanone	x	-	-	825	4

365	Dihydrokaempferol	x	-	-	2000	4
388	4-Hydroxybenzyl isothiocyanate rhamnoside	x	-	-	3750	5
393	Protocatechuic acid 4-glucoside	x	-	-	3750	5
409	Gallic acid 3-glucoside	x	-	-	2190	5
476	Flavogallol	x	-	-	945	4
497	Gallocatechin gallate	x	-	-	1000	4
206	2-oxo-5-methylthiopentanoate	-	x	-	3478	5
222	4-(1-Guanidino)butyric acid	-	x	-	12680	6
359	7,4'-Dimethoxyflavone	-	-	x	4000	5
384	3-(Methylseleno)alanine	-	-	x	660	4
400	Glutathioselenol	-	-	x	5000	5

Key: + = present; - = not present

Analysis of the metabolites of the normal pancreatic tissues revealed the presence of amino acids, vitamins, glycolytic, nucleotide, sugar, pyruvate and inosine metabolic intermediates as shown in **Table 3.3.3**. Induction of oxidative stress led to depletion of most of these metabolites, with the generation of oxalacetic acid, 2-keto-glutaramic acid, 2-phospho-d-glyceric acid, kynurenic acid, iduronic acid, 3-sulfopyruvic acid, d-erythro-imidazole-glycerol-phosphate and 2-carboxyarabinitol 5-phosphate metabolites. Treatment with the palm wine samples led to the generation of the lipid and adenosine metabolic intermediates, sugar and inositol phosphorylation intermediates, as well as generation of enzyme co-factors, selenium and ascorbic acid metabolites.

Table 3.3.3: LC-MS identified pancreatic metabolites

Key: + = present; - = not present

Metabolites	**Normal**	**Untreated**	**Unfermented palm wine Treated**	**24 h fermented palm wine Treated**	**48 h fermented palm wine Treated**
Lactaldehyde	X	–	–	–	–
L-Cystine	X	X	–	–	–
Carbamoyl phosphate	X	–	–	–	–
Glycolaldehyde	X	–	–	–	–
2'-deoxycytidine 5'-triphosphate	X	–	–	–	–
6-Thiourate	X	–	–	–	–
Uridine 2',3'-cyclic phosphate	X	–	–	–	–
3-Sulfopyruvic acid	X	–	–	–	–
Pamidronate	X	X	–	–	–
3-Hydroxyphenylpyruvic acid	X	–	–	–	–
Phosphatidylcholine O-34:2	X	–	–	–	–
6-Methylnicotinamide	X	X	–	–	–
Phosphoenolpyruvic acid	X	–	–	–	–

L-Selenocystathionine	X	–	–	–	–
Uridine triphosphate	X	X	–	–	–
O-Phosphohomoserine	X	X	–	–	–
Glycyl-Threonine	X	X	–	–	–
Inosine triphosphate	X	X	–	–	–
3-Iodopropanoic acid	X	X	X	–	–
Phosphohydroxypyruvic acid	X	–	–	–	–
N-Acetylglutamine	X	–	–	–	–
Mevalonic acid-5P	X	X	–	–	–
Cysteinyl-Cysteine	X	X	–	–	–
Dopamine 4-sulfate	X		–	–	–
Linoleic acid	X	X	–	–	–
5-Methylthioribulose 1-phosphate	X	X	–	–	–
Pyridoxine 5'-phosphate	X	X	–	–	–
PC(o-18:0/22:0)	X	X	–	–	–
Cysteinyl-Proline	X	X	–	–	–
D-Glucuronic acid 1-phosphate	X	X	–	–	–
N-Acetyl-L-tyrosine	X	X	–	–	–
Oxalacetic acid	–	X	–	–	–

2-Keto-glutaramic acid	–	X	–	–	–
2-Phospho-D-glyceric acid	–	X	–	–	–
Kynurenic acid	–	X	–	–	–
Iduronic acid	–	X	–	–	–
3-Sulfopyruvic acid	–	X	–	–	–
D-Erythro-imidazole-glycerol-phosphate	–	X	–	–	–
2-Carboxyarabinitol 5-phosphate	–	X	–	–	–
Nicotinate D-ribonucleoside	–	–	X	–	X
Uridine 5'-diphosphate	–	–	X	X	X
Selenomethionine se-oxide	–	–	X	X	X
Adenosine phosphosulfate	–	–	X	X	–
2'-deoxycytidine 5'-triphosphate (dCTP)	–	–	X	–	–
(Cardiolipin(82:18))	–	–	X	X	
TG(i-24:0/21:0/i-19:0)	–	–	X	X	X
TG(22:1(13Z)/20:4(5Z,8Z,11Z,14Z)/o-18:0)	–	–	X	X	X
Luteolin 3'-(4''-acetylglucuronide)	–	–	X	X	X
Adenosine tetraphosphate	–	–	X	X	X
UDP-L-iduronate	–	–	X	X	X
CL(i-13:0/i-22:0/i-12:0/i-14:0)	–	–	X		

CL(i-14:0/i-12:0/i-12:0/i-16:0)	–	–	X	X	X
1,4,6-Trigalloyl-beta-D-glucopyranose	–	–	X	–	–
1D-myo-inositol 1,4-bisphosphate (4-)	–	–	X	–	–
CL(20:4(5Z,8Z,11Z,14Z)/16:1(9Z)/ 20:4(5Z,8Z,11Z,14Z)/18:2(9Z,12Z))	–	–	X	X	X
Molybdopterin-AMP	–	–	X	X	X
cytidine 5'-diphosphate	–	–	X	X	X
3,4-Dihydro-3-hydroxy-4-S-glutathionyl bromobenzene	–	–	X	X	X
L-lysine	–	–	X	X	X
5-Phosphoribosylamine	–	–	–	X	X
Cysteic acid	–	–	–	X	–
L-Glutamic acid 5-phosphate	–	–	–	X	–
Diguanosine pentaphosphate	–	–	–	X	X
Ascorbic acid-2-sulfate	–	–	–	X	–
CL(i-13:0/i-21:0/i-17:0/i-14:0)	–	–	–	X	–
CL(16:1(9Z)/18:1(9Z)/16:0/16:0)	–	–	–	X	X
Dihydroneopterin triphosphate	–	–	–	–	X
3-Bromotyrosine	–	–	–	–	X
Molybdenum cofactor	–	–	–	–	X

Inositol 1,3,4,5,6-pentakisphosphate	–	–	–	–	X

Pathway enrichment of the identified metabolites revealed an alteration of the glycine and serine metabolic pathway on induction of oxidative stress, with concomitant activation of the malate-aspartate shuttle, citric acid cycle, transfer of acetyl groups into the mitochondria, and the alanine, tryptophan, tyrosine metabolic pathways (**Figure 3.3.6** and **Table 3.3.4**). Treatment with the palm wine deactivated of the oxidative induced metabolic pathways, with concomitant activation of biotin, carnitine synthesis, sulfate/sulfite, androstenedione, estrone, lysine degradation, androgen and estrogen, nicotinate and nicotinamide, porphyrin, and sphingolipid metabolic pathways (**Figure 3.3.6** and **Table 3.3.4**). Treatment with the 24 h fermented sample also led to the activation of taurine and hypotaurine metabolic pathway, while the 48 h fermented led to additional activation of the inositol phosphate, pterine biosynthesis, and inositol metabolic pathways.

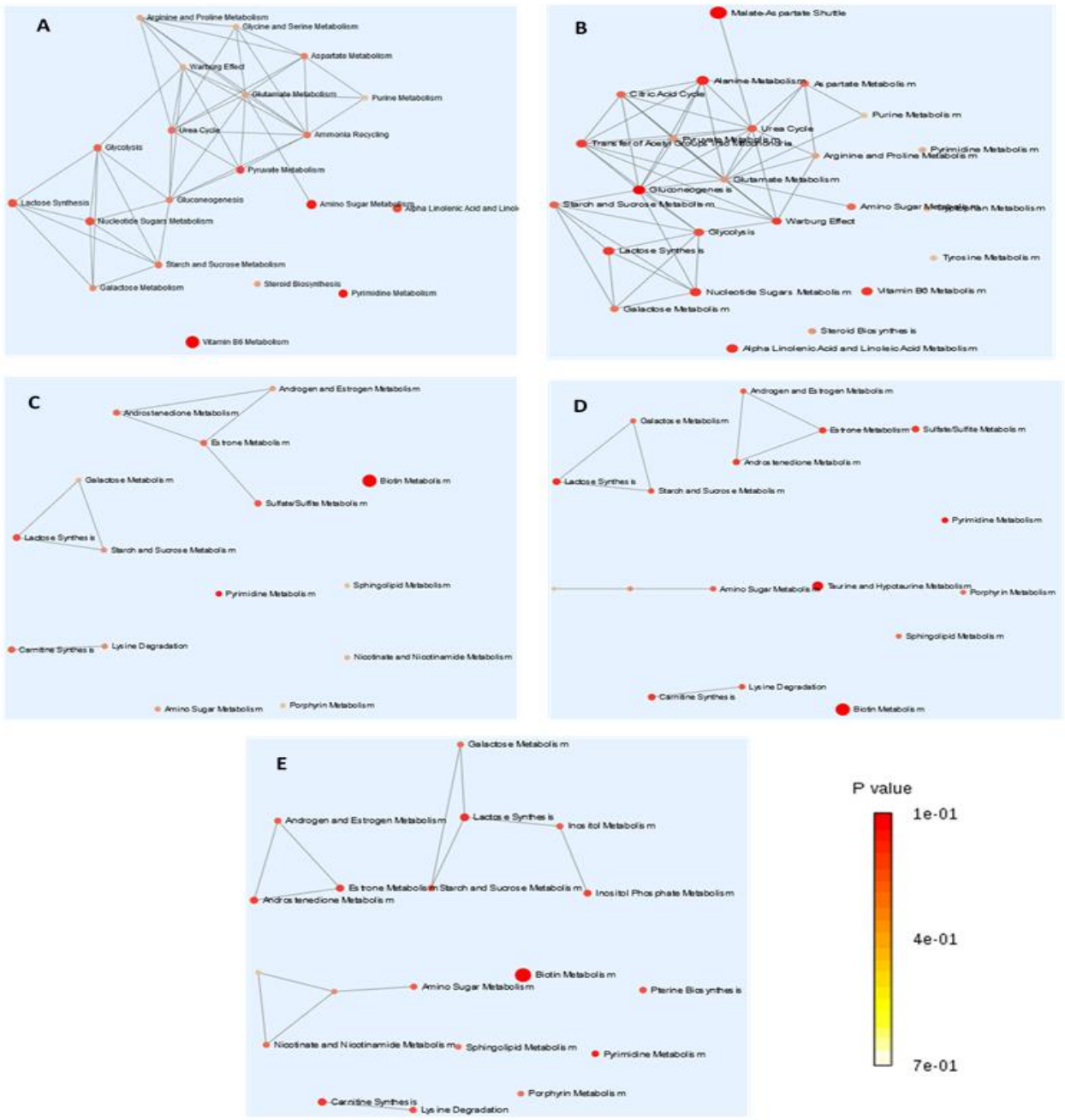

Figure 3.3.6: Identified metabolic pathways of (A) normal pancreatic tissue; (B) oxidative injured pancreatic tissue; (C) unfermented; (D) 24 h fermented; and (E) 48 h fermented raffia palm wine treated oxidative injure pancreatic tissues.

Table 3.3.4: Identified metabolic pathways

Metabolic Pathways	Normal	Untreated	Unfermented palm wine Treated	24 h fermented palm wine Treated	48 h fermented palm wine Treated
Vitamin B6 Metabolism	X	X	–	–	–
Pyrimidine Metabolism	X	X	X	X	X
Amino Sugar Metabolism	X	X	X	X	X
Pyruvate Metabolism	X	X	–	–	–
Alpha Linolenic Acid and Linoleic Acid Metabolism	X	X	–	–	–
Lactose Synthesis	X	X	X	X	X
Nucleotide Sugars Metabolism	X	X	–	–	–
Glycolysis	X	X	–	–	–
Urea Cycle	X	X	–		–
Sucrose Metabolism	X	X	X	X	X
Ammonia Recycling	X		–	–	–
Aspartate Metabolism	X	X	–	–	–
Gluconeogenesis	X	X	–	–	–
Galactose Metabolism	X	X	X	X	X

Steroid Biosynthesis	X	X	–		
Glutamate Metabolism	X	X	–	X	X
Arginine and Proline Metabolism	X	X	–	–	–
Warburg Effect	X	X	–	–	–
Glycine and Serine Metabolism	X	–	–	–	–
Purine Metabolism	X	X	–	X	X
Malate-Aspartate Shuttle	–	X	–	–	–
Alanine Metabolism	–	X	–	–	–
Transfer of Acetyl Groups into Mitochondria	–	X	–	–	–
Citric Acid Cycle	–	X	–	–	–
Tryptophan Metabolism	–	X	–	–	–
Tyrosine Metabolism	–	X	–	–	–
Biotin Metabolism	–	–	X	X	X
Carnitine Synthesis	–	–	X	X	X
Sulfate/Sulfite Metabolism	–	–	X	X	–
Androstenedione Metabolism	–	–	X	X	–
Estrone Metabolism	–	–	X	X	X
Lysine Degradation	–	–	X	X	X
Androgen and Estrogen Metabolism	–	–	X	X	X

Nicotinate and Nicotinamide Metabolism	–	–	X	–	X
Porphyrin Metabolism	–	–	X	X	X
Sphingolipid Metabolism	–	–	X	X	X
Taurine and Hypotaurine Metabolism	–	–	–	X	–
Inositol Phosphate Metabolism	–	–	–	–	X
Pterine Biosynthesis	–	–	–	–	X
Inositol Metabolism	–	–	–	–	X

Key: + = present; - = not present

3.3.5 Discussion

The epidemy of diabetes with its cost of treatment are huge economic burden to the world particularly sub-Saharan Africa, with poor health infrastructures. This may account for Africa having the highest rate of diabetes mortality for ages below 60 years in 2017 despite the lowest number of people living with diabetes (I.D.F., 2018). Hence, the search for and development of affordable treatments with little or no side effects. This has led to a paradigm shift to natural products. In this study, we investigated the ability of raffia palm wine to arrest glucose diffusion as well as mitigate pancreatic oxidative stress and the possible metabolic pathways that may be involved.

Glucose uptake particularly in muscles have been demonstrated to be a potent antidiabetic mechanism, as its stimulation leads to reduced blood glucose levels and improves insulin sensitivity (Satoh 2014). Several studies have used the yeast cell as an *in vitro* model for studying glucose uptake (Nirupama et al. 2014; Sairam and Urooj 2013). In these studies, the authors correlated the yeast glucose uptake with antidiabetic potentials. Thus, the ability of raffia palm wine to enhance glucose uptake in yeast cells (**Figure 3.3.1A**) demonstrates a muscle glucose uptake enhancing potential.

Inhibition of glucose transportation across the cell membrane has been shown as a potent mechanism for reducing blood glucose level (Nirupama et al. 2014), thus implying that the ability of the palm wine samples to inhibit glucose diffusion across the dialysis membrane (**Figure 3.3.1B**) demonstrates a hypoglycemic potential.

The pancreatic tissues have been reported to be very low in endogenous antioxidant concentration particularly the SOD and catalase enzymes (Pi et al. 2010; Tiedge et al. 1997), making them very prone to oxidative damage. The decreased and increased levels of GSH and MDA respectively, and activities of SOD and catalase in the untreated tissues indicates an occurrence of oxidative stress (**Figure 3.3.2**). This is further evident by the activation of the citric acid cycle (**Figure 3.3.6B** and **Table 3.3.4**) leading to the formation of the citric acid intermediates: oxalacetic acid and 2-Keto-glutaramic acid (**Table 3.3.3**). The continuous drive of the citric acid and the concomitant generation of these intermediates leads to an increased accumulation of the electron donors, NADH and FADH2 (Brownlee 2001). Activation of the transfer of acetyl groups into the

mitochondria in the untreated pancreatic oxidative tissue (**Figure 3.3.6B** and **Table 3.3.4**), also corroborates an occurrence of oxidative stress as electron donors are generated by acylCoA dehydrogenase (ACAD) and electron transfer flavoprotein (ETF) (Quijano et al. 2016). Metabolism of alanine, tryptophan, and tyrosine also fuels the citric acid cycle by providing intermediates, thus further driving the cycle with concomitant generation of electron donors. The activation of these pathways will lead to accumulation of electron donors and may be responsible for the activation of the malate-aspartate shuttle (**Figure 3.3.6B** and **Table 3.3.4**) via which the electrons are translocated to the mitochondria (Fernie et al. 2004). Once in the mitochondria, these electron donors trigger a high mitochondrial membrane potential, which inhibits electron transport at complex II with concomitant reduction of oxygen to (O_2) to superoxide ($O_2^{\cdot -}$) (Brownlee 2001; Du et al. 2001).

The generated superoxide radical ($O_2^{\cdot -}$) is dismutated to hydrogen peroxide (H_2O_2) by SOD, which is then converted to oxygen and water in a reaction catalyzed by catalase. If not broken down by catalase, the generated H_2O_2 will be broken down to hydroxy radicals ($^{\bullet}OH$). Hydroxyl radicals have been recognized as the key starter of membrane lipid peroxidation (Aslan et al. 2000). H_2O_2 also serves as a substrate for the enzyme, myeloperoxidase which converts it to $HOCl^-$ (Furtmüller et al. 2000). Nitric oxide can react with $O_2^{\cdot -}$ to generate peroxynitrate ($ONOO^-$). Accumulation of $HOCl^-$ and $ONOO^-$ will lead to proinflammation. Thus, implying that the increased NO level and myeloperoxidase activity in the untreated pancreatic oxidative tissues (**Figure 3.3.2**) portrays an occurrence of proinflammation.

The increased SOD, catalase, myeloperoxidase activities as well as decreased levels of MDA and NO in the palm wine treated tissues, therefore indicate antioxidative and anti-proinflammatory potentials of raffia palm wine (**Figures 3.3.2** and **3.3.3**). These activities can be attributed to the LC-MS identified compounds particularly the polyphenols and ascorbic acid which have been reported for their potent antioxidant and anti-inflammatory activities (Klimczak et al. 2007). This is further evident by the presence of their metabolites (**Table 3.3.3**) and deactivation of the electron donor generating pathways (**Table 3.3.4**).

Carnitine has been reported for its free radical and antioxidative activities in cells (Gülcin 2006; Kolodziejczyk et al. 2011; Ribas et al. 2014), thus activation of its synthesis in pancreatic tissues treated with the palm wine samples (**Table 3.3.4**) may portray an increased antioxidative effective

owing to concomitant increased level of cellular carnitine. The antioxidant protective effect of the palm wine samples against oxidative pancreatic tissues is also evident by the activation of metabolism of the sex hormones, androgen and estrogen. The receptors of these hormones have been reported in pancreatic tissues, and dysregulation of their metabolism have been implicated in pancreatitis and pancreatic β – cell proliferation in diabetes (Corbishley et al. 1986; Fernández-del et al. 1990; Li et al. 2008). These hormones have also been shown to modulate antioxidant enzyme activities (Bellanti et al. 2013). Similarly, activation of the B vitamins metabolism as well as that of sulfate/sulfite (**Figure 3.3.6** and **Table 3.3.4**) portrays increased antioxidant activities as well as generation of essential cofactors required for DNA repair and antioxidant enzyme activities (Kamat and Devasagayam 1999; Mukwevho et al. 2014; Sauve 2008). The antioxidant and antidiabetic properties of inositol and its phosphates have been demonstrated (Foster et al. 2017; Özturan et al. 2017; Phillippy and Graf 1997). Thus, activation of their metabolism by the 48 h fermented sample (**Figures 3.3.6E** and **Table 3.3.4**) further indicates an antioxidant and antidiabetic potential.

The increased ATPase activity in the untreated oxidative pancreatic tissue (**Figure 3.3.4**) indicates an impaired depolarization of the β – cell membrane (Erukainure et al. 2017). Depolarization of the β – cell membrane has been correlated with decreased ATPase activity, leading to the influx of Ca^{2+} which is a major mechanism involved in insulin secretion (Costa et al. 2010; Owada et al. 1999). Thus, the decreased activity on treating with palm wine samples may indicate a therapeutic benefit in the treatment and management of diabetes and its complications. The increasing activity in tissues treated with the 48 h fermented sample, may suggest caution in its usage at high concentrations.

3.3.6 Conclusion

These results suggest the antidiabetic and antioxidative effects of unfermented and fermented raffia palm wine as evident by their ability to inhibit glucose diffusion and promote glucose uptake, as well as increase the antioxidant enzyme activities while depleting the levels of MDA, NO and myeloperoxidase activity. This is further evident by the inhibition of metabolic pathways involved in free radical generation, and activation of pathways involved in antioxidant activities. Thus, giving credence to the traditional use of this drink in the treatment and management of diabetes and its complications amongst other diseases.

3.4 Raffia Palm (*Raphia hookeri*) Wine Modulates Glucose Homeostasis by Enhancing Insulin Secretion; Inhibiting Redox Imbalance, Glycolytic and Cholinergic Enzymes Activities; and Downregulates Pancreatic Nrf2 Expression in Type 2 Diabetic Rats

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Preface: This article investigated the antidiabetic effect of Raffia palm wine in type 2 diabetic rats. Its antioxidative, anti-proinflammatory, antilipemic and insulin stimulatory effects were investigated. Its inhibitory effect on glycolytic, cholinergic and carbohydrate digesting enzymes were also investigated. Its ability to inhibit Nrf2 expression was also investigated. The article presently under review for publication in Chemico-Biological Interactions (CHEMBIOINT_2018_1532).

3.4.1 Abstract

The antidiabetic effect of Raffia palm (*Raphia hookeri*) wine (RPW) and its possible mechanism of action were investigated in a type 2 diabetes (T2D) model or rats. Diabetes was induced in the four groups of male albino rats (Sprague-Dawley) by supplying 10% fructose solution *ad libitum* for first 2 weeks followed by an intraperitonial injection of streptozotocin (40 mg/kg bw). Two diabetic groups were administered RPW at 150 and 300 mg/kg bodyweight (bw) respectively, a

group were administered with metformin (200 mg/kg bw), while the other group was untreated and served as negative control. Two other groups of normal rats were administered water and RPW (300 mg/kg bw), with the former serving as normal control and the latter, normal toxicological group. After 5 weeks of intervention, treatment with RPW led to significant ($p<0.05$) increase in serum insulin and HDL-cholesterol levels with concomitant reduction in blood glucose, fructosamine, ALT, uric acid, triglycerides and LDL-cholesterol levels in T2D rats. Rats treated with RPW had elevated levels of GSH, SOD, catalase, ATPase and α-amylase activities, while reducing NO level and myeloperoxidase activity was observed in both serum and pancreatic tissues. RPW also caused an improved β-cell function as well as restored pancreatic β-cells and acinar cells morphology, and capillary networks. The activities of glycogen phosphorylase, fructose-1,6-biphosphatase, glucose-6-phosphatase, and acetylcholinesterase activities were also inhibited in RPW-treated T2D rats, with concomitant downregulation of Nrf2 expression. These results indicate the antidiabetic effect of RPW, which may be attributed to the synergetic influence of its phytochemical constituents identified in this study by GC-MS analysis.

Keywords: Dyslipidemia; Glycolytic enzymes; Nrf2; Pancreatic β-cell; Type 2 diabetes;

3.4.2 Introduction

Diabetes mellitus (DM) is amongst the fastest growing global epidemic, with over 425 million people estimated to be diabetic in 2017 (Cho et al. 2018; I.D.F. 2018). This estimation portrays a 2.35% increase from 2015 estimates (I.D.F. 2016; Ogurtsova et al. 2017). The International Diabetic Federation (IDF) further projected a 35.35% increase in the number of people that will be diabetic by 2045 (Cho et al. 2018; I.D.F. 2018).

Diabetes affects carbohydrate, protein and lipid metabolism, leading to increase blood glucose level. This has been attributed to inability of the pancreatic β-cells to secrete insulin and/or inability of the body to utilize the secreted insulin (Erukainure et al. 2018a). The former condition is referred to as type 1 diabetes (T1D), while the latter, type 2 diabetes (T2D). Of these diabetes types, T2D is the most prevalent as it accounts for more than 90% of all diabetic cases as well as morbidity and mortality (I.D.F. 2016; I.D.F. 2018). Pancreatic β-cell dysfunction and insulin resistance have been implicated in the progression of T2D, leading to chronic hyperglycemia and dyslipidemia (Bardini et al. 2012; DeFronzo and Ferrannini 1991; Kahn 2003). An increased oxidative stress

has been reported in chronic hyperglycemia, and has been recognized as a major player in the pathogenesis and progression of complications associated with T2D (Evans et al. 2002; Giacco and Brownlee 2010). Oxidative stress occurs when the body's endogenous antioxidant defense system is suppressed by increased generation of free radicals. In T2D, the generation of these free radicals is increased owing to continuous glucose oxidation (Maritim et al. 2003b; Tiwari et al. 2013a).

Antioxidants have been demonstrated in several pre-clinical and clinical studies to ameliorate and manage oxidative-complications in diabetes (Sabu and Kuttan 2002; Tiong et al. 2013). This has been attributed to their ability to scavenge free radicals, as well as enhance the endogenous antioxidant activities. Medicinal plants have been reported for their antioxidant and hypoglycemic activities, which have been attributed to their nutritional and phytochemical constituents (Erukainure et al. 2017b). Their availability and affordability, as well as little or no side effects compared to synthetic drugs have led to increase in the use of medicinal plants in the treatment and management of several ailments.

Although Raffia palm wine is used traditionally in treating DM and its complications, there are still scientific dearth of its antidiabetic properties. Thus, the study was undertaken to investigate its antidiabetic properties in fructose-streptozotocin induced T2D rats. Its inhibitory effect on key glycolytic, cholinergic and carbohydrate digesting enzymes were also investigated as well as its modulatory effect on pancreatic oxidative injury, and morphology. Additionally, its ability to alter the expressions of nuclear factor erythroid 2–related factor 2 (Nrf2) and caspase-3 were also investigated.

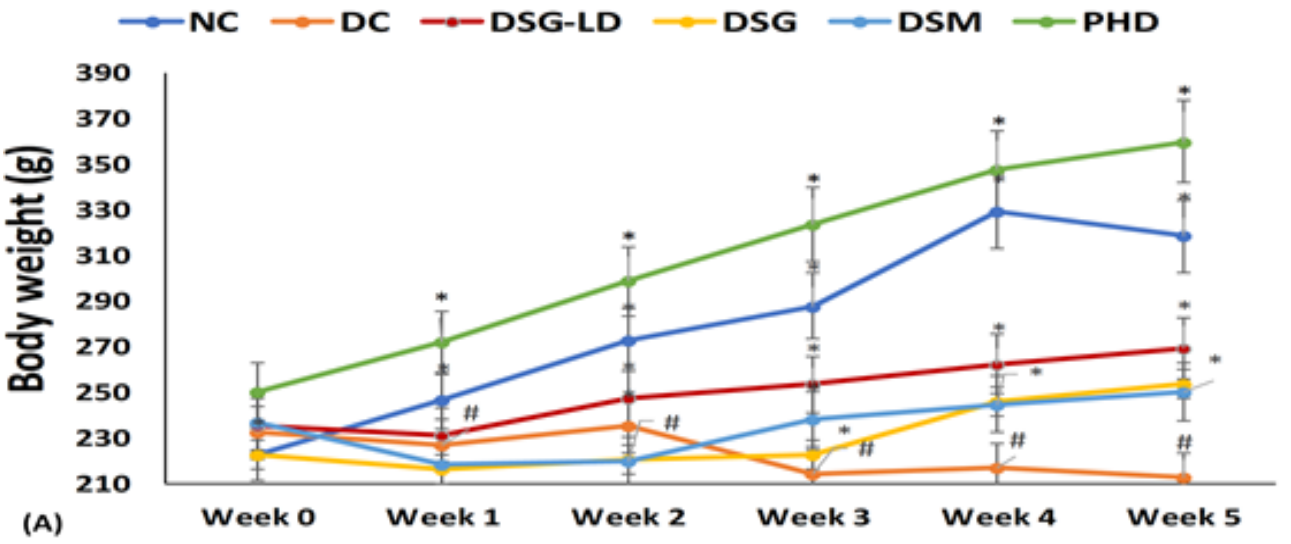
3.4.3 Materials and Methods

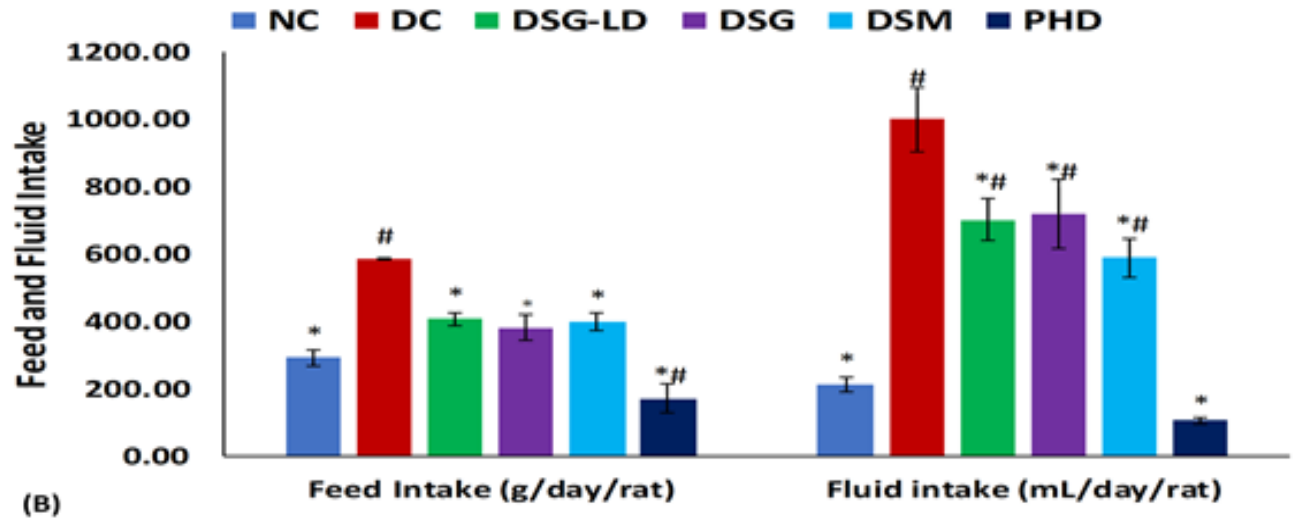
Kindly refer to Chapter 2, subsections 2.1.1; 2.1.3; 2.10.1; 2.12 – 2.13 for details materials and methods.

3.4.4 Results

As shown in **Figure 3.4.1A**, induction of T2D led to a significant ($p<0.05$) decrease in body weights of diabetic groups. Increase in body weights were observed in all treatment groups as the intervention period proceeded, with rats treated with low dose of Raffia palm wine having the

highest weight gain. The body weights of the diabetic control (DC) were significantly ($p<0.05$) reduced as the intervention period proceeded. There was a significant ($p<0.05$) increase in food and fluid intake in all diabetic groups, with the DC having the highest intake as depicted in **Figure 3.4.1B**. There was a significant ($p<0.05$) decrease in the weight and relative weight of the pancreas in the untreated diabetic rats (**Figure 3.4.1C**). These were slightly increased in diabetic rats treated with Raffia palm wine.





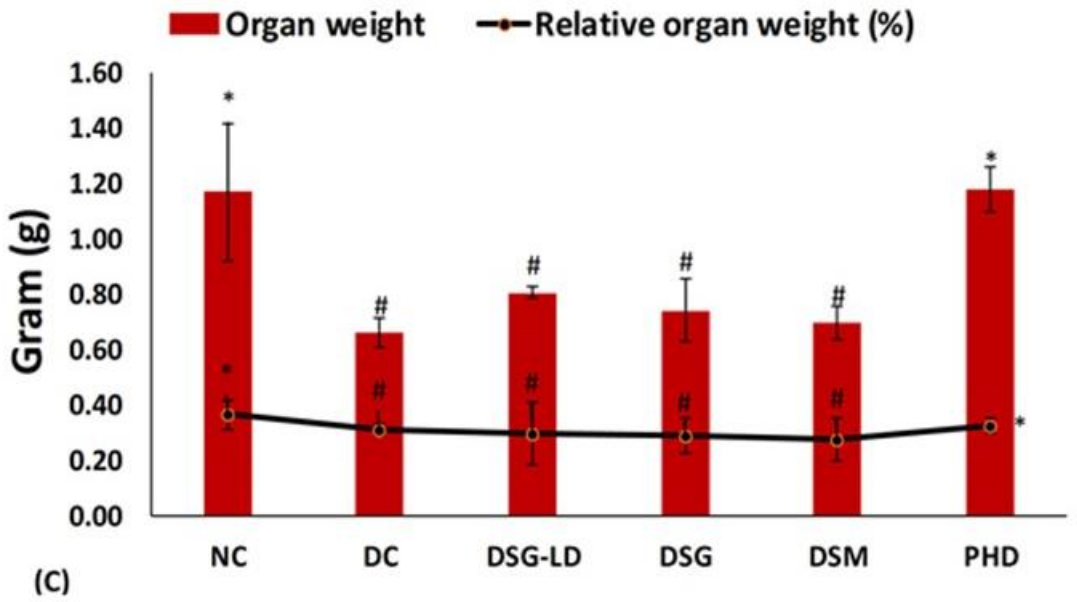


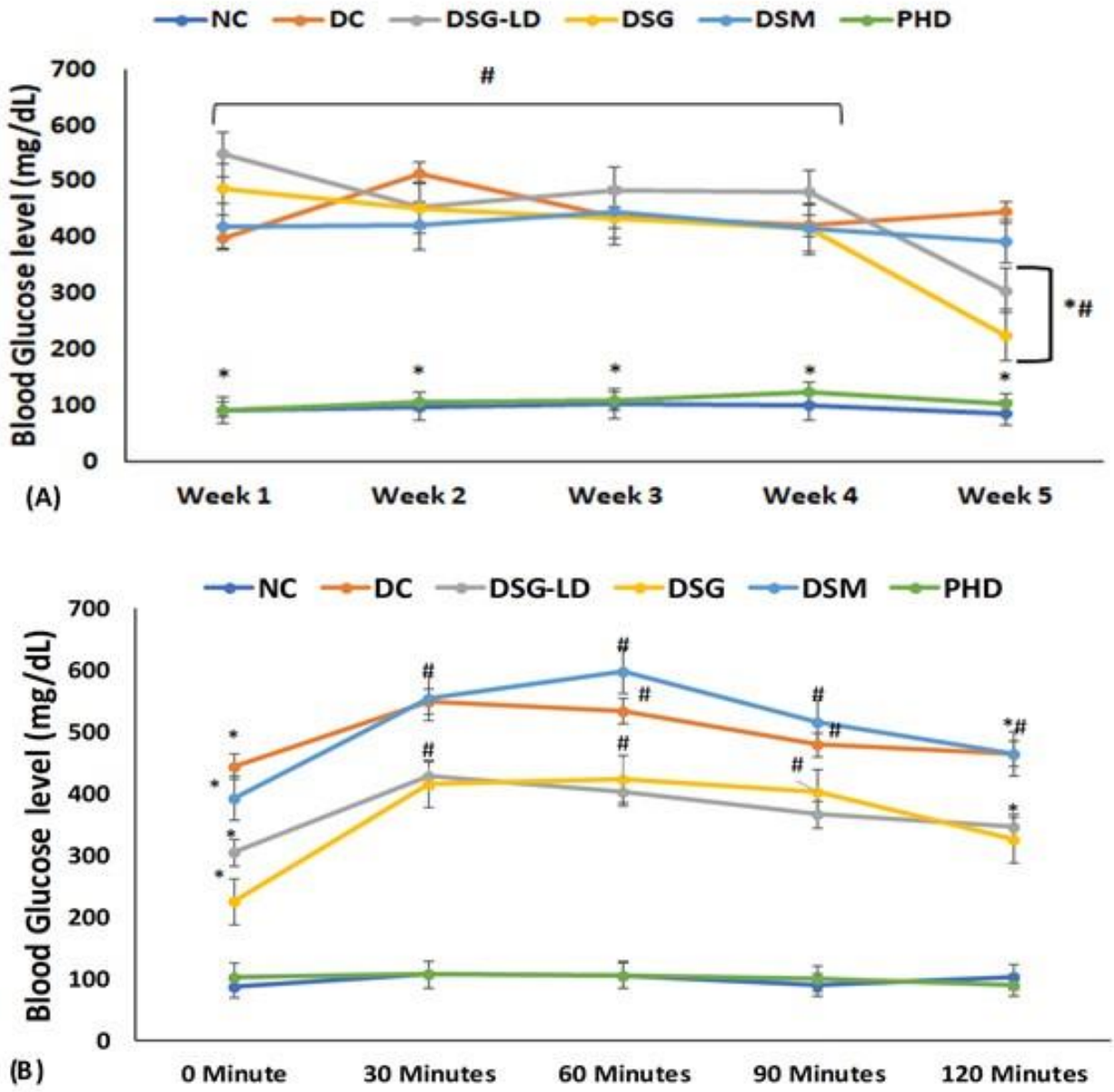
Figure 3.4.1: (A) Body weight; (B) feed and fluid intake; and (C) weight and relative weight of pancreas of experimental groups. Values = mean ± SD; n = 5 (NC and PHD) and 7 (DC, DSG-LD, DSM, and DG). *Statistically ($p<0.05$) significant to DC, #statistically significant ($p<0.05$) to NC. **NC** = normal rats, **DC** = diabetic control, **DSG-LD** = diabetic rats + Raffia palm wine (150 g/kg bw), **DSG** = diabetic rats + Raffia palm wine (300 g/kg bw), **DSM** = diabetic rats + metformin (200 g/kg bw), and PHD = Normal rats + Raffia palm wine (300 g/kg bw).

Induction of T2D led to significant increase in blood glucose level as shown in **Figure 3.4.2A**. These were reduced in all treatment groups, with Raffia palm wine showing significant ($p<0.05$) reduction as both doses led to 44.29 and 53.57% reduction respectively, with fasting blood glucose was measured only at week 5.

There was a significant ($p<0.05$) increase in blood glucose levels of all the experimental animals at 30 min after oral dosing with glucose (2 g/kg BW), indicating a peak rise (**Figure 3.4.2B**). The glucose level reduced with increasing time for all groups, with the lowest levels recorded at 120 min.

Induction of T2D led to significant ($p<0.05$) reduction in serum insulin level, with concomitant reduction in pancreatic β-cell function as shown in Fig. 2C. The insulin level was significantly ($p<0.05$) increased to near normal in rats treated with low dose of Raffia palm wine, with a 93.14%

increase in pancreatic β-cell function (**Figure 3.4.2C**). Treatment with high dose of Raffia palm wine also led to a significant increase in serum insulin level, with concomitant increase (88.29%) in pancreatic β-cell function. Both serum insulin and pancreatic β-cell function were slightly but significantly ($p<0.05$) increased in metformin-treated rats. Administration of Raffia palm wine to normal rats, led to a significant ($p<0.05$) reduction in serum insulin level (51.40%) and pancreatic β-cell function when compared to the normal control (NC).



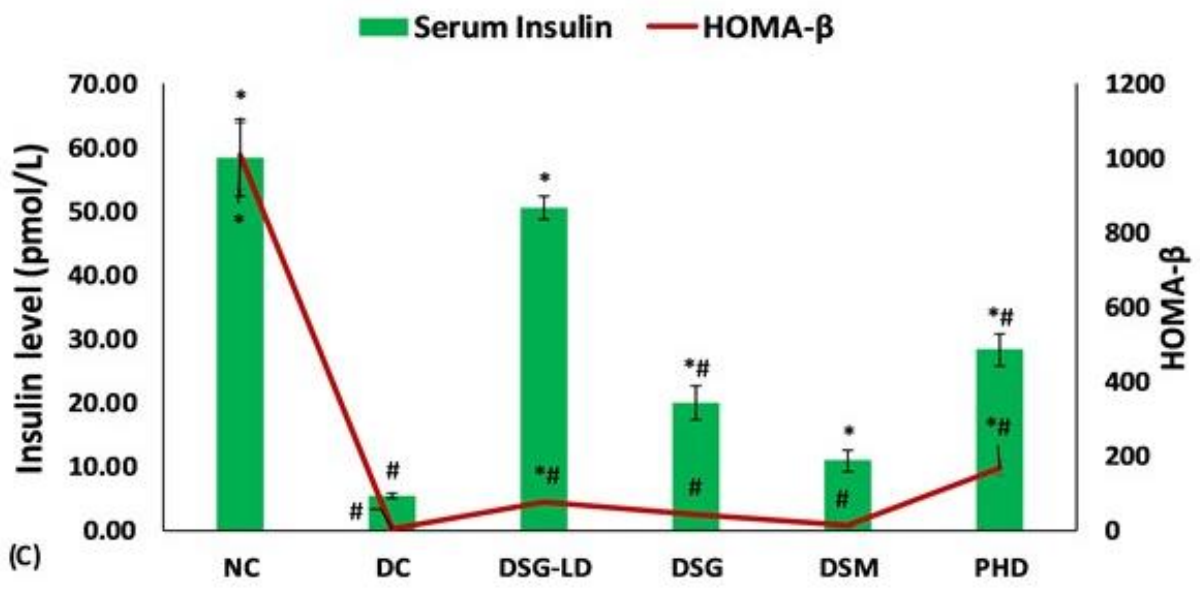


Figure 3.4.2: (A) Blood glucose level; (B) oral glucose tolerance; and (C) serum insulin and β-cell function of experimental groups. Values = mean ± SD; n = 5 (NC and PHD) and 7 (DC, DSG-LD, DSM, and DG). *Statistically ($p<0.05$) significant from DC, #statistically significant ($p<0.05$) from NC. **NC** = normal rats, **DC** = diabetic control, **DSG-LD** = diabetic rats + Raffia palm wine (150 g/kg bw), **DSG** = diabetic rats + Raffia palm wine (300 g/kg bw), **DSM** = diabetic rats + metformin (200 g/kg bw), and PHD = Normal rats + Raffia palm wine (300 g/kg bw).

As shown in **Figure 3.4.3**, there was no significant difference in the total cholesterol (TC) levels of all the experimental groups. However, induction of T2D led to a significant ($p<0.05$) increase in triglycerides (TG), LDL-C, and concomitant decrease in HDL-C levels. These were significantly ($p<0.05$) reversed in all the treatment groups, with rats treated with low dose of Raffia palm wine showing the best results.

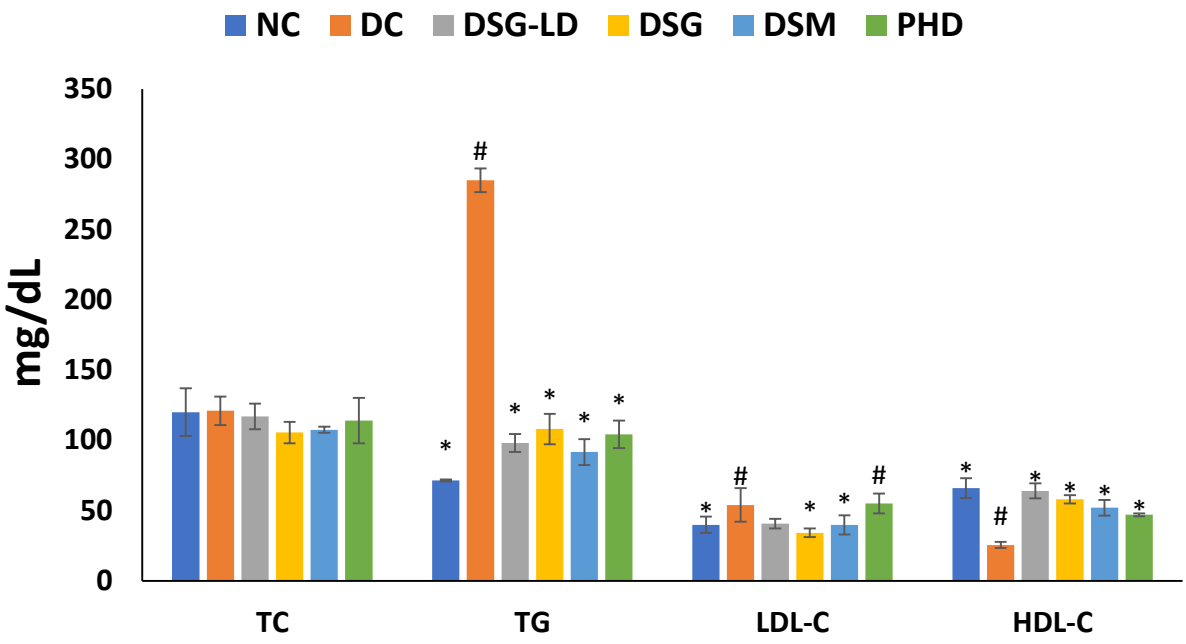


Figure 3.4.3: Serum lipid profile of experimental groups. Values = mean ± SD; n = 5 (NC and PHD) and 7 (DC, DSG-LD, and DG). *Statistically ($p<0.05$) significant from DC, #statistically significant ($p<0.05$) from NC. **NC** = normal rats, **DC** = diabetic control, **DSG-LD** = diabetic rats + Raffia palm wine (150 g/kg bw), **DSG** = diabetic rats + Raffia palm wine (300 g/kg bw), **DSM** = diabetic rats + metformin (200 g/kg bw), and PHD = Normal rats + Raffia palm wine (300 g/kg bw).

Induction of T2D led to significant increase in serum and pancreatic α-amylase activities as depicted in **Table 3.4.1**. These were significantly ($p<0.05$) decreased in all treatment groups, with metformin showing the best result. Among the Raffia palm wine treated diabetic groups, the high dose had the best decreasing activity for serum α-amylase, while the low dose was best for pancreatic α-amylase.

There was also a significant ($p<0.05$) decrease in serum ATPase activity, with concomitant and significant ($p<0.05$) increase in pancreatic ATPase activity (**Table 3.4.1**). These were significantly ($p<0.05$) reversed in all treatment group, with low dose of Raffia palm wine and metformin having the best activities for serum and pancreatic ATPase respectively.

As shown in **Table 3.4.1**, the significant ($p<0.05$) increased acetylcholinesterase activity on induction of T2D was significantly decreased in all treatment group, except for rats treated with low dose of Raffia palm wine which had little or no effect on the activity.

Induction of T2D led to significant increase in the activities of the studied glycolytic enzymes as shown in **Table 3.4.1**. Their activities were ($p<0.05$) significantly decreased in all the treatment groups, with the Raffia palm wine treated groups having the best results.

There was a significant ($p<0.05$) increase in NO and MDA levels and myeloperoxidase activity, with concomitant decrease in SOD and catalase activities and GSH level in both serum and pancreatic tissues on induction of T2D as shown in **Table 3.4.2**, indicating an occurrence of oxidative stress. These were significantly ($p<0.05$) reversed in all the treatment groups.

Table 3.4.1: Enzyme activities of experimental groups.

Enzyme Activities	NC	DC	DSG-LD	DSG	DSM	PHD
Serum α-amylase	25.38±3.50*	72.36±4.25#	59.41±5.56#	64.73±3.19#	41.57±9.53*	37.42±4.39*
Pancreatic α-amylase	46.90±4.25*	89.72±4.21#	56.80±2.16#	41.81±4.80*	28.02±3.37*	19.44±3.27*
Serum ATPase	279.18±5.60*	201.24±17.89#	340.33±4.52*	287.86±15.08*	241.92±12.42*	259.67±6.12*
Pancreatic ATPase	144.35±21.59*	234.49±7.781#	136.24±15.86*	194.76±14.36#	103.95±14.28*	84.85±13.75*
Acetylcholinesterase	29.56±3.24*	44.20±3.24#	41.33±3.25#	24.68±3.03*	22.96±3.76*	21.81±2.84*
Glycogen Phosphorylase	174.22±9.98*	209.89±17.50#	146.92±6.10*	164.76±12.98*	169.35±24.65*	152.19±7.98*
Fructose 1,6 Biphosphatase	177.19±7.93*	247.46±9.90#	163.41±6.03*	155.57±1.15*	191.78±19.49*	149.35±10.32*
Glucose 6 Phosphatase	153.68±3.06*	223.14±12.03#	164.22±7.62*	168.54±4.87*	176.92±23.96*	139.08±15.29*

Values = mean ± SD; n = 5 (NC and PHD) and 7 (DC, DSG-LD, DSM, and DG). *Statistically ($p<0.05$) significant from DC, #statistically significant ($p<0.05$) from NC. **NC** = normal rats, **DC** = diabetic control, **DSG-LD** = diabetic rats + Raffia palm wine (150 g/kg bw), **DSG** = diabetic rats + Raffia palm wine (300 g/kg bw), **DSM** = diabetic rats + metformin (200 g/kg bw), and PHD = Normal rats + Raffia palm wine (300 g/kg bw).

Table 3.4.2: Antioxidant status of (A) serum and (B) pancreas of experimental groups

(A)

	GSH (mol/L)	SOD (U/mg protein)	Catalase (U/mg protein)	MDA (mol/L)	NO (µml)	Myeloperoxidase (U/mg protein)
NC	64.28±11.90*	86.21±8.13*	636.37±29.48*	63.09±4.57*	0.075±0.006*	100.27±9.68*
DC	36.65±2.47#	40.23±3.32#	490.64±42.75#	71.19±4.52#	0.102±0.007#	121.08±18.63#
DSG-LD	55.35±3.24*	71.84±2.44*	584.35±29.37*	53.11±6.48*	0.04±0.007*	67.32±5.75*
DSG	51.16±7.29*	64.66±3.5*	636.37±29.48*	52.54±7.16*	0.044±0.007#	35.99±6.36*
DSM	53.31±2.27*	68.97±8.78*	650.50±4.82*	50.09±9.53*	0.053±0.008*	47.44±1.53*
PHD	40.19±3.55#	34.48±8.30#	687.69±21.84*	41.24±6.79*	0.039±0.004#	66.67±5.60*

(B)

	GSH (mol/L)	SOD (U/mg protein)	Catalase (U/mg protein)	MDA (mol/L)	NO (µml)	Myeloperoxidase (U/mg protein)
NC	104.87±3.09*	659.96±57.9*	783.32±28.21*	40.96±5.79*	0.24±0.02*	20.31±5.75*
DC	54.06±5.01#	150.86±10.6#	711.48±34.46#	71.75±3.60#	0.70±0.02#	35.35±3.61#
DSG-LD	72.45±5.47*	580.46±20.31*	779.71±26.9*	42.66±5.59*	0.27±0.02*	19.49±1.83*
DSG	85.35±7.75*	647.99±58.92*	750.02±59.56*	48.02±7.69*	0.27±0.01*	21.29±1.89*
DSM	51.80±6.38#	583.33±31.8*	754.29±40.8*	55.08±5.99*	0.34±0.03*	17.85±1.81*
PHD	46.64±3.94*#	655.17±6.09*	772.80±17.76*	43.64±7.39*	0.17±0.02*	16.30±2.89*

Values = mean ± SD; n = n = 5 (NC and PHD) and 7 (DC, DSG-LD, DSM, and DG). *Statistically ($p<0.05$) significant from DC, #statistically significant ($p<0.05$) from NC. **NC** = normal rats, **DC** = diabetic control, **DSG-LD** = diabetic rats + Raffia palm wine (150 g/kg bw), **DSG** = diabetic rats + Raffia palm wine (300 g/kg bw), **DSM** = diabetic rats + metformin (200 g/kg bw), and PHD = Normal rats + Raffia palm wine (300 g/kg bw).

Table 3.4.3: Toxicity biomarkers of experimental groups

Enzyme Activities	**NC**	**DC**	**DSG-LD**	**DSG**	**DSM**	**PHD**
ALP U/L	101.00±5.55*	1235.00±29.88#	558.00±10.89*	523.00±46.67*	582.33±10.67*	133.00±1.41*
ALT U/L	91.00±4.24	87.50±0.71	84.00±2.58	86.00±1.41	88.00±3.20	87.33±1.15
CK-MB U/L	625.00±15.80*	959.00±25.10#	572.75±14.42*	813.70±68.45#	739.40±12.89#	478.00±10.73*
Fructosamine mg/dL	656.00±12.6*	743.00±15.56#	702.00±17.21*	686.00±21.11#	658.50±12.02*	506.67±12.728*
Urea mg/dL	58.00±0.71	46.00±5.67	37.00±4.51#	36.00±4.8#	31.50±6.81#	61.00±2.89
Uric acid mg/dL	2.46±0.24	3.48±0.70	2.85±0.475	2.87±0.18	1.60±0.23*#	2.81±0.66

Values = mean ± SD; n = n = 5 (NC and PHD) and 7 (DC, DSG-LD, and DG). *Statistically ($p<0.05$) significant to DC, #statistically significant ($p<0.05$) to NC. **NC** = normal rats, **DC** = diabetic control, **DSG-LD** = diabetic rats + Raffia palm wine (150 g/kg bw), **DSG** = diabetic rats + Raffia palm wine (300 g/kg bw), **DSM** = diabetic rats + metformin (200 g/kg bw), and PHD = Normal rats + Raffia palm wine (300 g/kg bw).

Induction of T2D led to increase serum level of ALP, CK-MB, fructosamine and uric acid as depicted in **Table 3.4.3**. These levels were significantly ($p<0.05$) reduced in all the treatment groups. Induction of T2D had a little or no effect on serum levels of ALT and urea. However, the urea levels of the treatment groups were significantly reduced compared to the normal and diabetic control groups, respectively (**Table 3.4.3**). However, the administration of Raffia palm wine to normal rats (PHD) caused an increase in serum urea level.

Figure 3.4.4A reveals intact β-cells, acinar cells and intralobular ducts in the pancreatic tissue of the normal control (NC). The intact morphology was however altered on induction of T2D as depicted by the decreased number of acinar and normal β-cells, and increased number of inflamed β-cells (red coloured) (**Figure 3.4.4B**). Treatment with the Raffia palm wine led to the restoration of normal pancreatic tissue morphology (**Figures 3.4.4C and 3.4.4D**), with the low dose treated rats showing near normal intactness (**Figure 3.4.4C**). Treatment with metformin led to a slight increase in the number of β-cells but its morphology was altered (**Figure 3.4.4E**).

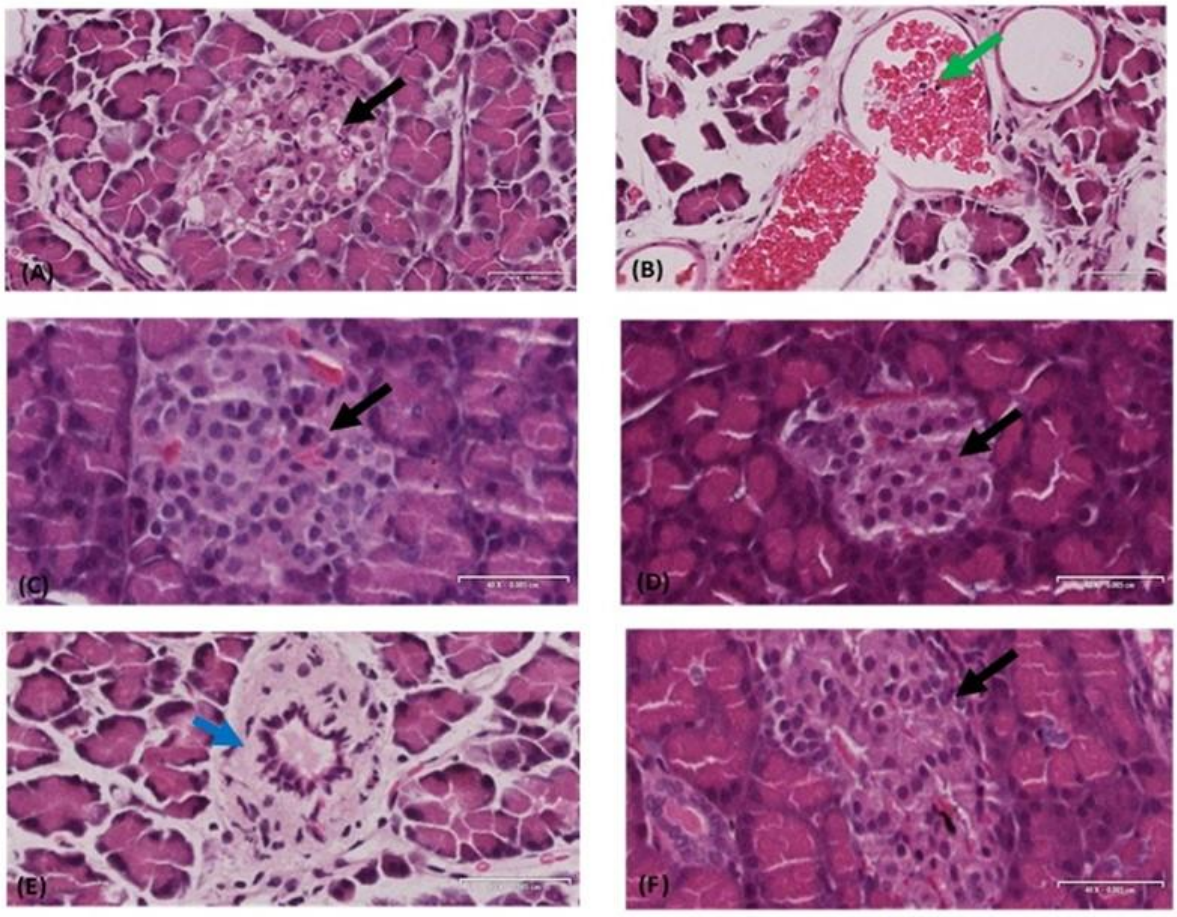


Figure 3.4.4: Morphological changes in pancreatic tissues of experimental groups. Magnification: 10x. (A) = NC, (B) = DC, (C) = DSG-LD, (D) = DSG, (E) = DSM, and (F) = PHD. **NC** = normal rats, **DC** = diabetic control, **DSG-LD** = diabetic rats + Raffia palm wine (150 g/kg bw), **DSG** = diabetic rats + Raffia palm wine (300 g/kg bw), **DSM** = diabetic rats + metformin (200 g/kg bw), and PHD = Normal rats + Raffia palm wine (300 g/kg bw). Black arrow: normal β-cells; Green arrow: inflamed β-cells; and Blue arrow: slightly altered β-cells morphology

SEM analysis of the pancreatic tissue revealed clusters of the exocrine cells, acini as shown in Fig. 5A. On the surface of the cell clusters were rich networks of capillaries. These networks were however altered in the diabetic tissue, making the acini cell clusters conspicuous as depicted in Fig. 5B. The acini cells of the diabetic tissue were also enlarged compared to the normal. Treatment with Raffia palm wine led to restoration of the capillaries network (**Figures 3.4.5C** and **3.4.5D**), with the high dose showing a reduced acini cell size (**Figure 3.4.5D**). The tissue from the metformin treated group (DSM) revealed a much-restored capillary network (**Figure 3.4.5E**).

While the pancreatic acini cells of normal rats administered Raffia palm wine were enlarged and conspicuous, with less capillary networks (**Figure 3.4.5F**).

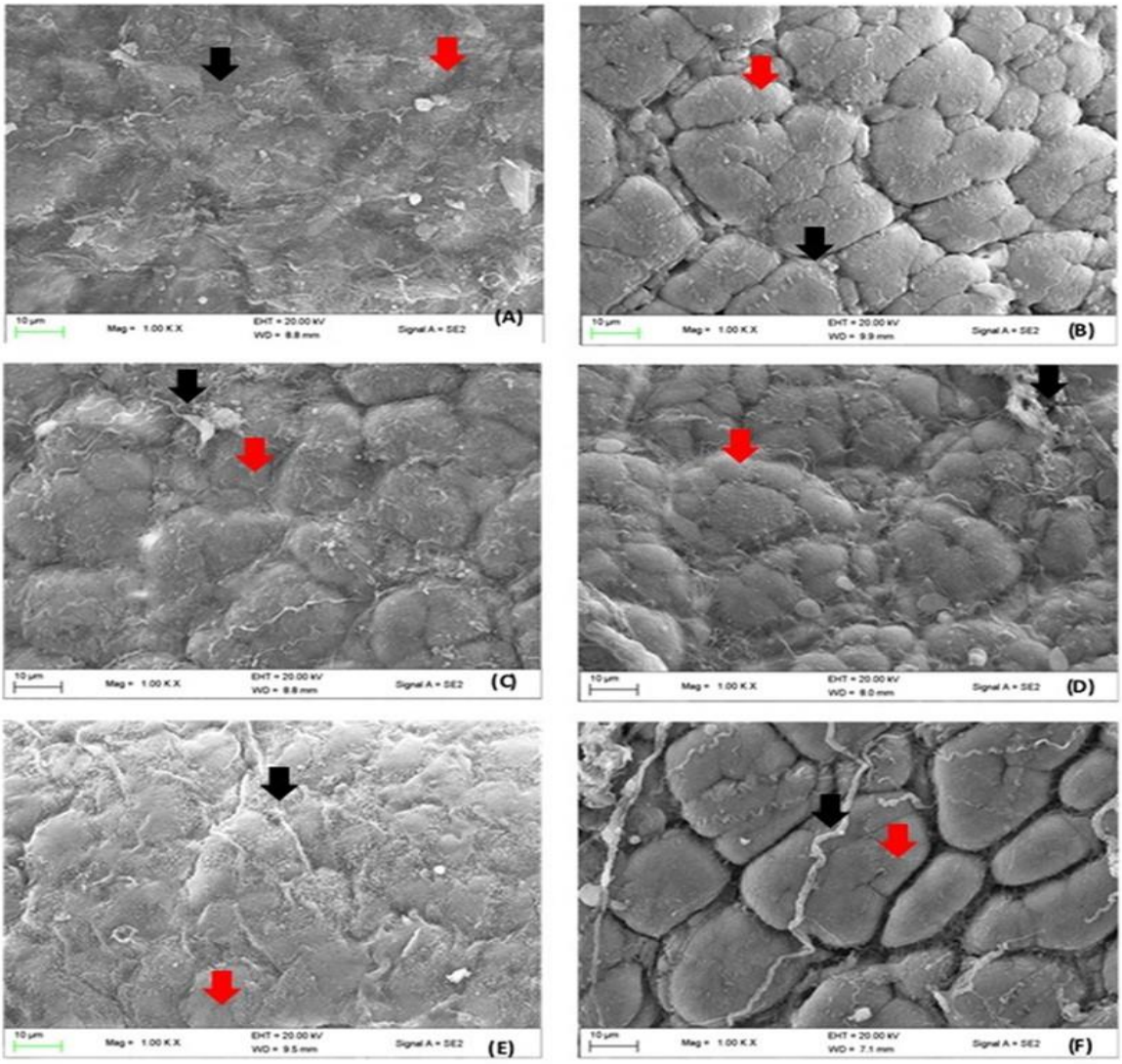
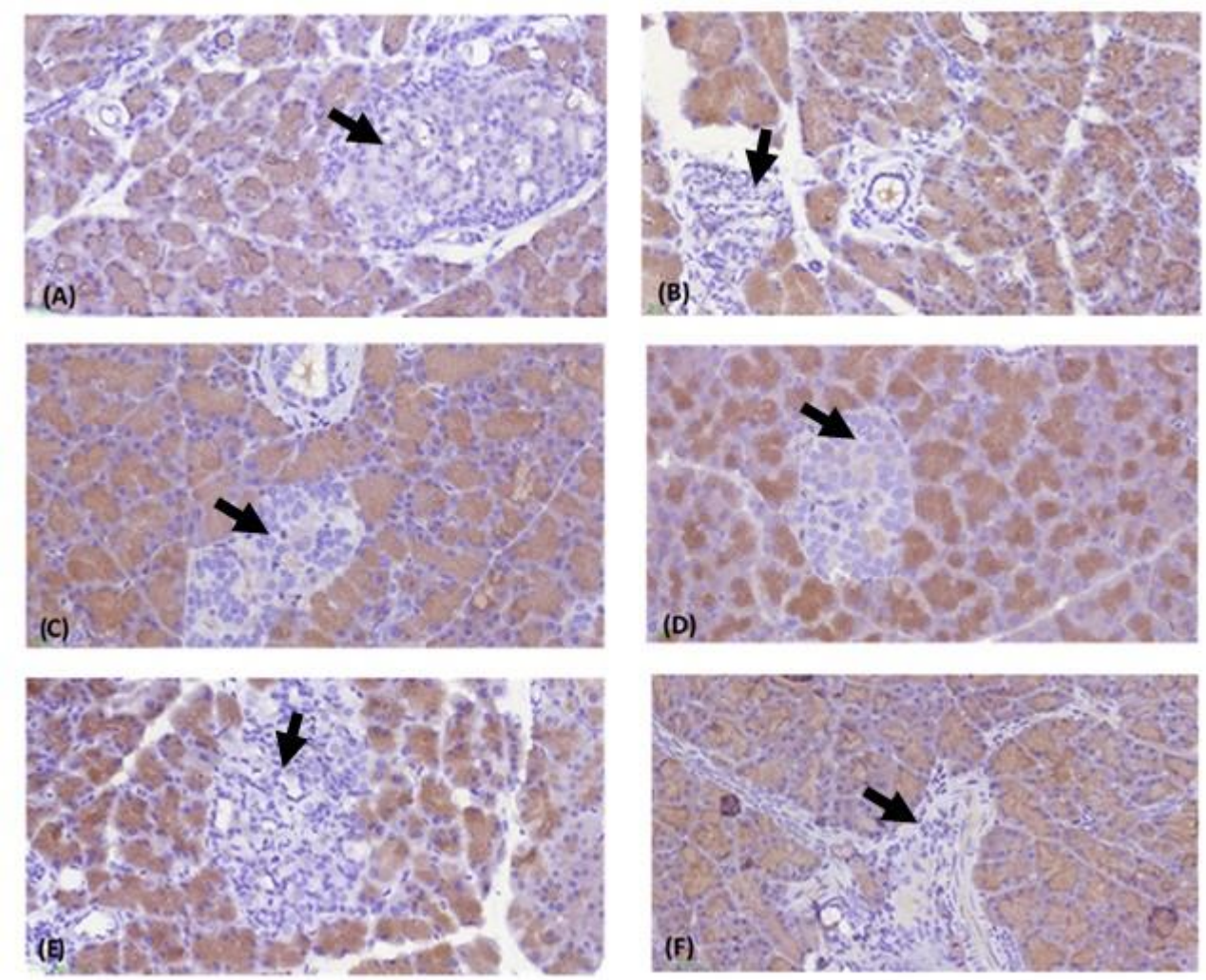


Figure 3.4.5: Electron microscopy of pancreatic tissues of experimental groups. Magnification = 1000x. (A) = NC, (B) = DC, (C) = DSG-LD, (D) = DSG, (E) = DSM, and (F) = PHD. **NC** = normal rats, **DC** = diabetic control, **DSG-LD** = diabetic rats + Raffia palm wine (150 g/kg bw), **DSG** = diabetic rats + Raffia palm wine (300 g/kg bw), **DSM** = diabetic rats + metformin (200 g/kg bw), and PHD = Normal rats + Raffia palm wine (300 g/kg bw). Arrow: Black = blood vessels; Red = acini

Induction of T2D led to upregulation of caspase-3 expression although not significant as shown in **Figures 3.4.6B and 3.4.6G**. The expression was rather slightly increased (not significant) in diabetic rats treated with Raffia palm wine (**Figures 3.4.6C, 3.46D** and **3.4.6G**).



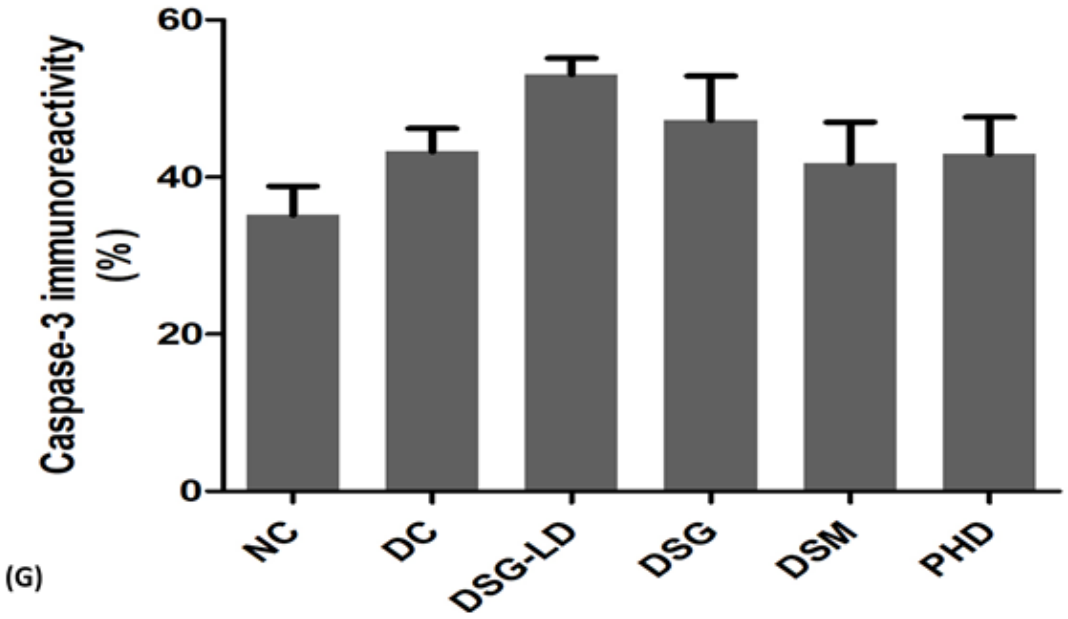


Figure 3.4.6: Immunohistochemistry photomicrographs of caspase3 expression in pancreatic tissues of experimental groups. Magnification = 200x; (A) = NC, (B) = DC, (C) = DSG-LD, (D) = DSG, (E) = DSM, and (F) = PHD. **NC** = normal rats, **DC** = diabetic control, **DSG-LD** = diabetic rats + Raffia palm wine (150 g/kg bw), **DSG** = diabetic rats + Raffia palm wine (300 g/kg bw), **DSM** = diabetic rats + metformin (200 g/kg bw), and **PHD** = Normal rats + Raffia palm wine (300 g/kg bw). Arrow: β-cells

There was also an upregulation of Nrf2 expression in the pancreatic tissues on induction of T2D as shown in **Figures 3.4.7B** and **3.4.7G**. This was however significantly ($p<0.05$) inhibited in diabetic rats treated with both doses of Raffia palm wine as shown in **Figures 3.4.7C, 3.4.7D** and **3.4.7G**. Administration of Raffia palm wine did not increase the expression in pancreatic tissues of normal rats (**Figure 3.4.7F** and **3.4.7G**).

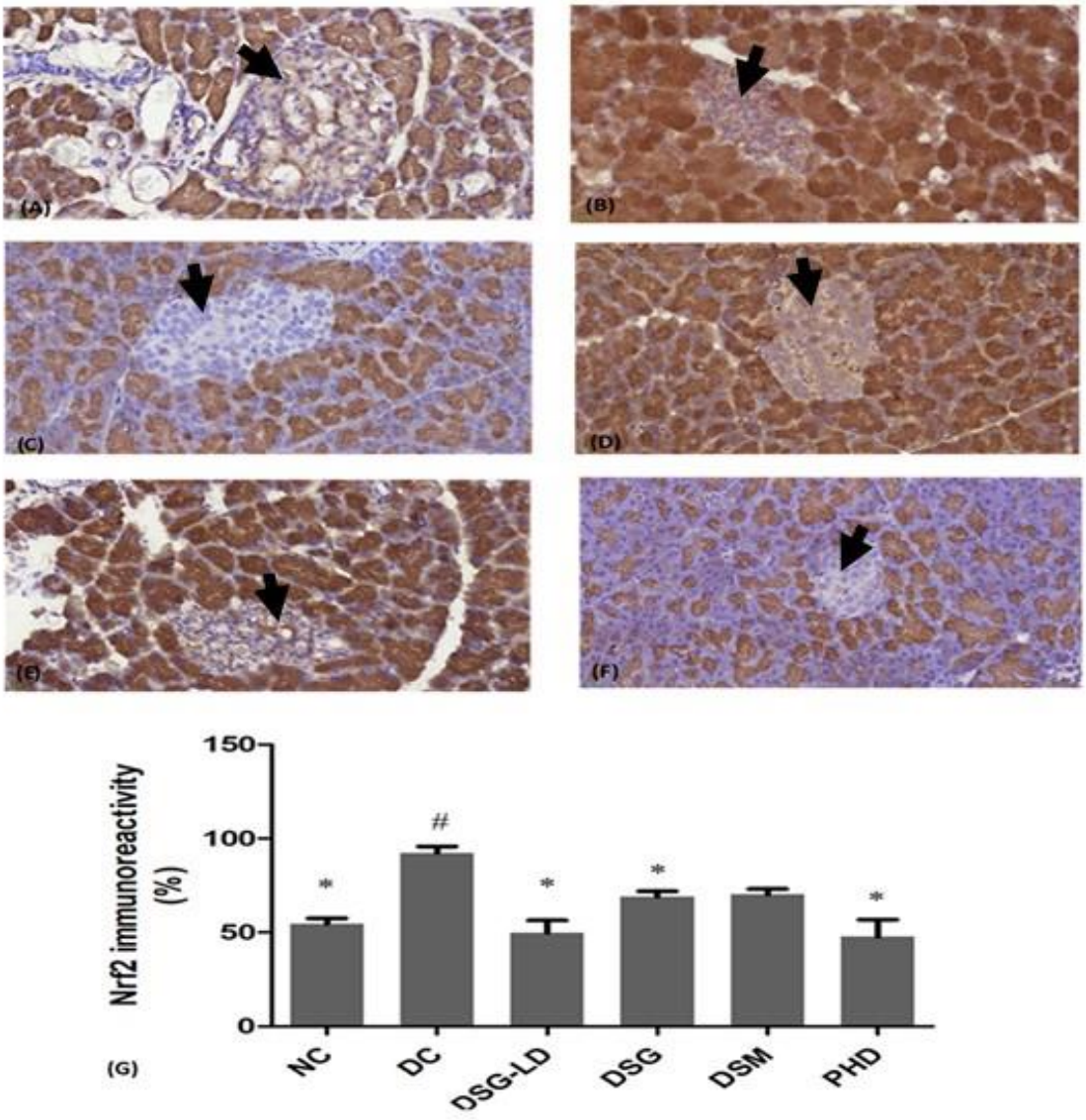


Figure 3.4.7: Immunohistochemistry photomicrographs of Nrf2 expression in pancreatic tissues of experimental groups. Magnification = 200x; (A) = NC, (B) = DC, (C) = DSG-LD, (D) = DSG, (E) = DSM, (F) = PHD, and (G) = Immunoreactivity of Nrf2 expression (Values = mean ± SD; n = 5 (NC and PHD) and 7 (DC, DSG-LD, and DG); *Statistically ($p<0.05$) significant to DC, #statistically significant ($p<0.05$) to NC). **NC** = normal rats, **DC** = diabetic control, **DSG-LD** = diabetic rats + Raffia palm wine (150 g/kg bw), **DSG** = diabetic rats + Raffia palm wine (300 g/kg bw), **DSM** = diabetic rats + metformin (200 g/kg bw), and **PHD** = Normal rats + Raffia palm wine (300 g/kg bw). Arrow: β-cells

GC-MS analysis of Raffia palm wine revealed the presence of sugars, phenolics, glycosides alkaloids, and fatty acids as shown in **Table 3.4.4**.

Table 3.4.4: GC-MS identified compounds in Raffia palm wine

Retention time	Compounds	A/H
6.59	Ethyl 1-thio-alpha-d-arabinofuranoside	2.15
6.69	Glycerin	6.73
7.96	Valeric acid, 3,5-dihydroxy-2,4-dimethyl-, .delta.-lactone	3.50
8.83	5-Keto-D-fructose	5.22
9.05	L-Erythro-ascorbate	3.4
9.33	Catechol	2.50
10.27	Nonadecanoic acid	1.78
10.77	beta.-d-Lyxofuranoside, O-nonyl-	4.77
11.29	1-Deoxy-d-arabitol	7.03
11.67	2-Hydroxyoctanoic acid	3.48
12.02	Biphenyl	1.82
12.81	Methyl 8-(5-octyl-1,2,4-trioxolan-3-yl)octanoate	3.24
12.87	Echinopsine	1.79
13.15	Cymarin	2.70
14.13	Dodecanoic aci	1.59
15.31	3-Deoxy-d-mannoic lactone	7.65
16.43	Tetradecanoic acid	1.79
17.35	d-Methyl-1-fucoside	3.90
17.49	Quinic acid	2.78
17.87	d-Mannitol, 1-O-hexyl-	5.89
18.32	Palmitoleic acid	2.53
18.54	l-(+)-Ascorbic acid 2,6-dihexadecanoate	1.74
18.90	Myo-inositol	0.83

20.45	Octadecanoic acid	1.77
25.67	Squalene	1.55

*A = peak area; H = peak height

3.4.5 Discussion

The cost of treating diabetes is of tremendous concern coupled with its rising epidemic. These can be described as a double-edged sword particularly for most developing countries who are struggling with other non-communicable diseases such as malaria and HIV/AIDS, with little or no proper health infrastructures. Hence, the search and development of novel and/or traditionally-recognized treatment alternatives particularly from plants. Africa is well known for the richness of her indigenous flora, which have vastly contributed to her traditional health system. Despite the endemicity of this flora and the traditional health system, some of these plants are yet to be scientifically authenticated for their acclaimed folkloric use. In this study, we investigated the ability of Raffia palm wine to reduce blood glucose level in T2D rats as well as the possible mechanism involved.

Studies have reported polyphagia, polydipsia, and body weight loss as major symptoms and signs of DM (Ibrahim et al. 2016; Okon et al. 2012), which is consistent with the weight loss and concomitant increased food and fluid intake after induction of T2D in the present study (**Figures 3.4.1A** and **3.4.1B**). The reduced food and fluid intake, and weight gain in the treatment groups, may indicate a therapeutic role of Raffia palm wine against T2D-induced polyphagia, polydipsia, and weight loss.

Chronic hyperglycemia has been recognized as the hallmark of T2D and has also been attributed to decreased pancreatic β-cell mass and number leading to β-cell dysfunction and insulin insufficiency (Donath and Halban 2004). This corresponds with the increased blood glucose level (**Figure 3.4.2A**), decreased pancreas weight (**Figure 3.4.1C**), reduced serum insulin level and pancreatic β-cell function (**Figure 3.4.2C**) in the diabetic control (DC). This also corroborates the depleted β-cells as revealed by histological analysis of the pancreas (**Figure 3.4.4B**), thus demonstrating an occurrence of T2D. The reduced blood glucose levels in the treatment groups, particularly the Raffia palm wine treated rats portrays an anti-hyperglycemia activity. The ability of both doses of Raffia palm wine to increase serum insulin level (**Figure 3.4.2C**), improve

pancreatic β-cell function (**Figure 3.4.2C**), as well as regeneration of pancreatic β-cells (**Figure 3.4.4B**) further demonstrates their therapeutic effect in the treatment and management of T2D. This is further evident by the improved glucose tolerance in the Raffia palm wine treated rats (DSG-LD and DSG) (**Figure 3.4.2B**). This corroborates previous report on the low glycemic index of Raffia palm wine (Oboh et al. 2011). This corresponds with reported antidiabetic mechanisms of several medicinal plants which bring about their activity by instigating pancreatic β-cell regeneration and/or insulinotropic action (Erukainure et al. 2015b; Ibrahim et al. 2016; Islam and Choi 2007).

Increased glycogenolysis, gluconeogenesis and glycolysis have been reported in T2D (Clore et al. 2000; Guo et al. 2012; Wu et al. 2005). This has been attributed to activation of key enzymes involved in these pathways. Increased glycogenolysis and gluconeogenesis in T2D leads to the continuous production of glucose from glycogen and non-carbohydrate, thereby exacerbating hyperglycemia. While increased glycolysis could lead to the generation of metabolites for the hexosamine, polyol, protein kinase C, and AGE pathways which have been linked to the pathogenesis of T2D (Luo et al. 2016; Rolo and Palmeira 2006). The elevated activities of glycogen phosphorylase and fructose 1,6 biphosphatase in the diabetic control (DC) group (**Table 3.4.1**), insinuates glycogenolytic and gluconeogenetic activities respectively, which correlates with the elevated blood glucose level (**Figure 3.4.2A**). While an elevated glycolysis in DC is portrayed by the increased activities of glucose 6 phosphatase. The increased activities of these enzymes can also be attributed to insufficient insulin secretion by the pancreatic β-cell as seen in DC (**Figure 3.4.2C**), as insulin has been reported for its glycogenic activity (Cersosimo et al. 2018; Gardner et al. 1993). The decreased activities of these enzymes in the treatment groups correlates with the reduced blood glucose levels (**Figure 3.4.2A**) and serum insulin level (**Figure 3.4.2C**). These also portrays an antidiabetic potential of Raffia palm wine.

The role of pancreatic α-amylase in elevated postprandial glucose level is well documented (Adisakwattana et al. 2011; Kim et al. 2005). It breaks down dietary carbohydrate to glucose, leading to increased postprandial blood glucose level. The increased α-amylase activities in the pancreas and serum of DC (**Table 3.4.2**), insinuate an elevated postprandial blood glucose level which may also contribute to the elevated blood glucose level (**Figure 3.4.2A**). This agrees with previous reports on elevated α-amylase activities in T2D (Aughsteen et al. 2005; Ewadh et al.

2014; Yadav et al. 2013). The reduced activities in diabetic rats treated with the Raffia palm wine portrays an inhibitory potential, which also corroborates the reduced blood glucose level (**Figure 3.4.2A**). This also demonstrates a therapeutic effect of Raffia palm wine against T2D, as some commercial antidiabetic drugs such as acarbose and miglitol bring about their activity by inhibiting carbohydrate enzymes (Chelladurai and Chinnachamy 2018; Rahimzadeh et al. 2014).

The role of pancreatic ATPase in insulin secretion has been reported (Costa et al. 2010; Owada et al. 1999). A decreased activity leading to depolarization of the pancreatic β – cell membrane, and influx of Ca^{2+} have been suggested as a major mechanism by which glucose stimulates insulin secretion (Owada et al. 1999). Thus, the increased pancreatic ATPase in DC (**Table 3.4.1**), reflects a decrease in insulin secretion which correlates with the reduced serum insulin level (**Figure 3.4.2C**). The decreased serum ATPase activity in DC is in correlation with previous reports on decreased activities in T2D (Kiziltunç et al. 1997; Zadhoush et al. 2015). The reversed activities of these enzymes in diabetic rats by Raffia palm wine, further insinuates its antidiabetic potential, which also correlates with other studies on the ability of medicinal plant to inhibit and stimulate pancreatic and serum ATPase activities respectively (Baxi et al. 2010; Erukainure et al. 2017b).

Alteration of lipid metabolism, characterized by elevated levels of triglyceride, LDL-cholesterol and low HDL-c level, with normal or elevated total cholesterol have been reported in T2D (Boden and Laakso 2004; Parhofer 2015; Sugden and Holness 2011). This is often described as diabetic dyslipidemia, and has been attributed to hyperglycemia and insulin resistance (Parhofer 2015; Vijayaraghavan 2010). Thus, the elevated levels of triglyceride, LDL-c and low HDL-c level, despite the normal level of total cholesterol in the untreated diabetic rats depicts diabetic dyslipidemia as shown in **Figure 3.4.3**. The reduced levels of triglyceride, LDL-c and elevated levels of HDL-c in the treatment groups, particularly DSG-LD and DSG insinuates an antidyslipidemic activity by Raffia palm wine. This correlates with previous studies on the use of medicinal plants in the treatment of dyslipidemia (Erukainure et al. 2018a; García-Carrasco et al. 2015; Puri et al. 2007).

The toxic effect of chronic hyperglycemia on pancreatic islets in the progression of DM have been linked to redox imbalance caused by elevated production of ROS (Acharya and Ghaskadbi 2010; Donath 2014). This redox imbalance can be attributed to the extremely low levels of the pancreatic endogenous antioxidant enzymes (Pi et al. 2010; Robertson and Harmon 2007), thus leading to

oxidative damage of the pancreatic β-cells. This is evidenced by the depleted GSH level, SOD and catalase activities in the serum and pancreatic tissues of the untreated diabetic rats (**Table 3.4.2**). The elevated MDA levels indicate an occurrence of lipid peroxidation which can be attributed to the reduced catalase activity. Reduced catalase activity has been implicated in excessive accumulation of hydrogen peroxide (H_2O_2) arising from SOD dismutation of superoxide anion ($O_2^{\bullet-}$), leading to generation of hydroxyl radical (•OH). The generated •OH binds with the membrane lipids, triggering peroxidative chain reactions. The accumulated H_2O_2 can also be converted to the potent radical, hypochlorous acid (HOCl) in a reaction catalyzed by myeloperoxidase in the presence of react with hydrochloric acid (HCl). High concentrations of NO can react with $O_2^{\bullet-}$ to produce peroxynitrite ($ONOO^-$), also a potent free radical. Elevated NO level and myeloperoxidase activity have also been recognized as proinflammatory markers (Bedoya et al. 2012; Erukainure et al. 2018b). Their elevation in the serum and pancreatic tissue (Table 2), therefore indicates an occurrence of proinflammation. This corroborates with the increased pancreatic acetylcholinesterase activity (**Table 3.4.1**), as its increased activity has been linked to inflammatory effects (Zhang et al. 2012). Elevation of these biomarkers in the untreated diabetic rats may be responsible for their β-cell dysfunction and reduced insulin secretion (**Figure 3.4.2C**) and inflamed pancreatic β-cells (**Figure 3.4.4B**). The ability of Raffia palm wine to elevate the GSH level, SOD and catalase activities, while reducing NO and MDA levels, and myeloperoxidase activity in diabetic rats, indicates its therapeutic potential against hyperglycemia-induced oxidative and proinflammatory injuries. These effects correlate with previous studies on the antioxidant and anti-inflammatory properties of antidiabetic medicinal plants (Erukainure et al. 2018a; Grzanna et al. 2005; Modak et al. 2007).

Multiple arteries derived from the abdominal aorta have been reported to perfuse the pancreas, and they play major roles in the maintenance of the internal milieu of the islets and normal endocrine physiology (Honka et al. 2014; Jansson et al. 2016). This perfusion is however altered in the onset and progression of T2D resulting from β-cell dysfunction (Honka et al. 2014). In this study, the decreased capillary networks on the pancreatic surfaces of the untreated diabetic rats (**Figure 3.4.5B**) may portray an altered perfusion, which may also be responsible for the significant β-cell dysfunction and reduced serum insulin level (**Figure 3.4.2C**). The increased network in the treatment groups (Fig. 5C – 5E), particularly in rats treated with Raffia palm wine (DSG-LD and

DSG) reflects an increased perfusion which corroborates with the elevated serum insulin levels and decreased significant β-cell dysfunctions (**Figure 3.4.2C**).

Apoptosis of the pancreatic β-cells have been recognized as a major contributor to the pathogenesis, pathophysiology and progression of T2D (Rojas et al. 2018; Tomita 2016). Often characterized by chromatic condensation, DNA fragmentation and cell shrinkage, β-cell apoptosis can be triggered by prolonged inflammation and oxidative stress arising from persistent hyperglycemia in T2D (Acharya and Ghaskadbi 2010; Kaneto et al. 1996; Tomita 2016). The caspase family have been recognized as a major orchestrator of the extrinsic apoptotic pathway, with caspase 3 being a major effector in β-cell apoptosis (Liadis et al. 2005; Tomita 2016). The elevated expression of caspase-3 in the pancreatic tissue of the untreated diabetic rats (**Figures 3.4.6B**) therefore indicates an occurrence of apoptosis. This may be attributed to the elevated levels of NO, MDA, and myeloperoxidase activities and concomitant depletion of GSH level, SOD and catalase activities in the pancreas (**Table 3.4.2**). Although these biomarkers were improved in the pancreatic tissues of the treatment groups, the Raffia palm wine showed a rather insignificant increased effect on caspase-3 expression (**Figures 3.4.6C** and 3**.4.6D**). Thus, implying that its antidiabetic mechanism may not involve the caspase-3 apoptotic pathway.

The increased expression of Nrf2 in pancreatic tissues of untreated diabetic rats (**Figure 3.4.7B**) correlates with reported exacerbated Nrf2 expression on the onset of T2D (He et al. 2012; Miao et al. 2012). Although increased Nrf2 has been reported to be beneficial in the recruitment of antioxidants and a therapeutic target in T2D (David et al. 2017; Dieter 2014), its activation of oxidative stress have however been reported (Wrighten et al. 2009; Zucker et al. 2014). Thus, implying that its increased expression in this study may be a major contributor to the pancreatic redox imbalance (**Table 3.4.2**). The reduced expressions in diabetic rats treated with Raffia palm wine (Fig. 7C, 7D and 7G), further portrays a therapeutic effect against T2D.

Fructosamine has been recognized as a glycemic biomarker for diabetes indication and control respectively (Malmström et al. 2014). It is a product of early stage glycated protein which is oxidatively cleaved to form advanced glycation end (AGE) products (Ibrahim et al. 2016; Sen et al. 2011). Its increased level in the untreated diabetic rats indicates a glycation cascade (**Table 3.4.1**). Thus, the reduced levels in the Raffia palm wine treated rats, suggests an interruption of the glycation cascade by the palm wine.

Although several studies have acknowledged the relative safety of natural products, there are however concerns on their toxic effects on tissues (Ezuruike and Prieto 2014). The elevated levels of ALP, CK-MB and uric acids in the untreated diabetic rats (**Table 3.4.4**), reflects hepato, cardio, and nephro – toxicities (Erukainure et al. 2018a; Mortazavi et al. 2016). Elevation of these biomarkers have been reported in T2D (Čaušević et al. 2010; Erukainure et al. 2017a; Fazel et al. 2005), and has been attributed to inflammation of the liver, heart, and kidney leading to their leakage to the serum (Giordano et al. 2015; Kim et al. 2008; Peppes et al. 2008). The depleted levels of these biomarkers in diabetic and normal rats treated with Raffia palm wine, do not only indicate a protective effect of Raffia palm wine against diabetic hepato, cardio and nephro - toxicities but also the safety of its consumption on healthy tissues.

These activities by Raffia palm wine in T2D can be attributed to the synergetic effect of the GC-MS identified compounds (**Table 3.4.4**). The antioxidative and proinflammatory activities can be attributed to the presence of catechol, quinic acid, echinopsine and cimarin, as they have been reported for their antioxidant, anti-inflammatory and antidiabetic properties (Halim et al. 2011; Sarian et al. 2017; Wojdyło et al. 2016). The ascorbic acid derivatives, l-Erythro-ascorbate and l-(+)-Ascorbic acid 2,6-dihexadecanoate derivatives have also been reported for their antioxidant activities (Okwu and Emenike 2006). Although its high concentration of sugars maybe a question of concern, the identified sugars are however non-caloric and have been reported for their *ex vivo* and *in vivo* antidiabetic activities (Bobiş et al. 2018; Chukwuma and Islam 2017b; Nichol et al. 2018).

3.4.6 Conclusion

These results suggest the therapeutic and protective effects of Raffia palm wine against T2D and its complications. This is evidenced by its ability to improve pancreatic β-cell function and stimulate insulin secretion with concomitant inhibition of glycolytic and carbohydrate-digesting enzymes, leading to reduced blood glucose level while attenuating oxidative pancreatic injury and proinflammatory, dyslipidemia and Nrf2 expression. Its reduction of serum, liver, cardiac, and kidney biomarkers suggests its safety on diabetic and healthy organs. These activities can be attributed to the synergetic effect of the GC-MS identified compounds.

3.5 Type 2 Diabetes Induced Oxidative Brain Injury involves Altered Cerebellar Neuronal Integrity and Elemental distribution, and Exacerbated Nrf2 Expression: Therapeutic Potential of Raffia Palm (*Raphia hookeri*) Wine

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Preface: This article investigated the antioxidative protective effect of Raffia palm wine on type 2 diabetes induced brain injury. Its antioxidative and anti-proinflammatory effects were investigated. Its inhibitory effect on cholinergic enzyme activity and Nrf2 expression were investigated. Its ability to protect against oxidative induced neuronal damages was also investigated as well as its ability to stimulate brain glucose uptake. The article presently under review for publication in Nutritional Neuroscience (NNS1452).

3.5.1 Abstract

Neurodegenerative diseases, such as Alzheimer's disease have been recognized as one of the microvascular complications of type 2 diabetes (T2D). In this study, the effect of T2D on neuronal integrity and elemental distribution in the cerebellar cortex, as well as the therapeutic effect of Raffia Palm *(Raphia hookeri)* wine (RPW) were investigated in male albino rats. T2D was induced in 4 groups of rats using the fructose and streptozotocin. One group served as negative control which was administered water, the second and third group were administered with 150 and 300 mg/kg bodyweight of RPW, while the fourth group was administered with metformin (200 mg/kg bodyweight). Two other groups of normal rats were administered distilled water (control) and of

RPW (300 mg/kg bodyweight). The rats were sacrificed after 5 weeks of treatment, and brains were collected. The cerebellum was removed, and several parts analyzed by immunochemistry, histology and scanning electron microscope (SEM) analyses. Other parts of the brain were analyzed for oxidative stress biomarkers and acetylcholinesterase activity. These analyses revealed oxidative damage with concomitant increased in acetylcholinesterase activity and upregulation of Nrf2 expression in the diabetic brain cerebellar cortexes. Histological and microscopic analysis also revealed altered distribution of neurons and axonal nodes with concomitant elevation of several heavy metals. Treatment with RPW significantly elevated glutathione (GSH) level, superoxide dismutase (SOD) and catalase activities, as well as depleted acetylcholinesterase and malondialdehyde (MDA) level, while concomitantly inhibiting Nrf2 expression. It also led to improved neuronal integrity and reduction in the levels of heavy metals. Taken together, these studies suggest RPW may afford a novel neuroprotective potential against diabetic neurodegeneration.

Keywords: EDX; Neurodegeneration; Nrf2; Raffia Palm Wine; and Type 2 diabetes

3.5.2 Introduction

Being a major global epidemic, diabetes mellitus (DM) is a metabolic disease characterized by increased blood glucose level due to the incapability of pancreatic β-cells to synthesize insulin as seen in type 1 diabetes (T1D) and/or insufficient utilization of synthesized insulin as seen in type 2 diabetes (T2D) (Unnikrishnan et al. 2017; van Crevel et al. 2017). T2D is the predominant of all DM types, and it is characterized by insulin resistance and β-cell dysfunction, leading to chronic hyperglycaemia. Chronic hyperglycemia, in turn, leads to excess production of free radicals, which can initiate oxidative stress when they surpass the tissue's endogenous antioxidant capacity (Erukainure et al. 2017b; Maritim et al. 2003b). Hyperglycaemia-induced oxidative stress has been associated with the pathogenesis and progression of micro- and macro- vascular complications in T2D.

Neurodegeneration has been recognized as one of the microvascular complications in T2D (Vagelatos and Eslick 2013; Whitlow et al. 2015; Wrighten et al. 2009), with oxidative stress playing a key role in mediating this effect (Kim et al. 2015; Uttara et al. 2009). The brain utilizes glucose as its predominant source of energy and it is transported by glucose transporters across the

blood-brain barrier (BBB) (McEwen and Reagan 2004; Reagan et al. 2008; Wrighten et al. 2009). However, this homeostasis is disrupted in T2D due to impaired signaling and downregulation of glucose transporters (GLUTs) (Gejl et al. 2017; Hwang et al. 2017; Pardridge et al. 1990; Wrighten et al. 2009), leading to neuronal loss, structural alterations, and metal accumulation (Folarin et al. 2017; Kim et al. 2015; Wrighten et al. 2009).

The role of nuclear factor erythroid 2–related factor 2 (Nrf2) have been correlated with redox homeostasis as it mediates the activation and recruitment of endogenous antioxidants in response to oxidative stress (Dieter 2014; Ma 2013). Its beneficiary role has been reported in cerebellar function and neurodegenerative diseases such as Alzheimer and Parkinson (Ma 2013; Sandberg et al. 2014), cardiovascular diseases (Barančík et al. 2016), and hepatoxicity (Ma 2013). Its role in the activation of oxidative stress has been reported (Wrighten et al. 2009; Zucker et al. 2014). The activation of Nrf2 on the onset of T2D with concomitant generation of ROS, leading to downstream of antioxidants has also been reported (He et al. 2012; Miao et al. 2012).

This study aims to investigate the effect of Raffia palm wine on the redox status, ATPase and acetylcholinesterase activities in whole brain, as well as the morphology, neuronal distribution, elemental distribution, and Nrf2 expression in the cerebellar cortex of T2D rats. Brain glucose uptake have been reported to be altered in T2D due to downregulation of GLUTs (Gejl et al. 2017; Hwang et al. 2017; Pardridge et al. 1990), thus the ability of Raffia palm wine to stimulate glucose uptake in brain tissues was also investigated *ex vivo*.

3.5.2 Materials and Methods

Kindly refer to Chapter 2, subsections 2.1.1; 2.1.3; 2.10.5; 2.12 – 2.13 for details materials and methods.

3.5.3 Results

As shown in **Figures 3.5.1A – 3.5.1D**, induction of T2D led to a significant ($p<0.05$) decrease in GSH levels, SOD and catalase activities, with concomitant increase in MDA levels. These were reversed on treatment with Raffia palm wine and metformin to levels indistinguishable from controls. Administration of Raffia palm wine to normal rats had no significant effect on GSH levels and SOD activities but reduced the catalase activity compared to the control.

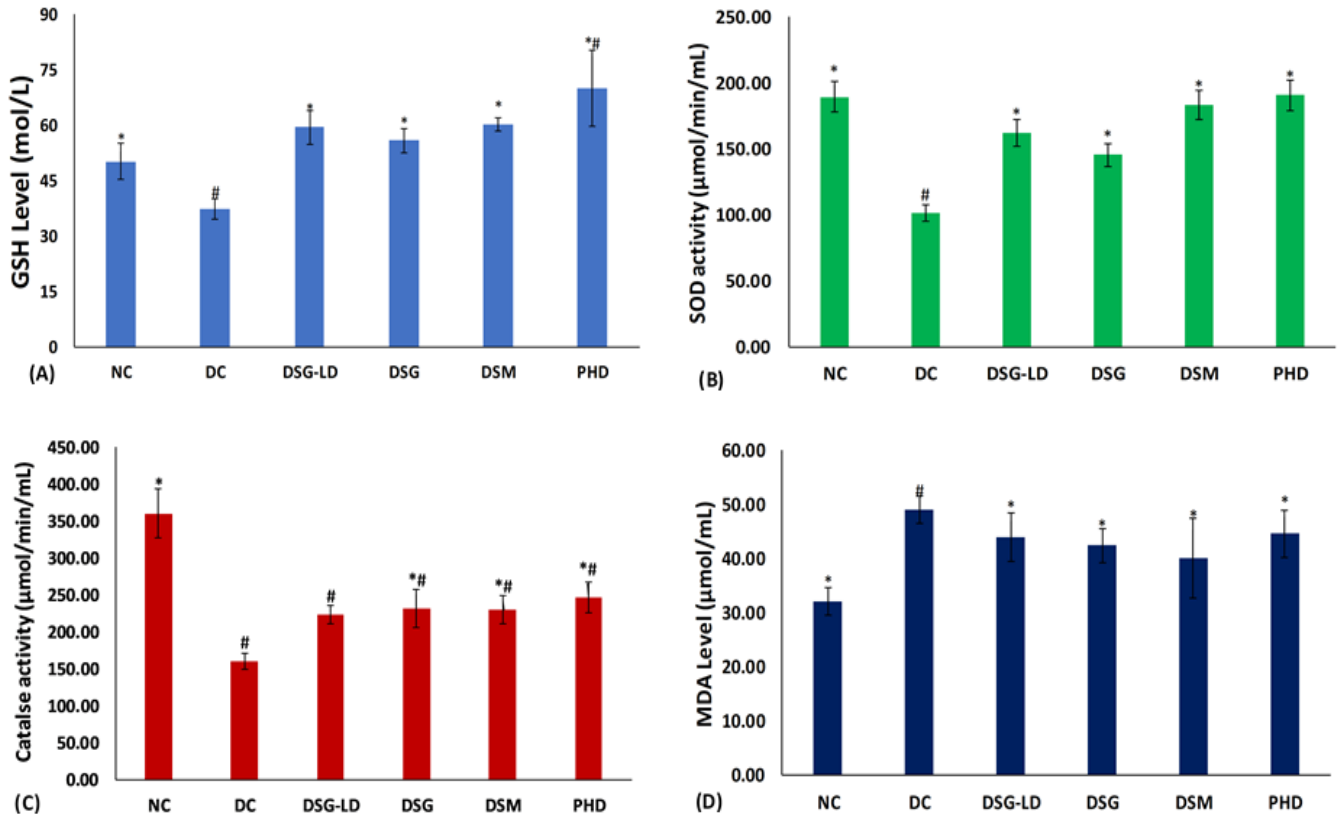


Figure 3.5.1: Antioxidant status of experimental groups. Values = mean ± SD; n = 5 (NC and PHD) and 7 (DC, DSG-LD, DG, and DSM). *Statistically ($p<0.05$) significant from DC, #statistically significant ($p<0.05$) from NC. **NC** = normal rats, **DC** = diabetic control, **DSG-LD** = diabetic rats + Raffia palm wine (150 g/kg bw), **DSG** = diabetic rats + Raffia palm wine (300 g/kg bw), **DSM** = diabetic rats + metformin (200 g/kg bw), and **PHD** = Normal rats + Raffia palm wine (300 g/kg bw).

Induction of T2D led to a significant ($p<0.05$) increase in myeloperoxidase activity as shown in **Figure 3.5.2**, indicating a proinflammatory response. This was significantly reduced in the Raffia palm wine treated groups, with the low dose showing a better activity, and comparable with metformin. Raffia palm wine had no significant effect on the activity in normal rats compared to the control.

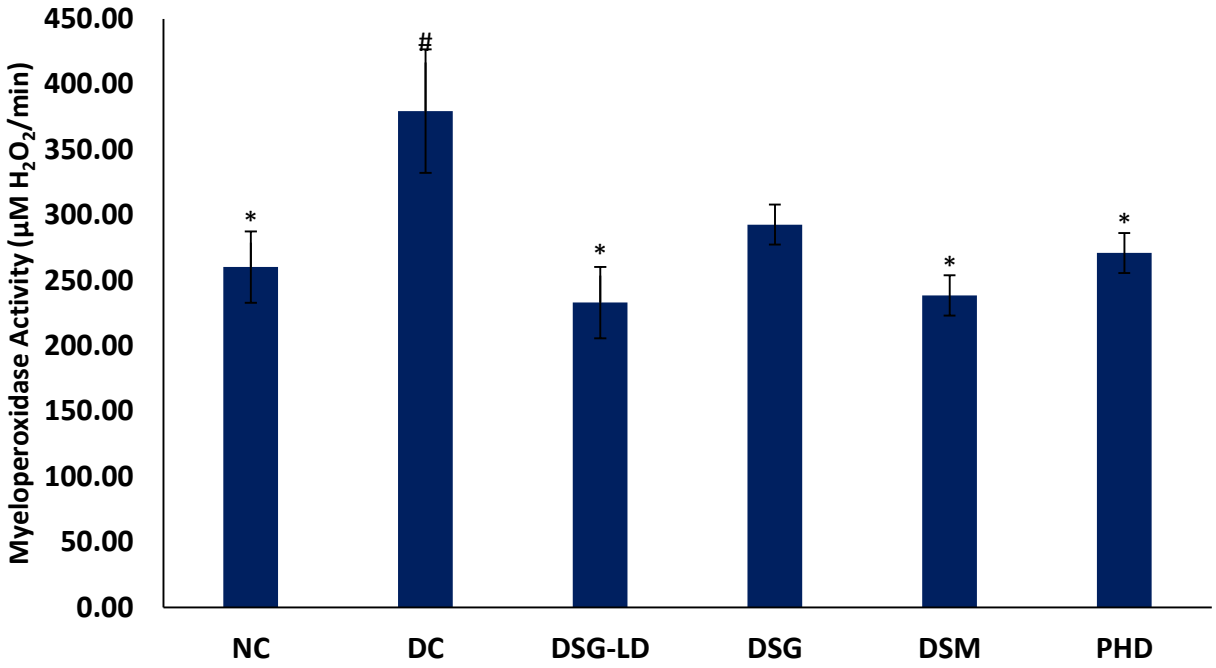


Figure 3.5.2: Myeloperoxidase activities of experimental groups. Values = mean ± SD; n = 5 (NC and PHD) and 7 (DC, DSG-LD, DG, and DSM). *Statistically ($p<0.05$) significant from DC, #statistically significant ($p<0.05$) from NC. **NC** = normal rats, **DC** = diabetic control, **DSG-LD** = diabetic rats + Raffia palm wine (150 g/kg bw), **DSG** = diabetic rats + Raffia palm wine (300 g/kg bw), **DSM** = diabetic rats + metformin (200 g/kg bw), and **PHD** = Normal rats + Raffia palm wine (300 g/kg bw).

As shown in **Figure 3.5.3**, ATPase activity in the diabetic group was significantly ($p<0.05$) higher compared to the control. This effect was attenuated in response to treatment with Raffia Palm wine, with the high dose showing a significant ($p<0.05$) reduction reaching levels indistinguishable from controls. Metformin had no significant effect on the activity. The activity significantly increased in normal rats administered Raffia palm wine compared to controls.

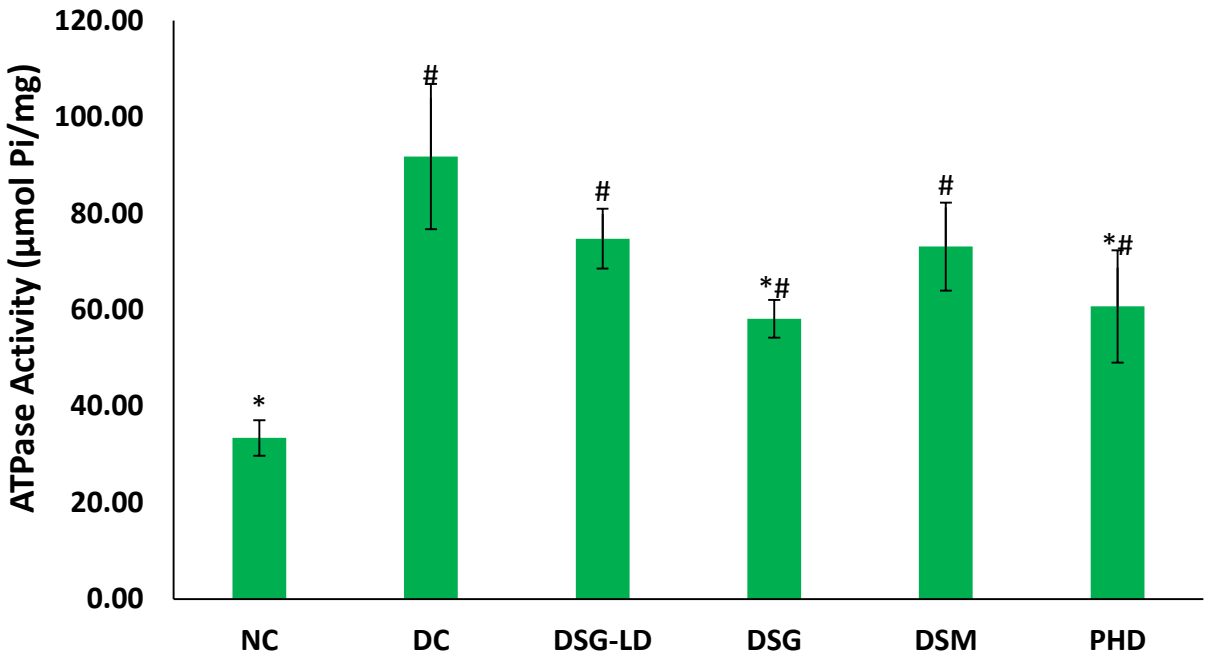


Figure 3.5.3: ATPase activities of experimental groups. Values = mean ± SD; n = 5 (NC and PHD) and 7 (DC, DSG-LD, DG, and DSM). *Statistically ($p<0.05$) significant from DC, #Statistically significant ($p<0.05$) from NC. **NC** = normal rats, **DC** = diabetic control, **DSG-LD** = diabetic rats + Raffia palm wine (150 g/kg bw), **DSG** = diabetic rats + Raffia palm wine (300 g/kg bw), **DSM** = diabetic rats + metformin (200 g/kg bw), and **PHD** = Normal rats + Raffia palm wine (300 g/kg bw).

Induction of T2D led to significant ($p<0.05$) increase in acetylcholinesterase activity as depicted in **Figure 3.5.4**. This effect was significantly reduced in rats treated with low dose of Raffia palm wine. The metformin treated group, DSM also showed a significant reduction in acetylcholinesterase activity. Acetylcholinesterase activity in normal rats administered Raffia palm wine was indistinguishable from the controls.

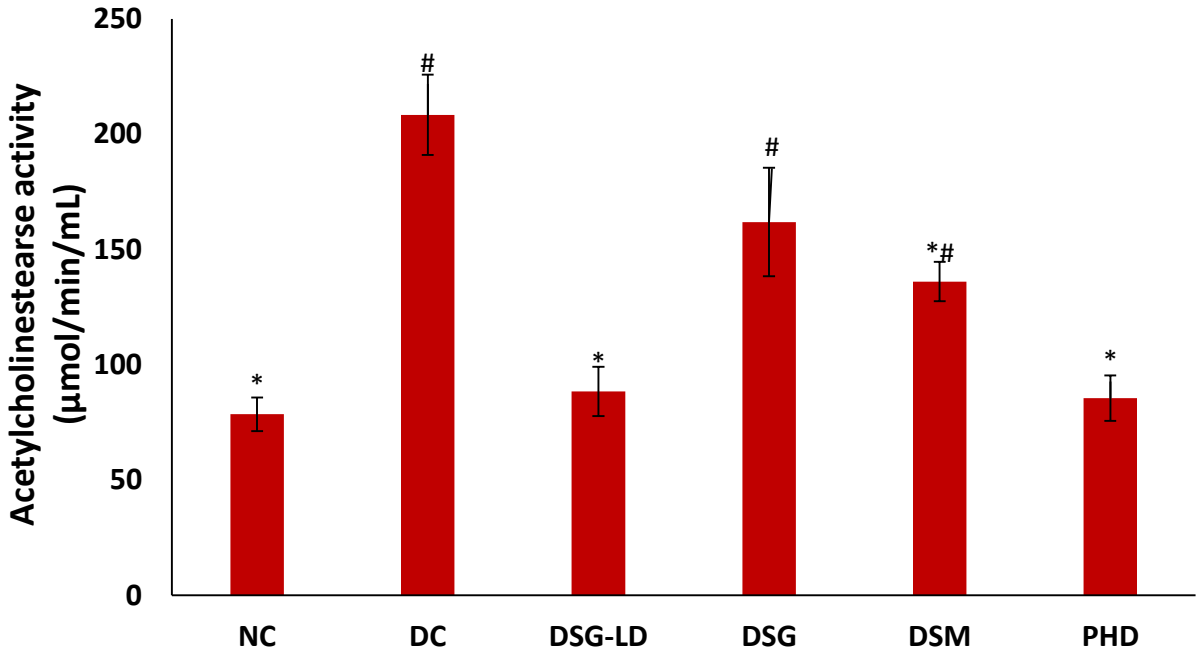


Figure 3.5.4: Acetylcholinesterase activities of experimental groups. Values = mean ± SD; n = 5 (NC and PHD) and 7 (DC, DSG-LD, DG, and DSM). *Statistically ($p<0.05$) significant from DC, #statistically significant ($p<0.05$) from NC. **NC** = normal rats, **DC** = diabetic control, **DSG-LD** = diabetic rats + Raffia palm wine (150 g/kg bw), **DSG** = diabetic rats + Raffia palm wine (300 g/kg bw), **DSM** = diabetic rats + metformin (200 g/kg bw), and **PHD** = Normal rats + Raffia palm wine (300 g/kg bw).

Histological analysis of the cerebellar cortex revealed intact Purkinje and granular cell layers as shown in **Figure 3.5.5A**. The intactness of these layers was distorted upon induction of T2D (**Figure 3.5.5B**), with evidence for compromised neuronal integrity. These layers appeared normal in rats treated with low dose of Raffia palm wine and metformin (**Figures 3.5.5C** and **3.5.5E**), indicating a protective activity and maintenance of optimal neuronal integrity.

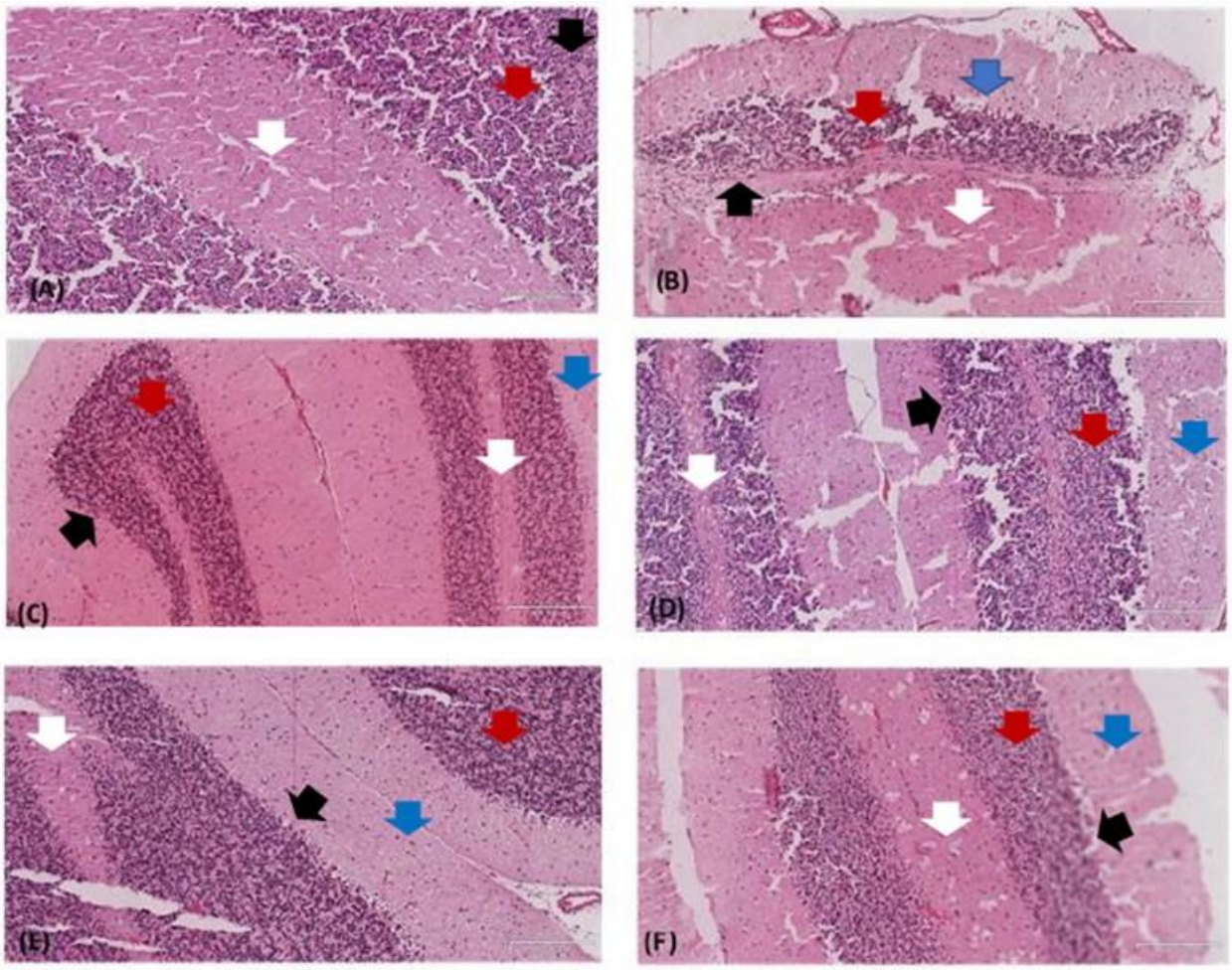
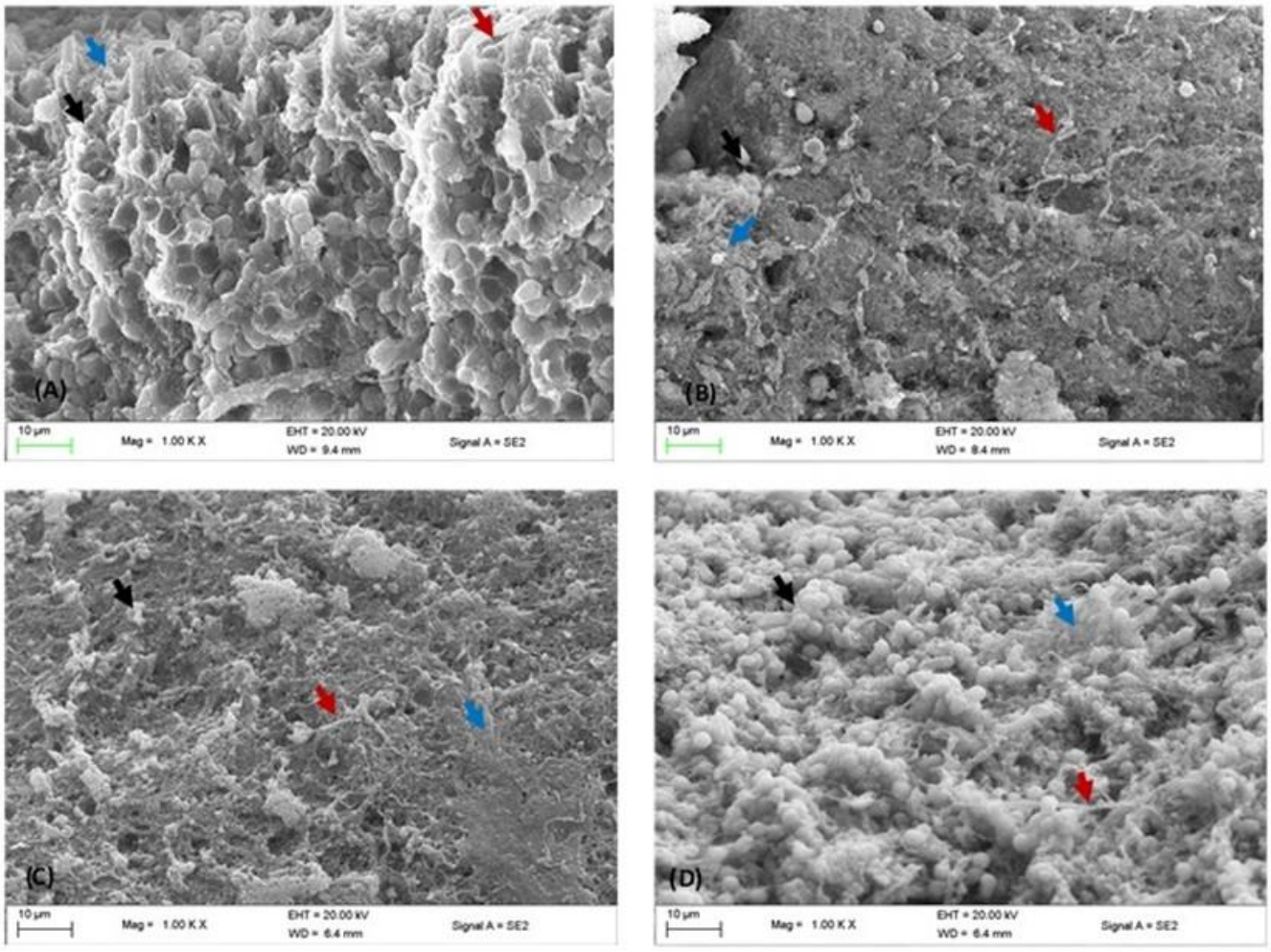


Figure 3.5.5: Morphological changes in cerebellar tissues of experimental groups. Magnification: 10x. (A) = NC, (B) = DC, (C) = DSG-LD, (D) = DSG, (E) = DSM, and (F) = PHD. **NC** = normal rats, **DC** = diabetic control, **DSG-LD** = diabetic rats + Raffia palm wine (150 g/kg bw), **DSG** = diabetic rats + Raffia palm wine (300 g/kg bw), **DSM** = diabetic rats + metformin (200 g/kg bw), and **PHD** = Normal rats + Raffia palm wine (300 g/kg bw). Arrows: Black = Purkinje cell layer; Red = Granule cell layer; White = white matter; Blue = Molecular layer

SEM analysis of the cerebellar cortex revealed intact Purkinje and glial cells, with a vast network of dendrites and axons (**Figure 3.5.6A**). This intactness was also distorted in the untreated diabetic group (**Figure 3.5.6B**) as evidenced by a depleted number of Purkinje and glial cells, and altered network of dendrites and axons. The dendrites and axon networks were more conspicuous in the low dose Raffia Palm wine treated group (**Figure 3.5.6C**), while the Purkinje and glial cells, and nerve networks were more intact in the high dose treated group (**Figure 3.5.6D**). These cells and

networks were altered in metformin treated rats (**Figure 3.5.6E**). Administration of Raffia palm wine to normal rats had no discernible effect on the Purkinje and glial cells as well as dendrites and axons networks (**Figure 3.5.6F**).



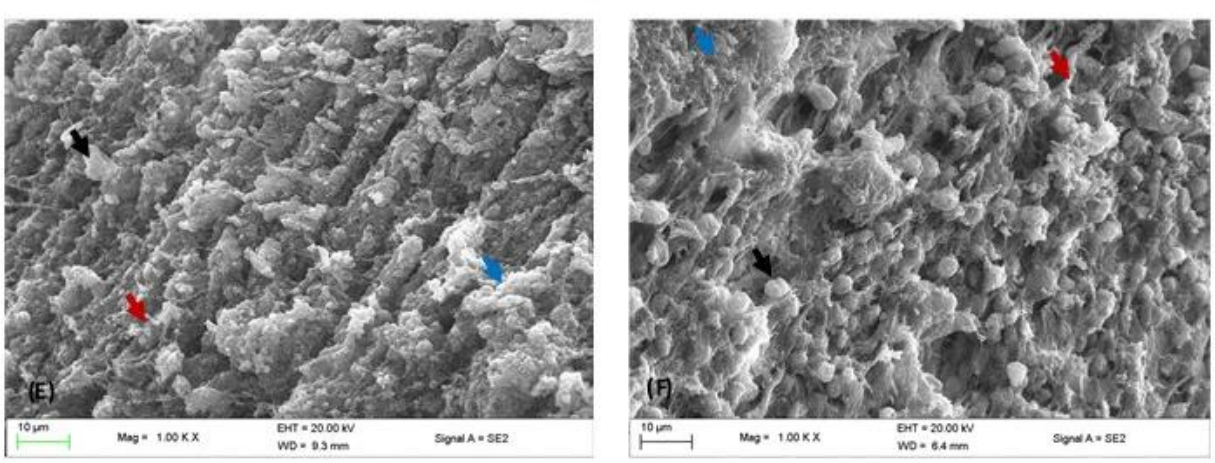
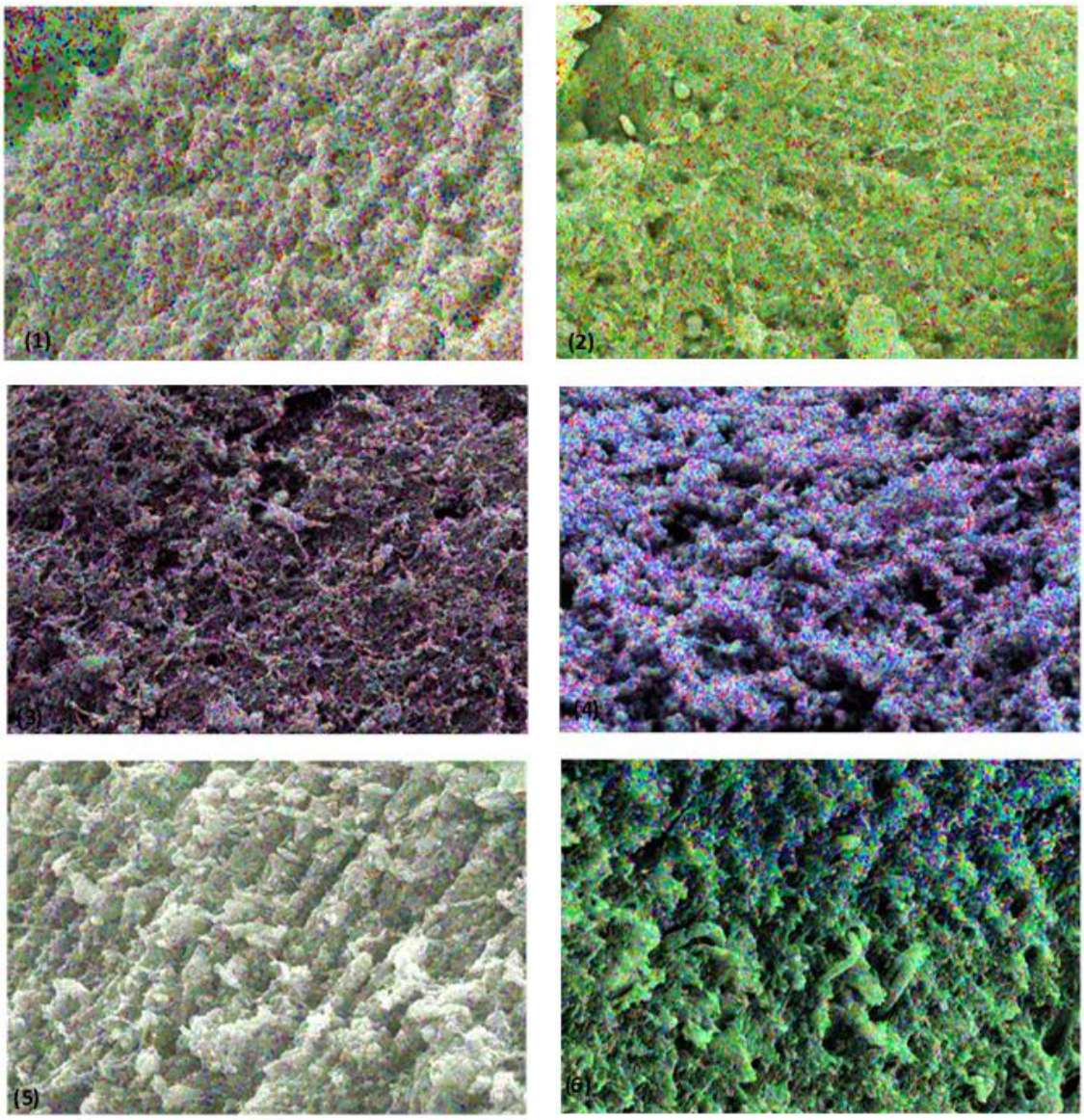


Figure 3.5.6: SEM analysis of cerebellar tissues of experimental groups. Magnification = 1000x. (A) = NC, (B) = DC, (C) = DSG-LD, (D) = DSG, (E) = DSM, and (F) = PHD. **NC** = normal rats, **DC** = diabetic control, **DSG-LD** = diabetic rats + Raffia palm wine (150 g/kg bw), **DSG** = diabetic rats + Raffia palm wine (300 g/kg bw), **DSM** = diabetic rats + metformin (200 g/kg bw), and **PHD** = Normal rats + Raffia palm wine (300 g/kg bw). Arrows: Black = Purkinje cells; Blue = dendrite network; Red = axon

Figure 3.5.7A (1 – 6) reveals the EDX mapping of the cerebellar cortex, showing elemental distribution in the cortex. Quantitative analysis revealed depleted levels of carbon, nitrogen and iron, with concomitantly elevated levels of oxygen, magnesium, aluminum and calcium **Figure 3.5.7B**). Induction of T2D had little or no effect on the cerebellar potassium content. These were reversed in the Raffia palm wine treated rats, with the low dose showing elevated levels of carbon and iron, and depleted levels of potassium. While the high dose showed higher levels of nitrogen and potassium compared to the control. Magnesium, calcium and iron were largely depleted in the cerebellar cortex of diabetic rats treated with high dose of Raffia palm wine. The cerebellar aluminum contents were highly depleted in diabetic rats treated with both doses of Raffia palm wine.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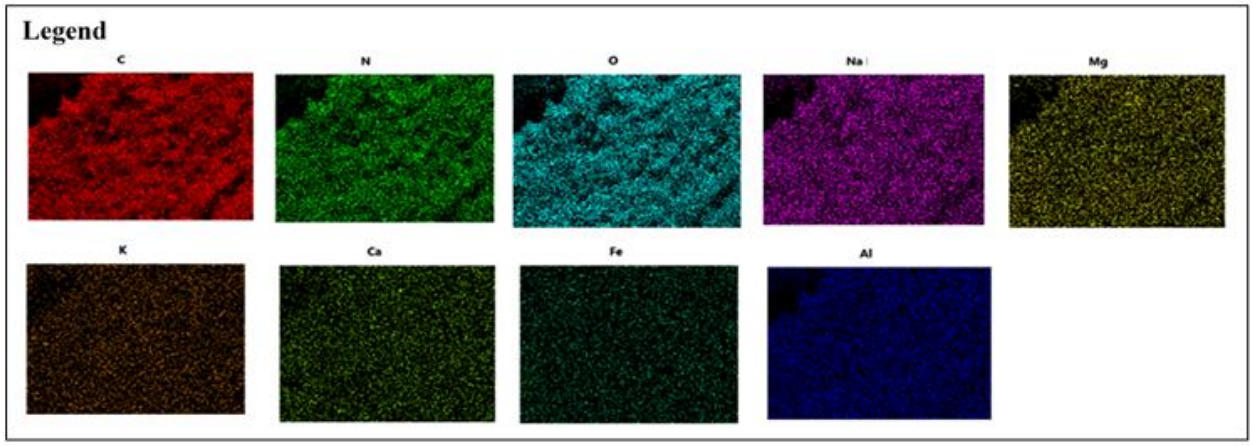


Figure 3.5.7A: EDX mapping of cerebellar tissues of experimental groups. Magnification = 1000x. (1) = NC, (2) = DC, (3) = DSG-LD, (4) = DSG, (5) = DSM, and (6) = PHD. Legends: C = carbon, N = nitrogen, O = oxygen, Na = sodium, Mg = magnesium, K = potassium, Ca = calcium, Fe = iron, and Al = aluminum

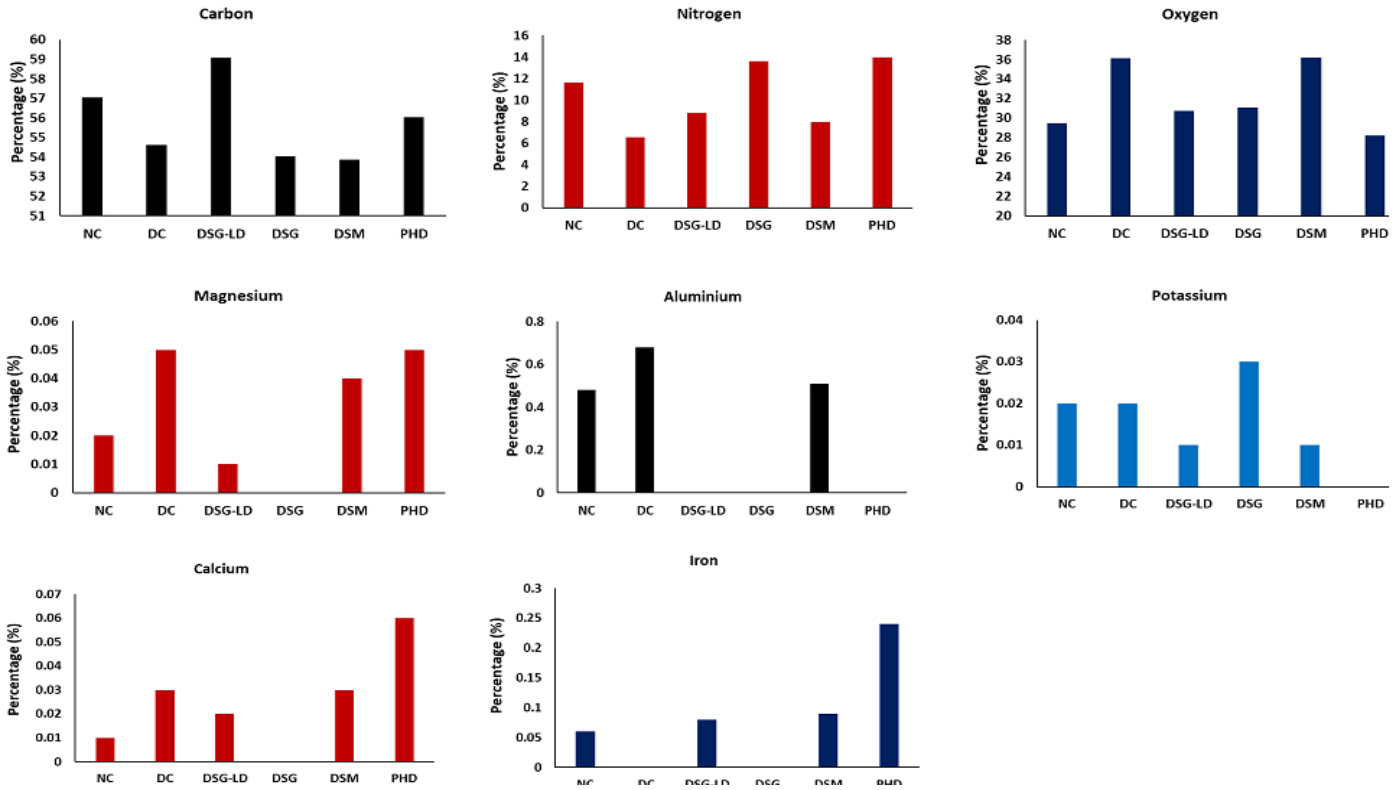
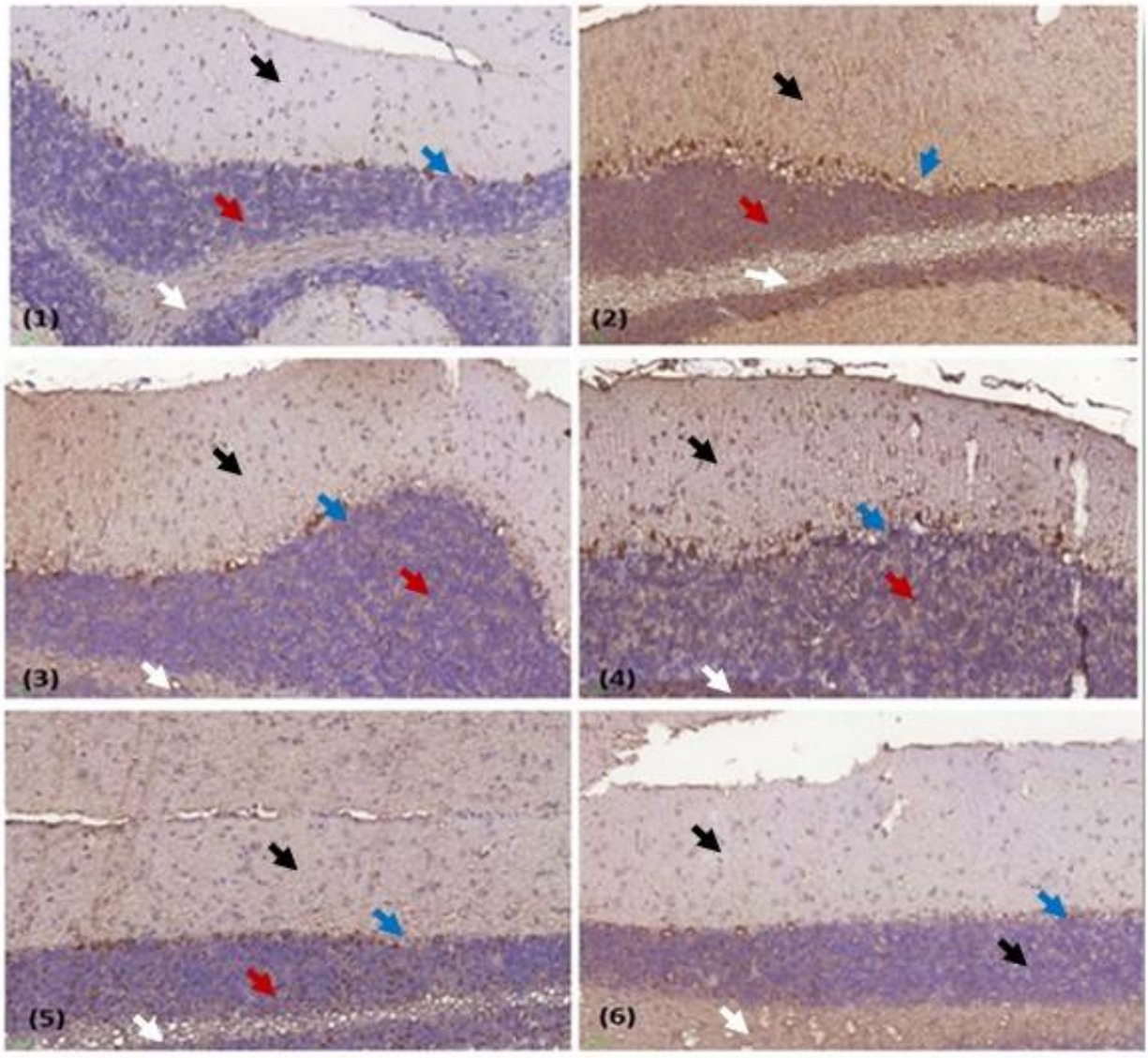


Figure 3.5.7B: Quantitative analysis of EDX scans

Induction of T2D led to significantly ($p < 0.05$) increased expression of Nrf2 in the cerebellar cortex, particularly in the Purkinje and granular cell layers compared to the normal controls, (NC). Treatment with Raffia palm wine attenuated the increase in Nrf2 expression in T2D rats (**Figure 3.5.8**).



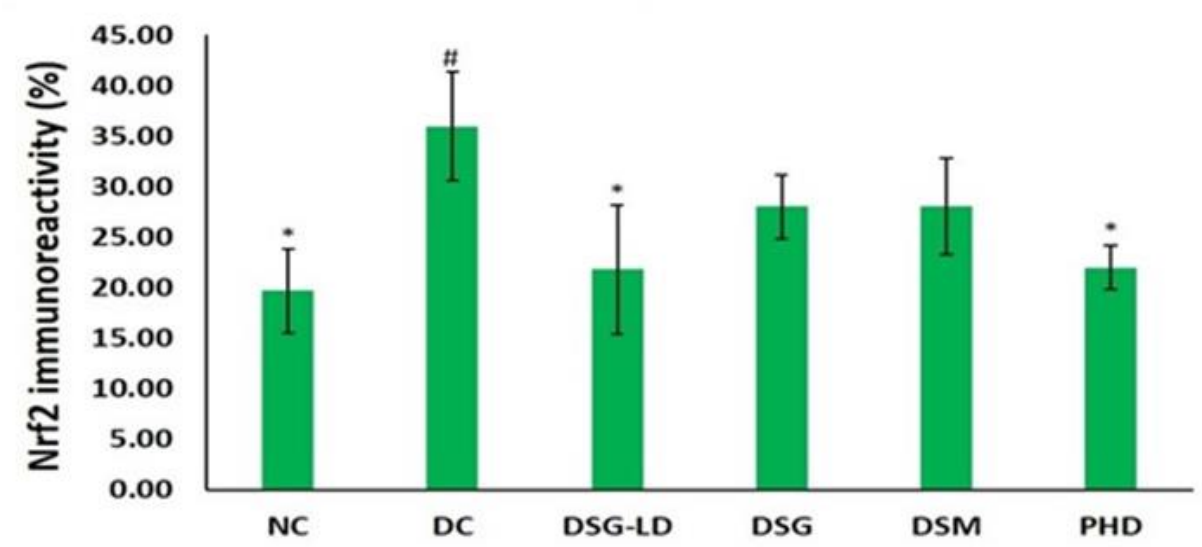


Figure 3.5.8: (A) Immunohistochemistry photomicrographs of Nrf2 expression in cerebellar cortex of experimental groups. Magnification = 200x; (1) = NC, (2) = DC, (3) = DSG-LD, (4) = DSG, (5) = DSM, and (6) = PHD. B; Nrf2 immunoreactivity in cerebellar cortex of experimental groups. *Statistically ($p<0.05$) significant from DC, #statistically significant ($p<0.05$) from NC. **NC** = normal rats, **DC** = diabetic control, **DSG-LD** = diabetic rats + Raffia palm wine (150 g/kg bw), **DSG** = diabetic rats + Raffia palm wine (300 g/kg bw), **DSM** = diabetic rats + metformin (200 g/kg bw), and **PHD** = Normal rats + Raffia palm wine (300 g/kg bw). Arrows: Black = Molecular layer; Red = Granule cell layer; White = White matter; Blue = Purkinje cell layer

Incubation of rat brain with Raffia palm wine led to decrease glucose level of the incubating buffer, suggesting increased glucose uptake, as depicted in **Figure 3.5.9**. This enhanced glucose uptake activity increased in a concentration-dependent manner. Incubation with metformin did not alter glucose uptake vs. control.

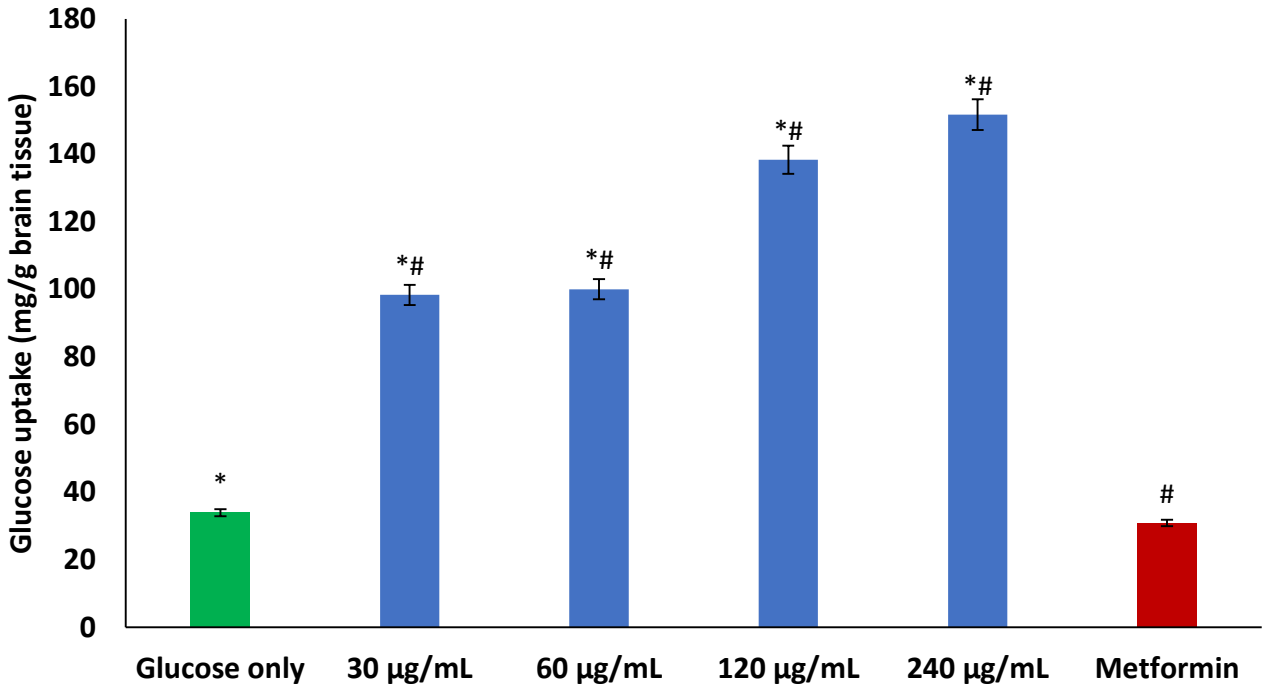


Figure 3.5.9: Effect of Raffia palm wine on brain glucose uptake ex vivo. Values = mean ± SD. *Statistically ($p<0.05$) significant from metformin, #Statistically significant ($p<0.05$) from glucose only.

3.5.4 Discussion

The brain dependence on glucose as its primary source of energy is well established, with neurons being the highest consumers owing to their high energy demand (Howarth et al. 2012; Mergenthaler et al. 2013). Hence, the continuous need for a constant supply of glucose from the blood across the BBB to the brain via glucose transporters. This supply has been reported to be altered in T2D (Vagelatos and Eslick 2013; Whitlow et al. 2015), and has been attributed to downregulation of glucose transporters at the BBB (Gejl et al. 2017; Hwang et al. 2017). Diminished brain supply of glucose might, in turn, lead to neurodegenerative diseases characterized by oxidative stress, defective physiology, neuronal loss and dysfunction. In this study, the therapeutic effect of Raffia palm wine on T2D-induced oxidative brain injury as well as its ability to promote brain glucose uptake *ex vivo* were investigated.

The high consumption of O_2 and concomitant dependence on glucose by the brain has been attributed for its high susceptibility to oxidative stress, which has been recognized as a major mechanism of T2D-induced neurodegeneration (Das et al. 2009; Patel 2016). This susceptibility can also be attributed to its low endogenous antioxidative system, high polyunsaturated fatty acid content, as well as the excitotoxic and auto-oxidizable activities of neurotransmitters (Patel 2016). The depleted GSH level, SOD and catalase activities upon induction of T2D (**Figure 3.5.1A** – **3.5.1C**) portray a distorted pro-oxidant/antioxidant equilibrium, reflected by increased ROS production. This corroborates earlier reports on the increased activities of these biomarkers in brain tissues of STZ-induced diabetic rats (Mastrocola et al. 2005; Moreira et al. 2003). ROS, such as $O_2^{\cdot-}$ and $^{\cdot}OH$ are toxic to neuronal cells (Uttara et al. 2009). SOD catalyzes the dismutation of $O_2^{\cdot-}$ to H_2O_2 and O_2. Catalase further catalyzes the breakdown of H_2O_2 to H_2O and O_2 (Erukainure et al. 2017b). In the absence of its breakdown by catalase, H_2O_2 generates hydroxyl ions ($^{\bullet}OH$). Both H_2O_2 and $^{\bullet}OH$ which can initiate peroxidation of polyunsaturated fatty acids. The elevated MDA level in brain tissues of the diabetic control (DC) rats (**Figure 3.5.1D**) indicate the occurrence of lipid peroxidation, which correlates with the depleted GSH levels, SOD and catalase activities. H_2O_2 can also be converted to hypochlorite (HOCl) in a reaction catalyzed myeloperoxidase in the presence of hydrochloric acid (HCl) (Patel 2016). HOCl is known to have proinflammatory activities (Erukainure et al. 2018b). The increased myeloperoxidase activity in the untreated diabetic group (DC) (**Figure 3.5.2**), may thus reflect a proinflammatory activity. The reversal of these levels and activities to near normal in the Raffia palm wine-treated groups, indicates an antioxidative and anti-proinflammatory potential of the palm wine.

Altered ATP levels have been reported in neurodegenerative diseases (Mochel et al. 2012; Zhang et al. 2015), as a consequence of increased ATPase activity. Increased brain ATPase activity, particularly the Na^+/K^+ ATPase have also been implicated in impaired glucose facilitative transportation across the BBB (Falkowska et al. 2015; Magistretti and Allaman 2015). In this study, the increased ATPase activity in the diabetic control (DC) (**Figure 3.5.3**) may insinuate an impaired glucose transport across the BBB and/or altered brain ATP concentration. The decreased activity in the treated groups, particularly DSG likely reflects increased ATP concentration as well as increased glucose uptake. This corroborates the increased glucose uptake in rat brains incubated with Raffia palm wine in the presence of glucose (**Figure 3.5.9**). The slight decrease in ATPase activities in brains of metformin-treated rats might be attributed to the fact that metformin activates

GLUTs 2 and 4, which are less concentrated at the BBB (Kellett and Brot-Laroche 2005; Rice et al. 2011). This may also explain its lesser effect on brain glucose uptake (**Figure 3.5.9**).

The role of acetylcholinesterase in exacerbating neurodegenerative diseases have been well established (Kuhl et al. 1999; Mushtaq et al. 2014; Pavlov et al. 2009). Its hydrolyzing effect on the neurotransmitter, acetylcholine has been implicated in impaired cognition, pathogenesis and progression of Alzheimer disease, and motor neuron dysfunction amongst other neurodegenerative diseases (Hwang et al. 1999; Kuhad et al. 2008; Tabet 2006). Increased acetylcholinesterase activity has been reported in diabetic rat brains (Ghareeb and Hussen 2008; Kuhad et al. 2008), correlating with the increased activity in the diabetic control in our present study (**Figure 3.5.4**). The increased activity further corroborates an occurrence of brain injury due to induction of T2D. The increased activity also corroborates the increased myeloperoxidase activity (**Figure 3.5.2**), as acetylcholinesterase activity has been correlated with the inhibition of the cholinergic anti-inflammatory pathways (Martelli et al. 2014; Pavlov et al. 2009). Increased oxidative stress has also been correlated with elevated acetylcholinesterase activity (Melo et al. 2003; Mushtaq et al. 2014), consistent with increased oxidative stress (**Figure 3.5.1**). The decreased acetylcholinesterase activities in treated diabetic rats, particularly the low dose Raffia palm wine treated group, DSG-LD indicate an acetylcholinesterase-inhibitory activity as well as a protective effect against hyperglycemia-induced neurodegeneration by Raffia palm wine.

Changes in brain morphology have been correlated with the pathogenesis and progression of most neurodegenerative diseases, with oxidative stress playing a major role (Ojo et al. 2014; Sidhu and Nehru 2004). The cerebellum controls motor and muscular activities in the brains as well as body balance (Imosemi 2013), with Purkinje cells as the main neurons present (Lopez et al. 2009). The morphological changes in the cerebellar cortex of the diabetic control rats (**Figure 3.5.5**) as depicted by distorted Purkinje and granular cell layers indicate brain injury, which can be attributed to the T2D induced oxidative stress (**Figure 3.5.1**). The vulnerability of Purkinje cells to oxidative stress has been reported (Chen et al. 2003; Kern and Jones 2006; Lopez et al. 2009). Their altered morphology and distribution has also been reported in diabetes (Solmaz et al. 2017; Yamano et al. 1986). This is further evident the altered distribution and loss of Purkinje and glial cells as well as dendrites and axons networks as revealed in **Figure 3.5.6B**. These changes indicate an altered neuronal integrity, which is common in most neurodegenerative diseases such as Alzheimer's

disease. These results are in consent with reports on the alteration of cerebellar cortex on induction of DM in rats (Hernández-Fonseca et al. 2009; Nagayach et al. 2014; Ozdemir et al. 2016). The near-intactness of these cells and networks in the treated groups (**Figure 3.5.5C – 3.5.5F** and **3.5.6C – 3.5.6F**), particularly diabetic rats treated with Raffia palm wine correlates with the observed antioxidant activities (**Figure 3.5.1**). Thus, indicating a neuro-protective potential of Raffia palm wine against T2D-induced neurodegeneration.

Altered elemental distribution, particularly accumulation of metals has been implicated in oxidative neurodegeneration (Patel 2016). Unless altered, these metals are involved in the normal physiology of the brain as they act as co-factors for most enzymes and are integral component of most redox reactions (Uttara et al. 2009). Altered elemental distributions and accumulations have been reported in cerebellar toxicity (Folarin et al. 2017; Kamal and Kamal 2013). The increased O_2 content (**Figure 3.5.7B**) correlates with the induced oxidative stress in DC, as oxygen is the major precursor of ROS and its increased accumulation may lead to generation of $O_2^{\cdot -}$ (Patel 2016). The increased Ca and Al contents portrays toxic effect as their accumulation have been implicated in the pathogenesis and progression of neurodegeneration (Kamal and Kamal 2013; Wojda et al. 2008). The role of magnesium in neurodegeneration is still debatable. However, its aggregative and negative effect on neurodegeneration has been reported (Van Den Heuvel and Vink 2004). The modulation of these elemental levels to near normal in the treated groups, further portrays the neuro-protective effects of Raffia palm wine.

Exacerbated Nrf2 expression has been implicated in the onset of T2D and antioxidant suppression, leading to ROS generation (He et al. 2012; Miao et al. 2012). This correlates with the increased expression in Purkinje and granular layers in the cerebellar cortex of the diabetic control group (**Figure 3.5.8B**). The increased expression also corroborates the diminished antioxidant enzymes (SOD and catalase) activities and lipid peroxidation in the diabetic control (**Figure 3.5.1**). The downregulation of the expressions particularly in rats treated with Raffia palm wine, indicates an attenuative effect on cerebellar Nrf2 expression. This Attenuative effect also corroborates the exacerbated antioxidative activity in the rat brains (**Figure 3.5.1**).

3.5.5 Conclusion

These results indicate the neuroprotective potentials of Raffia palm wine against oxidative brain injury in T2D. This is evident by its ability to attenuate oxidative stress and proinflammation, inhibit acetylcholinesterase and ATPase activities, modulate elemental distribution, attenuation of Nrf2 expression, with concomitant maintenance of cerebellum neuronal integrity, while stimulating brain glucose uptake.

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3.6 Raffia Palm (*Raphia hookeri*) Wine Extenuates Redox Imbalance and Modulates Activities of Glycolytic and Cholinergic Enzymes in Hyperglycemia Induced Testicular Injury in Type 2 Diabetes Rats

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Preface: This article investigated the antioxidative protective effect of Raffia palm wine on type 2 diabetes induced testicular injury. Its antioxidative and anti-proinflammatory effects were investigated. Its inhibitory effect on glycolytic and cholinergic enzyme activities were investigated. The article presently under review for publication in Journal of Food Biochemistry (JFBC-10-18-0815).

3.6.1 Abstract

This study investigated the effects of Raffia palm wine (RPW) on redox imbalance, glycolytic and cholinergic enzymes, and ATPase activities in type 2 diabetic-induced oxidative testicular injury. Type 2 diabetes (T2D) was induced in male albino rats (Sprague-Dawley) by first administering 10% fructose for 14 days, before injecting with 40 mg/kg streptozotocin. RPW was administered to two T2D rat groups at 150 and 300 mg/kg bodyweight (bw), a T2D group without treatment served as a negative control, while metformin served as the standard drug for another group. Non-diabetic (ND) rats served as the normal control, while ND rats administered a 300 mg/kg bw RPW was served as a toxicology group. The rats were sacrificed after 5 weeks of treatment, and the testes were harvested. Analysis of the testes revealed elevated levels of nitric oxide, malondialdehyde and myeloperoxidase activity, with concomitant depleted level of reduced glutathione, superoxide dismutase and catalase activities on induction of T2D, indicating an oxidative stress and proinflammation. These were significantly ($p<0.05$) reversed after the treatment with RPW. Treatment with RPW led to decreased activities of glycogen phosphorylase,

glucose-6-phosphatase, and fructose-1,6-bisphosphatase in T2D rats. RPW also caused an elevation of ATPase activity and inhibition of acetylcholinesterase activity. Altered testicular morphology by T2D were restored to near-normal after the treatment with RPW. These results demonstrate the therapeutic potentials of RPW against the detrimental effects of T2D on testicular functions and morphology.

Keywords: Oxidative stress; Raffia palm wine; Testicular injury; and Type 2 diabetes

3.6.2 Introduction

Male infertility has been recognized as a neglected complication of diabetes mellitus (DM) (Dias et al. 2014; Kilarkaje et al. 2014; Xu et al. 2014). This has been attributed to the polyunsaturated fatty acids (PUFAs) rich content of the testicular tissues, which is highly susceptible to oxidative damage from hyperglycaemia-generated free radicals (Erukainure et al. 2012; Obode et al. 2015). Oxidative damage occurs when the generated free radicals overwhelms the testicular endogenous antioxidant system, leading to impaired spermatogenesis and steroidogenesis, and finally infertility (Alves et al. 2013; Xu et al. 2014). Hyperglycaemia accounts for the generation of free radicals in DM, owing to chronic increase in blood glucose level (Maritim et al. 2003b). The continuous increase in blood glucose level in DM have been attributed to the inability of the pancreatic β-cells to secrete insulin and/or inability of the body to utilize secreted insulin (Erukainure et al. 2017b). The former is referred to as type 1 diabetes (T1D) and the latter, type 2 diabetes (T2D) which accounts for over 90% of all diabetes types (I.D.F. 2016; I.D.F. 2018).

This study aimed to investigate the effects of Raffia palm wine on redox imbalance, glycolytic and cholinergic enzymes, and ATPase activities as well as tissue morphology in oxidative testicular injury in a type 2 diabetes model of rats.

3.6.3 Materials and Methods

Kindly refer to Chapter 2, subsections 2.1.1; 2.1.3; 2.12 – 2.13 for details materials and methods.

3.6.4 Results

Induction of T2D led to significant reduction in GSH level, SOD and catalase activities, with concomitant increase in MDA level depicting an occurrence of oxidative stress as shown in

Figures 3.6.1A – 3.6.1.1D. This was significantly reversed after the treatment with Raffia palm wine, with the high dose showing a better activity for GSH and MDA levels, and SOD activity. Treatment with metformin had a little or no effect on the MDA level.

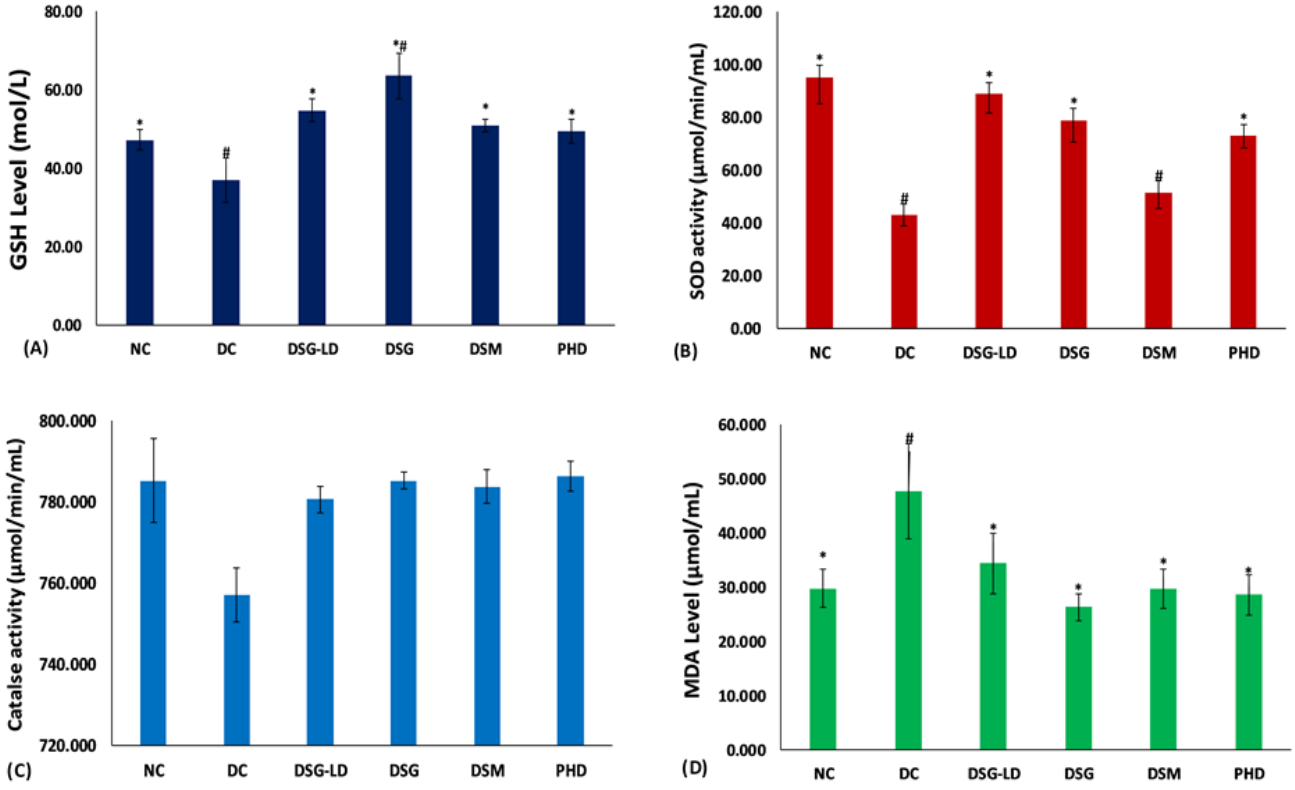

Figure 3.6.1: Effect of Raffia palm wine on (A) GSH level; (B) SOD activity; (C) catalase activity; and (D) MDA level in testicular tissues of experimental groups. Data = mean ± SD; n = 5 (NC & PHD) and 7 (DC, DSG-LD, DSG, & DSM). *Significantly different ($p<0.05$) from DC; #Significantly different from NC. **NC** = normal rats, **DC** = diabetic control, **DSG-LD** = diabetic rats + Raffia palm wine (150 g/kg bw), **DSG** = diabetic rats + Raffia palm wine (300 g/kg bw), **DSM** = diabetic rats + metformin (200 g/kg bw), and **PHD** = Normal rats + Raffia palm wine (300 g/kg bw).

There was a significant ($p<0.05$) increase in NO level and myeloperoxidase activity on the onset of T2D, indicating an occurrence of proinflammation as depicted in **Figure 3.6.2A** and **3.6.2B**. These were significantly reduced in all treatment groups, with metformin showing the least activity for NO.

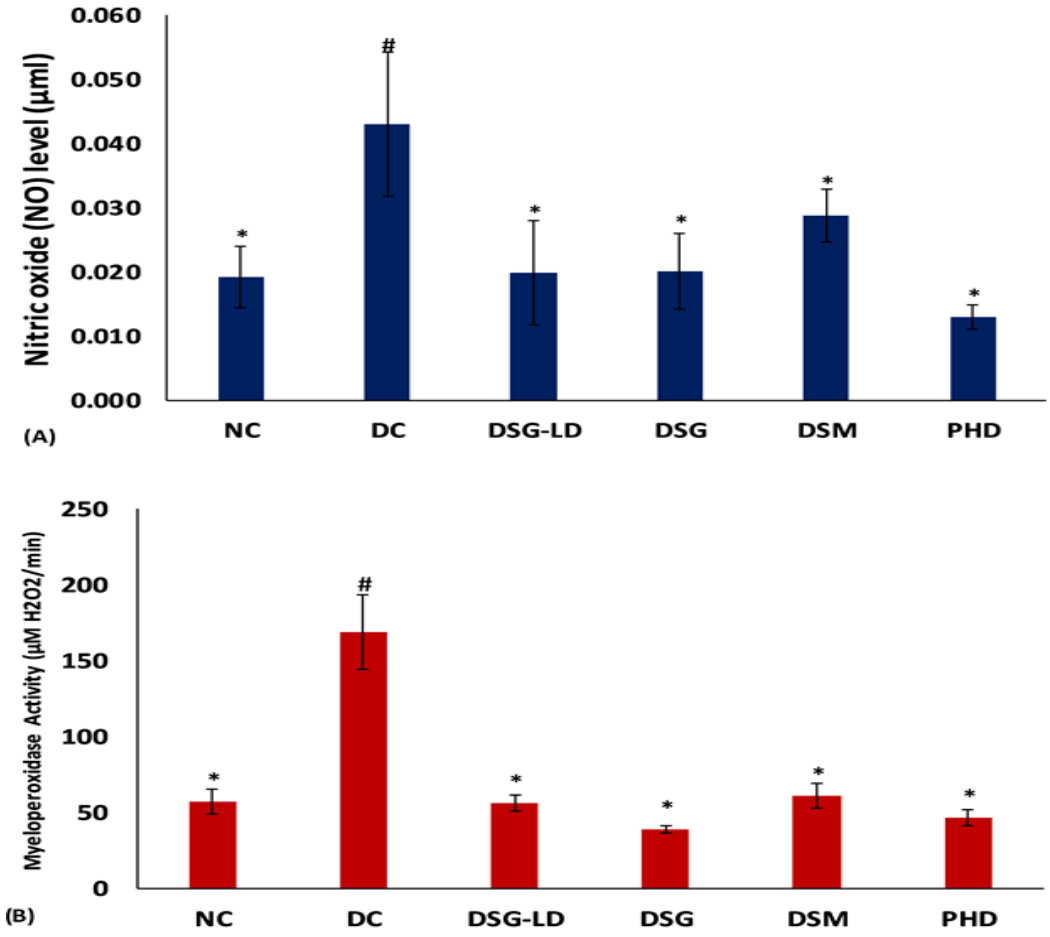

Figure 3.6.2: Effect of Raffia palm wine on (A) NO level; and (B) myeloperoxidase activity in testicular tissues of experimental groups. Data = mean ± SD; n = 5 (NC & PHD) and 7 (DC, DSG-LD, & DSG). *Significantly different ($p<0.05$) from DC; #Significantly different from NC. **NC** = normal rats, **DC** = diabetic control, **DSG-LD** = diabetic rats + Raffia palm wine (150 g/kg bw), **DSG** = diabetic rats + Raffia palm wine (300 g/kg bw), **DSM** = diabetic rats + metformin (200 g/kg bw), and **PHD** = Normal rats + Raffia palm wine (300 g/kg bw).

The studied glycolytic enzymes were significantly ($p<0.05$) elevated after the induction of T2D as shown in **Figures 3.6.3A – 3.6.3C**. These activities were depleted in all the treatment groups, with rats treated with high dose of Raffia palm wine and metformin showed the significant ($p<0.05$) depletion.

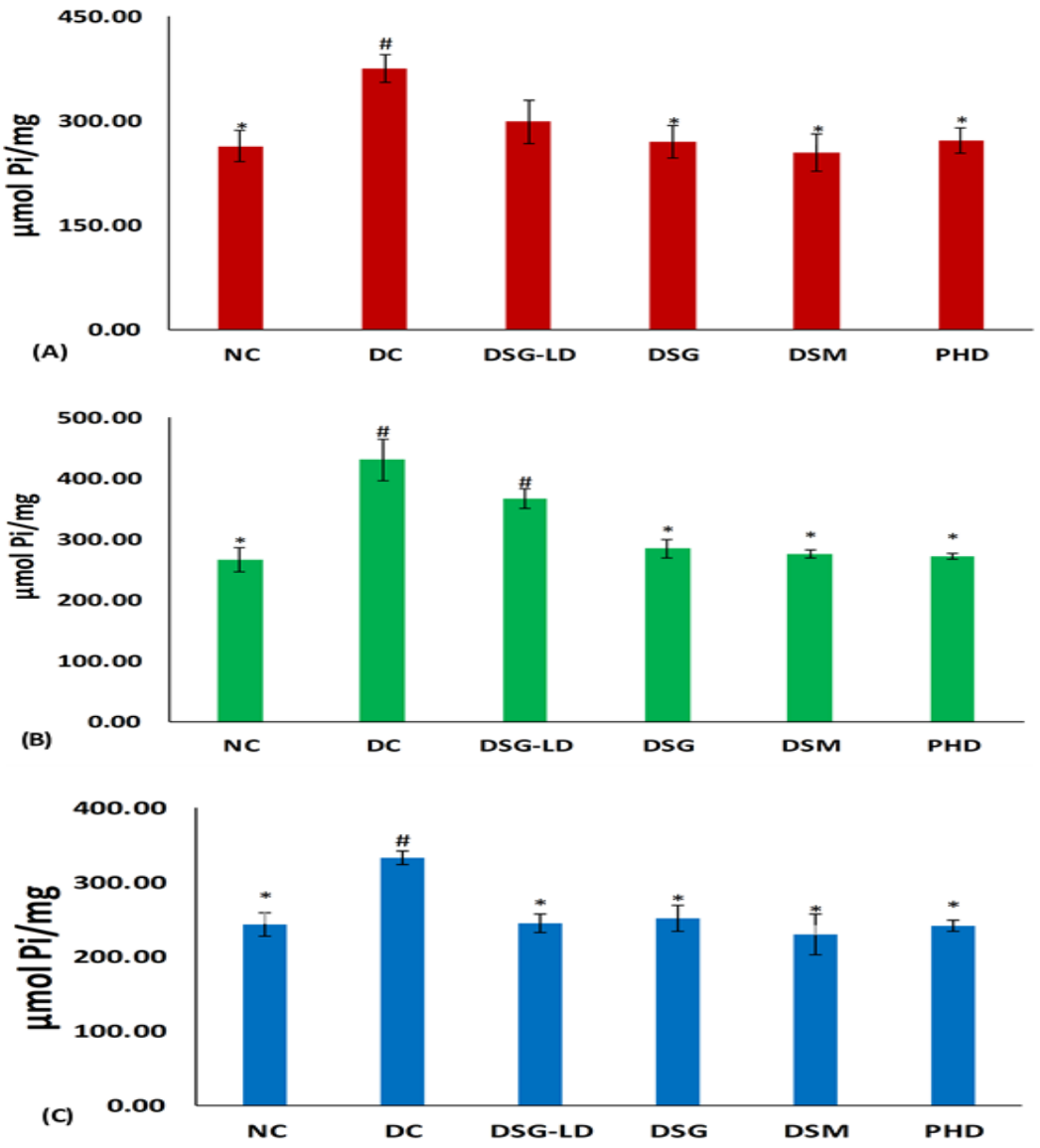

Figure 3.6.3: Effect of Raffia palm wine on (A) glycogen phosphorylase; (B) fructose 1,6 biphosphatase; and glucose 6 phosphatase activities in testicular tissues of experimental groups. Data = mean ± SD; n = 5 (NC & PHD) and 7 (DC, DSG-LD, DSG, & DSM). *Significantly different ($p<0.05$) from DC; #Significantly different from NC. **NC** = normal rats, **DC** = diabetic control, **DSG-LD** = diabetic rats + Raffia palm wine (150 g/kg bw), **DSG** = diabetic rats + Raffia palm wine (300 g/kg bw), **DSM** = diabetic rats + metformin (200 g/kg bw), and **PHD** = Normal rats + Raffia palm wine (300 g/kg bw).

Induction of T2D led to significant ($p<0.05$) decrease in ATPase activity as depicted in **Figure 3.6.4.** Treatment with Raffia palm wine at both doses led to a significant ($p<0.05$) increase in the

activity (Fig. 4A), while treatment with metformin as well as administration of Raffia palm wine to normal rats had a little or no effect.

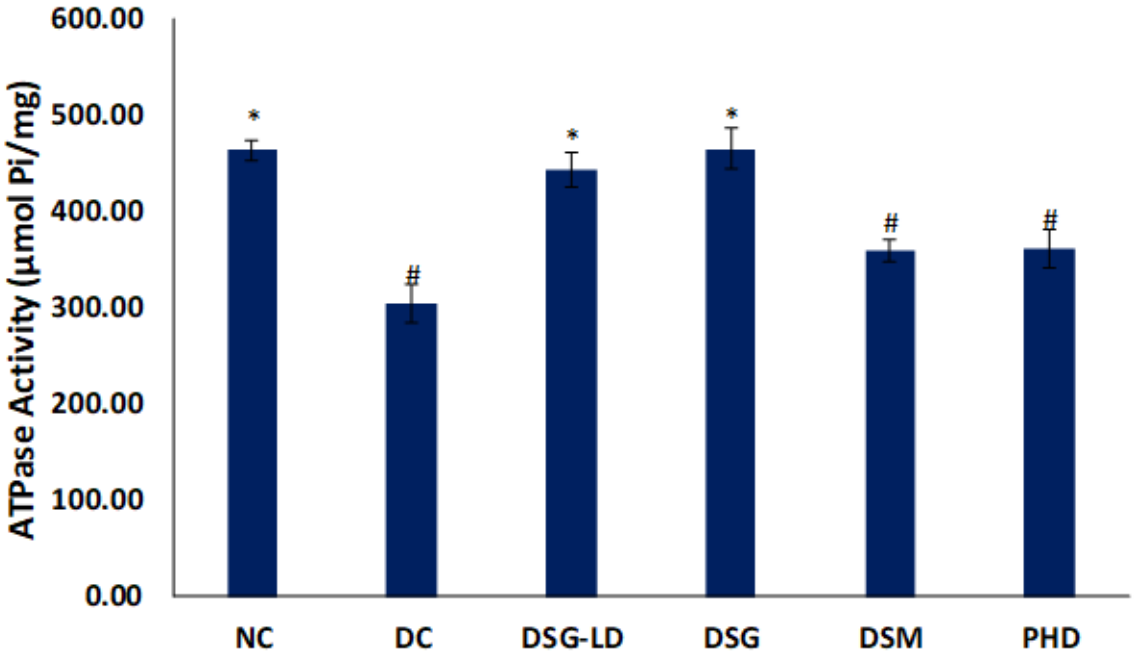

Figure 3.6.4: Effect of Raffia palm wine on ATPase activities in testicular tissues of experimental groups. Data = mean ± SD; n = 5 (NC & PHD) and 7 (DC, DSG-LD, DSG, & DSM). *Statistically significant ($p<0.05$) to DC; #statistically significant to NC. **NC** = normal rats, **DC** = diabetic control, **DSG-LD** = diabetic rats + Raffia palm wine (150 g/kg bw), **DSG** = diabetic rats + Raffia palm wine (300 g/kg bw), **DSM** = diabetic rats + metformin (200 g/kg bw), and **PHD** = Normal rats + Raffia palm wine (300 g/kg bw).

There was a significant ($p<0.05$) reduction in inhibitory effect of acetylcholinesterase activity after the induction of T2D, indicating an increased enzyme activity as depicted in **Figure 3.6.5**. This was significantly ($p<0.05$) increased in all treatment groups, depicting a depleted acetylcholinesterase activity.

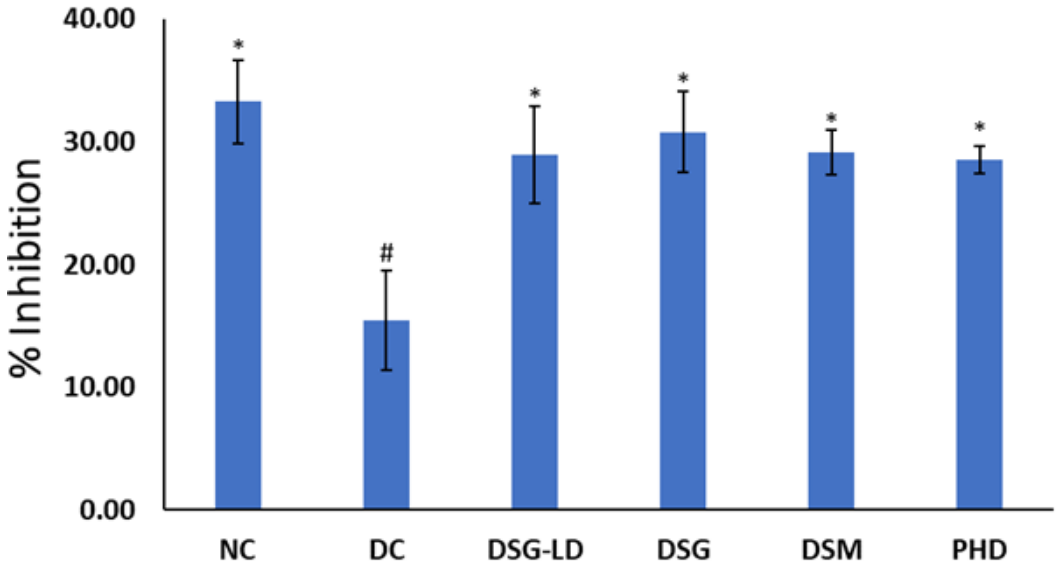

Figure 3.6.5: Inhibitory effect of Raffia palm wine on acetylcholinesterase activities in testicular tissues of experimental groups. Data = mean ± SD; n = 5 (NC & PHD) and 7 (DC, DSG-LD, & DSG). *Significantly different ($p<0.05$) to DC; #statistically significant to NC. **NC** = normal rats, **DC** = diabetic control, **DSG-LD** = diabetic rats + Raffia palm wine (150 g/kg bw), **DSG** = diabetic rats + Raffia palm wine (300 g/kg bw), **DSM** = diabetic rats + metformin (200 g/kg bw), and **PHD** = Normal rats + Raffia palm wine (300 g/kg bw).

Histological analysis of the testicular tissues revealed an intact spermatogonia, spermatocytes, spermatozoa, seminiferous epithelium, leydig cells, and a reduced lumen depicting an undistorted morphology (**Figure 3.6.6A(1 and 2)**). This morphology was however distorted on induction of T2D as portrayed by the wide lumen, decreased numbers of spermatogonia, spermatocytes, and spermatozoa depicting testicular injury (**Figure 3.6.6B(1 and 2)**). Treatment with low dose of Raphia palm wine led to increased number of these cells as well as a reduced lumen (**Figure 3.6.6C(1 and 2)**). The high-dose treated group (DSG) revealed an intact tissue morphology, with the numbers of cells and the width of the lumen close to that of the control (NC) (**Figure 3.6.6D(1 and 2)**). Treatment with metformin also led to distortion of the tissue morphology, with decreased number of spermatogonia, spermatocytes, and spermatozoa (**Figure 3.6.6E(1 and 2)**). Similarly, administration of Raffia palm wine to normal rats (PHD) showed a slight distorted morphology (**Figure 3.6.6F (1 and 2)**).

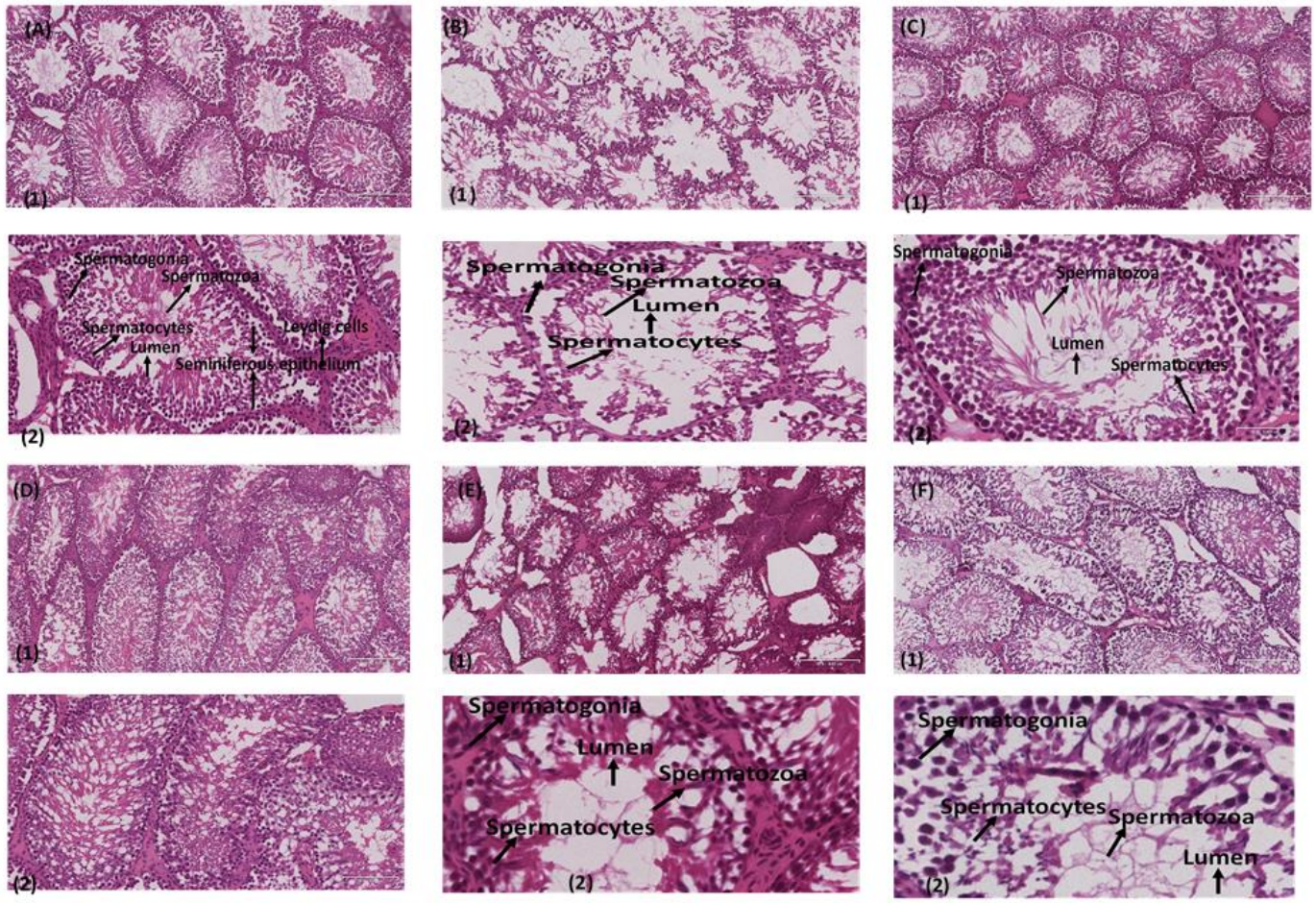

Figure 3.6.6: Effect of Raffia palm wine on testicular morphology of experimental groups. **Keys**: A(1 & 2) = NC; B(1 & 2) = DC; C(1 & 2) = DSG-LD; D(1 & 2) = DSG; E(1 & 2) = DSM; F(1 & 2) = PHD. **NC** = normal rats, **DC** = diabetic control, **DSG-LD** = diabetic rats + Raffia palm wine (150 g/kg bw), **DSG** = diabetic rats + Raffia palm wine (300 g/kg bw), **DSM** = diabetic rats + metformin (200 g/kg bw), and **PHD** = Normal rats + Raffia palm wine (300 g/kg bw).

Image analysis of the generated histological images revealed a decreased cell size on induction of T2D as shown in **Figure 3.6.7**. The low dose palm wine treated group (DSG-LD) showed little or no effect, while the high dose (DSG) had a significant ($p<0.05$) increase. Metformin (DSM) also showed a little or no significant effect, while PHD showed a slight increase in cell size.

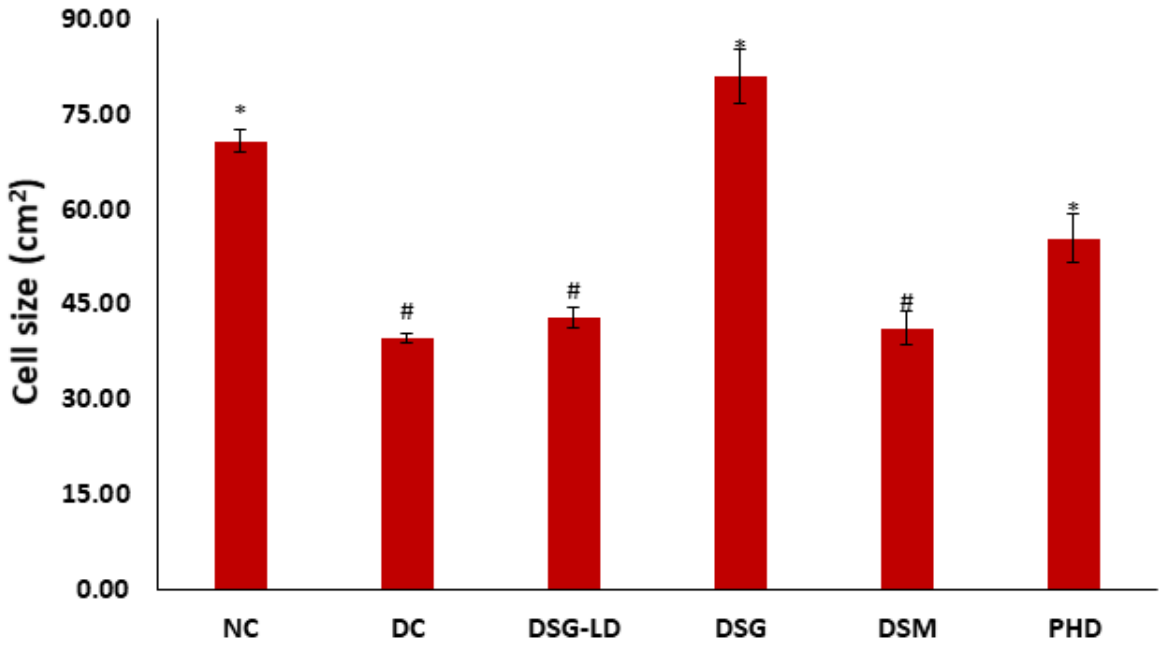

Figure 3.6.7: Cell size analysis of testicular tissues of experimental groups. Data = mean ± SD; n = 5 (NC & PHD) and 7 (DC, DSG-LD, & DSG). *Significantly different ($p<0.05$) from DC; #Significantly different from NC. **NC** = normal rats, **DC** = diabetic control, **DSG-LD** = diabetic rats + Raffia palm wine (150 g/kg bw), **DSG** = diabetic rats + Raffia palm wine (300 g/kg bw), **DSM** = diabetic rats + metformin (200 g/kg bw), and **PHD** = Normal rats + Raffia palm wine (300 g/kg bw).

3.6.5 Discussion

Hyperglycaemia -mediated oxidative stress has been linked to the pathogenesis and progression of T2D complications, owing to impairments of several intracellular signaling pathways (Kilarkaje et al. 2014; Maritim et al. 2003b). Alterations of these pathways have been implicated in the onset of macro- and microvascular diseases (Boden 2011; Kilarkaje et al. 2014). Of particular interest is the impairment of the male reproductive system, which is characterized by peroxidation of the testicular PUFAs constituents, morphological changes in the testes, and erectile dysfunction amongst others (Kilarkaje et al. 2014; Obode et al. 2015; Xu et al. 2014). In this study, the therapeutic effect of Raffia palm wine was investigated in hyperglycaemic-induced oxidative injury in T2D.

The depleted GSH level, SOD and catalase activities as well as increased MDA level in the untreated diabetic rats (DC) (**Figure 3.6.1**) connotes an occurrence of oxidative stress. GSH have been recognized as the first line component of the cellular antioxidant defense system, and plays a major role in testicular redox balance (Olayinka and Ore 2015). The reduced GSH level on induction of T2D may be attributed to overwhelming effect of the hyperglycaemia generated free radicals over its biosynthesis and turnover. The testes have been reported for having the highest cellular level of SOD (Aitken and Roman 2008; Mruk et al. 2002), which can be attributed to the catalytic dismutation of superoxide anion ($O_2{\cdot}^-$) to oxygen (O_2) and hydrogen peroxide (H_2O_2) by SODs (Abreu and Cabelli 2010). $O_2{\cdot}^-$ is also the major reactive oxygen species (ROS) generated by the spermatozoa (Hsieh et al. 2002). Under normal physiological conditions, spermatozoa produces superoxide ($O_2{\cdot}^-$) in smaller quantities for capacitation and acrosomal reaction (Agarwal and Prabakaran 2005). This physiological production is however compromised during disease states, leading to increased testicular $O_2{\cdot}^-$ levels with detrimental effect on male fertility (Hsieh et al. 2002; Mazzilli et al. 1994). The reduced testicular SOD activities on induction of T2D (**Figure 3.6.1B**) may therefore imply an increased testicular $O_2{\cdot}^-$ production. This also correlates with the increased NO level on the onset of T2D (**Figure 3.6.2A**), as NO reacts with $O_2{\cdot}^-$ to produce peroxynitrite ($ONOO^-$), which triggers nitrosative stress and proinflammation leading to testicular dysfunction, impaired sperm motility, decreased gonadotropin secretion, and finally infertility (Doshi et al. 2012; Uribe et al. 2014). The elevated SOD activities in the treatment groups (**Figure 3.6.1B**), therefore indicates a decreased testicular level of $O_2{\cdot}^-$ and its dismutation to H_2O_2 and O_2. It also implies a reduced $O_2{\cdot}^-$ availability for reaction with NO to produce $ONOO^-$, which correlates with the reduced NO level (**Figure 3.6.2A**). The generated H_2O_2 if not converted to H_2O and O_2 by catalase, will be broken down to hydroxyl ions ($^{\bullet}OH$). Both $^{\bullet}OH$ and H_2O_2 have been reported for their detrimental oxidative roles in male infertility, as they induce lipid peroxidation (Hsieh et al. 2002; Mruk et al. 2002). H_2O_2 is regarded as the main toxic ROS for spermatozoa (Hernández et al. 1995; Hsieh et al. 2002), owing to the susceptibility of the rich PUFAs constituents of the testicular tissue to peroxidation. Furthermore, H_2O_2 can also be converted to hypochlorous acid ($HOCl^-$) by myeloperoxidase in the presence of hydrochloric acid (HCl) (Furtmüller et al. 2000a). This correlates with the increased myeloperoxidase activity in the DC group (**Figure 3.6.2B**), indicating proinflammation. The increased MDA level on induction of T2D (**Figure 3.6.1D**), indicates an occurrence of lipid peroxidation which correlates with the

depleted SOD and catalase activities (**Figure 3.6.1B** and **3.6.1C**). Peroxidation of testicular lipids has been implicated in morphological changes of the testes, testicular dysfunction, decreased spermatogenesis and cell death (Asadi et al. 2017). The decreased level in the treatment groups indicates an antiperoxidative effect which corroborates the increased GSH level, SOD, and catalase activities (**Figures 3.6.1A – 3.6.1C**). Similarly, the decreased NO and myeloperoxidase activity in the treated groups connotes an anti-proinflammatory activity.

Maintenance of testicular glucose homeostasis have been reported to be of great importance to the male reproductive system, with glycogen playing an essential role (Rato et al. 2015a). Alterations of this homeodynamics have been reported in T2D, with implications on testicular functions and spermatogenesis (Alves et al. 2013; Rato et al. 2015a). The increased activities of glycogen phosphorylase, fructose 1,6 biphosphatase, and glucose 6 phosphatase on induction of T2D (**Figures 3.6.3A – 3.6.3C**) indicates a breakdown of testicular glycogen, leading to increased testicular glucose level. Previous studies correlated an increased testicular expression of glycogen phosphorylase with upregulation of GLUT1 and GLUT3 in testicular tissues of T2D rats (Rato et al. 2015a), implying an increased glucose uptake. In its enediol form, glucose is oxidized to an enediol radical in the presence of transition metal, leading to the production of reactive ketoaldehydes and $O_2^{\bullet -}$ (Maritim et al. 2003b). The increase in testicular glucose level coupled to the presence of the testicular transferrin synthesized by the seminiferous epithelium may thus imply an increased production of ROS, leading to oxidative stress. The decreased activities of the studied glycolytic enzymes in the treated groups therefore portrays a decreased testicular glucose level, thus reducing the amount of glucose available to produce ROS. Thereby reducing oxidative stress as corroborated by the increased antioxidant biomarkers (**Figures 3.6.1A – 3.6.1C**)

Several studies have linked to decrease testicular ATPase activity with testicular toxicity (El-Missiry and Shalaby 2000; Shen and Sangiah 1995). These studies correlated the decreased activity with oxidative stress, which corroborates this present study. Thus, the decreased activity on induction of T2D (**Figure 3.6.4**) portrays testicular toxicity, while the increased activity on treatment with Raffia palm wine reflects a therapeutic potential.

Testicular non-neuronal acetylcholine has been reported for its role in germ cell differentiation (Schirmer et al. 2011) and its deficiency has been demonstrated to reduce sperm motility and production (Bray et al. 2005; Schirmer et al. 2011; Yamamoto et al. 1998). Acetylcholinesterase

inhibits the actions of acetylcholine, which could have a negative effect on testicular functions. The decreased inhibition of acetylcholinesterase activity on induction of T2D (**Figure 3.6.5**) thus portrays a reduced acetylcholine function, further portraying the detrimental effect of T2D on male reproductive health. The increased inhibition in the treatment groups insinuates an increased acetylcholine function, which may demonstrate an improved testicular function.

The distorted testicular morphology on induction of T2D (**Figure 3.6.6B**) can be attributed to the peroxidation of the lipid membrane by hyperglycaemia-generated free radicals, leading to loss of cellular integrity (Armagan et al. 2006; Merker et al. 1996). The decreased numbers of spermatogonia, spermatocytes, and spermatozoa connotes impaired spermatogenesis, which correlates with previous reports on the detrimental effect of diabetes and oxidative stress on testicular functions (Asadi et al. 2017; Erukainure et al. 2012; Kaur and Bansal 2004; Turner and Lysiak 2008; Zhao et al. 2017). The near-intactness of the tissue morphology after treatment with high dose of Raffia palm wine indicates a therapeutic effect (**Figure 3.6.6D**). The restoration of the numbers spermatogonia, spermatocytes, and spermatozoa to near normal, further portrays a spermatogenic effect. The altered morphology and cells of DSM (**Figure 3.6.6E**) and PHD (**Figure 3.6.6F**), may portray toxic effects of metformin on diabetic-testicular injury and Raffia palm wine on healthy testicular tissues. These morphological changes correlate with the ImageJ analyzed cell size (**Figure 3.6.7**), with the increased size of DSG further reflecting the therapeutic effect of Raffia palm wine (high dose).

These therapeutic activities of Raffia palm win may be attributed to its reported mineral and vitamin constituents, particularly ascorbic acid (Bassir 1962; Cunningham and Wehmeyer 1988; Eze and Ogan 1988; Obahiagbon and Osagie 2007; Tuley 1965).

3.6.6 Conclusion

These results indicate the therapeutic potentials of Raffia palm wine against the detrimental effect of T2D on testicular functions and morphology. Thus, giving credence to its folkloric claims. However, its morphological effect on healthy tissues may require caution in its consumption.

3.7 Raffia (*Raphia hookeri*) Palm Wine Modulates Dysregulated Lipid Metabolic Pathways and Metabolites in Diabetic Testicular Toxicity

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Preface: This article investigated the effect of Raffia palm wine on lipid metabolites and metabolic pathways in type 2 diabetes induced testicular injury. The lipid metabolites, hormones and antioxidant markers and the pathways involved were reported. It is presently under review for publication in Biomedicine and Pharmacotherapy (BIOPHA_2018_5945).

3.7.1 Abstract

Testicular lipids play important functions on male fertility but also increases the susceptibility of the testes to peroxidative damage. This study investigates the effect of Raffia palm wine on dysregulated lipid metabolic pathways in testicular tissues of type 2 diabetic (T2D) rats. Raffia palm wine (150 and 300 mg/kg bodyweight) was administered to two T2D groups respectively, another T2D group was not administered treatment and served as negative control, while metformin served as the standard drug. After 6 weeks of treatment, the rats were sacrificed, and the testes collected. After weighing, the organs were homogenized in 20% methanol/ethanol and centrifuged at 20,000 g to extract the lipid metabolites. GC-MS analysis of the supernatants revealed an alteration of the metabolites on induction of T2D, with concomitant generation of 10 metabolites. Raffia palm wine inhibited the T2D-generated metabolites while replenishing cholesterol and squalene levels, with concomitant generation of 7 and 8 metabolites for low and high dose treatment respectively. Pathway enrichment analysis of the metabolites revealed a decreased level of steroid biosynthesis and increased level of fatty acid biosynthesis. Raffia palm

wine inactivated glycerolipid, fatty acid, and arachidonic acid metabolisms, fatty acid biosynthesis and fatty acid elongation in mitochondria pathways, and activated pathways for plasmalogen synthesis, mitochondrial beta-oxidation of long chain saturated fatty acids. The replenishment and generation of these metabolites and additional ones as well as activation of pathways involved in energy generation, phospholipids, antioxidant activity, steroidogenesis and spermatogenesis suggest a therapeutic effect of Raffia palm wine against hyperglycemic-induced testicular dysfunction.

Keywords: Lipid metabolites; Metabolic pathways; Testicular Dysfunction; Type 2 diabetes.

3.7.2 Introduction

The increasing incidence of diabetes mellitus (DM) is a major concern to global health practitioners. It is an endocranial disorder arising from inability of the pancreatic β-cells to secrete insulin (type 1 diabetes [T1D]) and/or inability of the tissues to utilized secreted insulin (type 2 diabetes [T2D]) (Erukainure et al. 2017b; Erukainure et al. 2018b). Of these types, T2D is the predominant and have been implicated in chronic hyperglycemia which compromises male fertility by impairing sperm quality, reducing fecundity, and fragmenting spermatozoa DNA (Dias et al. 2014; Rato et al. 2015b). This has been attributed to metabolic syndrome and hyperglycemic induced hormonal and metabolic dysfunction, concomitantly impairing spermatogenesis and steriodogensis (Dias et al. 2014; Ehala-Aleksejev and Punab 2018; Xu et al. 2014). The high lipid composition of the testicular tissue and sperm cells makes them susceptible to peroxidative damage by hyperglycaemic-induced oxidative stress (Erukainure et al. 2012; Obode et al. 2015). Oxidative stress occurs when the testicular antioxidant system is overwhelmed by reactive oxygen species (ROS) generated by hyperglycaemia (Erukainure et al. 2017b; Maritim et al. 2003b). Peroxidation of these lipids alters their normal homeostasis, leading to testicular and erectile dysfunction as testicular lipids play major roles in spermatogenesis and steriodogensis (Maqdasy et al. 2013; Sèdes et al. 2017).

Medicinal plants have been employed in the treatment and management of T2D and its complications (Ezuruike and Prieto 2014; Mohammed et al. 2014), as well as male infertility (Mbongue et al. 2005; Nantia et al. 2009) from time immemorial. This has been attributed to their phytochemical and nutritional constituents. These plants are readily available and affordable, with

little or no reported side effects. Amongst such plants is the Raffia palm (*Raphia hookeri*) and its constituent, Raffia palm wine.

This study aims to report the effect of T2D on testicular lipid metabolites and metabolic pathways and the therapeutic potentials of Raffia palm wine in male albino rats.

3.7.3 Materials and Methods

Kindly refer to Chapter 2, subsections 2.1.1; 2.1.3; 2.12 – 2.13 for details materials and methods.

3.7.4 Results

Induction of T2D led to a reduction in the weight of the testes organs as shown in **Figure 3.7.1**. There was an increase in weight after treatment with Raffia palm wine, with the lower dose having the highest gain compared to the high dose and metformin – treated groups.

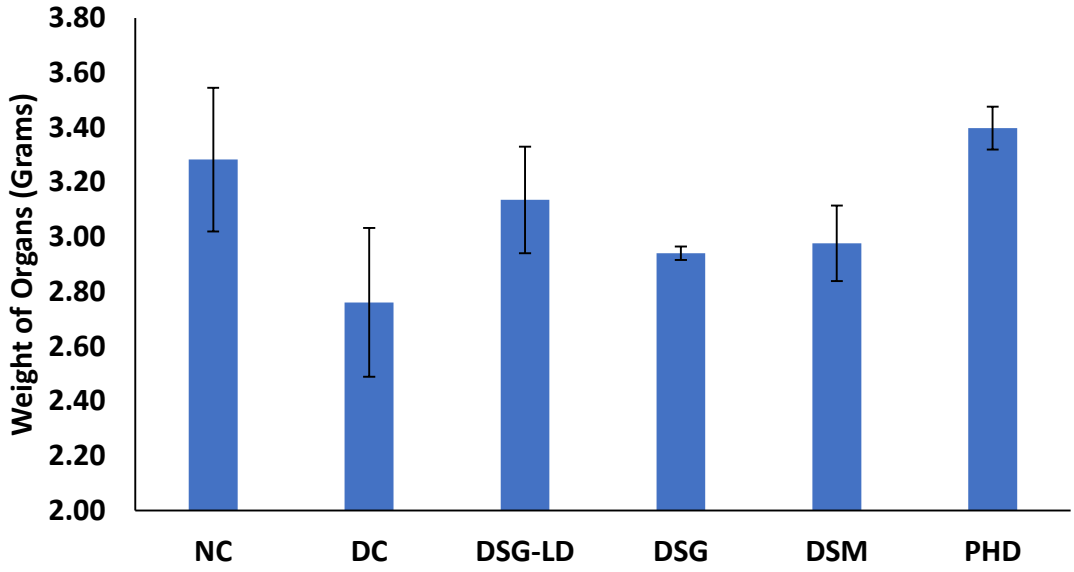

Figure 3.7.1: Weight of testes organs of experimental groups. Data = mean ± SD; n = 5 (NC & PHD) and 7 (DC, DSG-LD, DSG, & DSM). **NC** = normal rats, **DC** = diabetic control, **DSG-LD** = diabetic rats + Raffia palm wine (150 g/kg bw), **DSG** = diabetic rats + Raffia palm wine (300 g/kg bw), **DSM** = diabetic rats + metformin (200 g/kg bw), and **PHD** = Normal rats + Raffia palm wine (300 g/kg bw).

GC-MS analysis of the extracted testicular lipid metabolites from the normal rats (NC) revealed a very high level of cholesterol as depicted in **Table 3.7.1**. A high percentage of unsaturated fatty acids and their esters as well as low percentage of saturated fatty acids were also identified. The vitamin D metabolite, 24,25-dihydroxyvitamin D and the androsterone metabolite, androstane-3,11,17-triol were present in minute percentages. Other identified metabolites include 2-heptanone and cholesta-4,6-dien-3-one.

Induction of T2D led to inhibition of 82.1% of these metabolites, while retaining pentadecanoic acid, ethyl hexadecanoate, arachidonic acid, cholesterol and icosapentaenoic acid as shown in **Table 3.7.1**. The levels of arachidonic acid and cholesterol were significantly reduced, while the others increased. Onset of T2D also led to generation of 10 metabolites, with ethyl linoleate and (E)-9-octadecenoic acid ethyl ester being the most abundant (12.12 and 13.78% respectively), while eicosanoic acid (1.39%), methyl stearate (1.05%), and cholest-8-en-3-yl acetate (0.34%) were the least.

Treatment with low dose of Raffia palm wine led to the restoration of squalene and increased cholesterol level (**Table 3.7.1**). It also caused an inhibition of the T2D – generated metabolites except icosapentaenoic acid, methyl linoleate and eicosanoic acid, ethyl ester which were significantly reduced. They also led to concomitant generation of 7 metabolites particularly the antioxidant, vitamin A aldehyde and the eicosanoic acid triglyceride, triarachidin.

Treatment with high dose of Raffia palm wine led to significant increase in squalene and cholesterol levels, while inhibiting ethyl hexadecanoate and all T2D – generated metabolites (**Table 3.7.1**). It also led to concomitant generation of other 8 metabolites particularly the testosterone anabolic metabolite, 11.alpha.-Hydroxy-17.alpha.-methyl testosterone and glycoprotein, N-acetylgalactosamine. Octadecanoic acid was common to both Raffia palm wine treated groups, with DSG-LD having a higher level (2.79%).

Treatment with metformin also caused a significant increase in squalene and cholesterol levels and regeneration of pentadecanoic acid and ethyl icosanoate (**Table 3.7.1**). Except for 10-octadecenoic acid, methyl ester and methyl stearate, the T2D-generated metabolites were inhibited. There was concomitant generation of 10 metabolites particularly the antioxidant metabolite, ascorbic acid dipalmitate. The group had higher levels of methyl palmitate (1.85%) and 11,14-eicosadienoic

acid, methyl ester (3.12%) compared to that of DSG, while the ethyl oleate level (6.93%) was higher than that of DSG-LD.

Administration of Raffia palm wine to normal rats (PHD) led to increased levels of pentadecanoic acid (2.79%) and cholesterol (48.37%), while depleting that of ethyl icosanoate, arachidonic acid and squalene as compared to NC. It also led to the generation of 9 metabolites particularly the vitamin D metabolite, 1,25-Dihydroxyvitamin D3, TMS derivative.

Table 3.7.1: GC-MS identified testicular lipid metabolites of experimental groups

Metabolites	NC (%)	DC (%)	DSG-LD (%)	DSG (%)	DSM (%)	PHD (%)
Pentadecanoic acid	1.34	2.91	4.8	2.81	–	2.68
Palmitic acid	1.55	2.91	–	–	–	–
Ethyl hexadecanoate	2.15	9.09	2.88	–	4.38	–
Linoleic acid ethyl ester	1.02	–	–	–	–	–
9-Octadecenoic acid, ethyl ester	1.05	–	–	–	–	–
Ethyl icosanoate	1.48	–	–	–	2.02	1
Arachidonic acid	1.37	0.88	–	0.66	0.9	0.44
Docosahexaenoic acid	3.3	–	–	–	–	–
2-Heptanone	0.77	–	–	–	–	–
24,25-Dihydroxyvitamin D	0.52	–	–	–	–	–
Nonyl isovalerate	0.27	–	–	–	–	–
Squalene	1.35	–	0.61	1.04	0.88	0.7
Cholesterol	43.16	17.43	34.95	53.1	34.12	48.37
Androstane-3,11,17-triol	0.29	–	–	–	–	–
Cholesta-4,6-dien-3-one	0.63	–	–	–	–	–
Isosteviol	0.56	–	–	–	–	–

Icosapentaenoic acid	0.37	2.77	1.49	–	1.9	–
Glycerol 1,2-dipalmitate	–	1.9	–	–	–	–
Tetradecanoic acid	–	2.58	–	–	–	–
Methyl linoleate	–	4.17	2.54	–	–	–
10-Octadecenoic acid, methyl ester	–	4.51	–	–	3.27	–
Methyl stearate	–	1.05	–	–	1.11	–
Ethyl linoleate	–	12.12	–	–	–	0.95
(E)-9-Octadecenoic acid ethyl ester	–	13.78	–	–	–	
Eicosanoic acid	–	1.39	–	–	–	–
Eicosanoic acid, ethyl ester	–	3.37	1.71	–	–	–
Cholest-8-en-3-yl acetate	–	0.34	–	–	–	–
Methyl 14-methylpentadecanoate	–	–	1.25	–	–	–
Methyl 9-eicosenoate	–	–	2.42	–	–	–
9,12-Octadecadienoic acid, ethyl ester	–	–	8.11	–	–	–
Ethyl oleate	–	–	5.68	–	6.93	
Octadecanoic acid	–	–	2.79	1.27	–	–
Triarachidin	–	–	0.41	–	–	–
Vitamin A aldehyde	–	–	0.56	–	–	–
Ethyl docosanoate	–	–	–	0.85	–	–
Methyl palmitate	–	–	–	1.17	1.85	–
2-Dodecenoic acid	–	–	–	1.66	–	–
11,14-Eicosadienoic acid, methyl ester	–	–	–	1.32	3.12	–
Methyl 15-acetoxyhexadecanoate	–	–	–	1.22	–	–
N-Acetylgalactosamine	–	–	–	0.32	–	–

11.alpha.-Hydroxy-17.alpha.-methyl testosterone	–	–	–	0.42	–	–
Globulol	–	–	–	0.77	–	–
Dodecanolactone	–	–	–	–	1.25	–
Ascorbic acid dipalmitate	–	–	–	–	1.75	–
Ethyl octadeca-9,12-dienoate	–	–	–	–	7.41	–
Ethyl 9-tetradecenoate	–	–	–	–	1.05	–
Stearic acid	–	–	–	–	0.68	0.86
2,4-Dimethyladipic acid	–	–	–	–	0.3	–
Cholesterol, myristate	–	–	–	–	0.57	–
10,12-Docasadiyndioic acid	–	–	–	–	–	0.69
15-Hydroxypentadecanoic acid	–	–	–	–	–	1.47
Oleamide	–	–	–	–	–	0.34
1,25-Dihydroxyvitamin D3, TMS derivative	–	–	–	–	–	1.48
5-Hexylsulfanylpentane-1,2,3,4-tetrol	–	–	–	–	–	1.59
Cholesta-3,5-diene	–	–	–	–	–	0.6
Distearin	–	–	–	–	–	0.42
Methyl 5-eicosenoate	–	–	–	–	–	0.41

Key: – = not present. **NC** = normal rats, **DC** = diabetic control, **DSG-LD** = diabetic rats + Raffia palm wine (150 g/kg bw), **DSG** = diabetic rats + Raffia palm wine (300 g/kg bw), **DSM** = diabetic rats + metformin (200 g/kg bw), and **PHD** = Normal rats + Raffia palm wine (300 g/kg bw).

Pathway enrichment analysis of the GC-MS identified lipid metabolites revealed the activation of metabolic pathways for steroid biosynthesis, alpha linolenic and linoleic acids metabolism, bile acid biosynthesis, glycerolipid metabolism, fatty acid biosynthesis, metabolism and elongation in mitochondria, steroidogenesis, and arachidonic acid metabolism as depicted in **Table 3.7.2** and **Figure 3.7.2**. These pathways were not inactivated on induction of T2D but base on their p-values,

the level of fatty acid and steroid biosynthesis were respectively higher and lower on the onset of T2D (**Figure 3.7.2)**.

Table 3.7.2: Metabolic pathways of testicular lipid metabolites of experimental groups

Pathways	NC	DC	DSG-LD	DSG	DSM	PHD
Steroid Biosynthesis	X	X	X	X	X	X
Alpha Linolenic Acid and Linoleic Acid Metabolism	X	X	X	X	X	X
Bile Acid Biosynthesis	X	X	X	X	X	X
Glycerolipid Metabolism	X	X	–	–	–	–
Fatty Acid Biosynthesis	X	X	–	X	–	–
Fatty Acid Elongation in Mitochondria	X	X	–	–	–	–
Fatty acid Metabolism	X	X	–	–	–	–
Steroidogenesis	X	X	X	X	X	X
Arachidonic Acid Metabolism	X	X	–	X	X	X
Plasmalogen Synthesis	–	–	X	X	X	X
Mitochondrial Beta-Oxidation of Long Chain Saturated Fatty Acids	–	–	X	X	X	X
Retinol Metabolism	–	–	X	–	–	–

Key: X = present; – = not present. **NC** = normal rats, **DC** = diabetic control, **DSG-LD** = diabetic rats + Raffia palm wine (150 g/kg bw), **DSG** = diabetic rats + Raffia palm wine (300 g/kg bw), **DSM** = diabetic rats + metformin (200 g/kg bw), and **PHD** = Normal rats + Raffia palm wine (300 g/kg bw).

Treatment with low dose of Raffia palm wine led to inactivation of glycerolipid, fatty acid, and arachidonic acid metabolisms, fatty acid biosynthesis and mitochondria fatty acid elongation pathways (**Figure 3.7.2**). Base on the p-value (**Figure 3.7.2**), biosynthesis of bile acid was significantly reduced. There was an activation of 3 additional pathways for plasmalogen synthesis, mitochondrial beta-oxidation of long chain saturated fatty acids, and retinol metabolism.

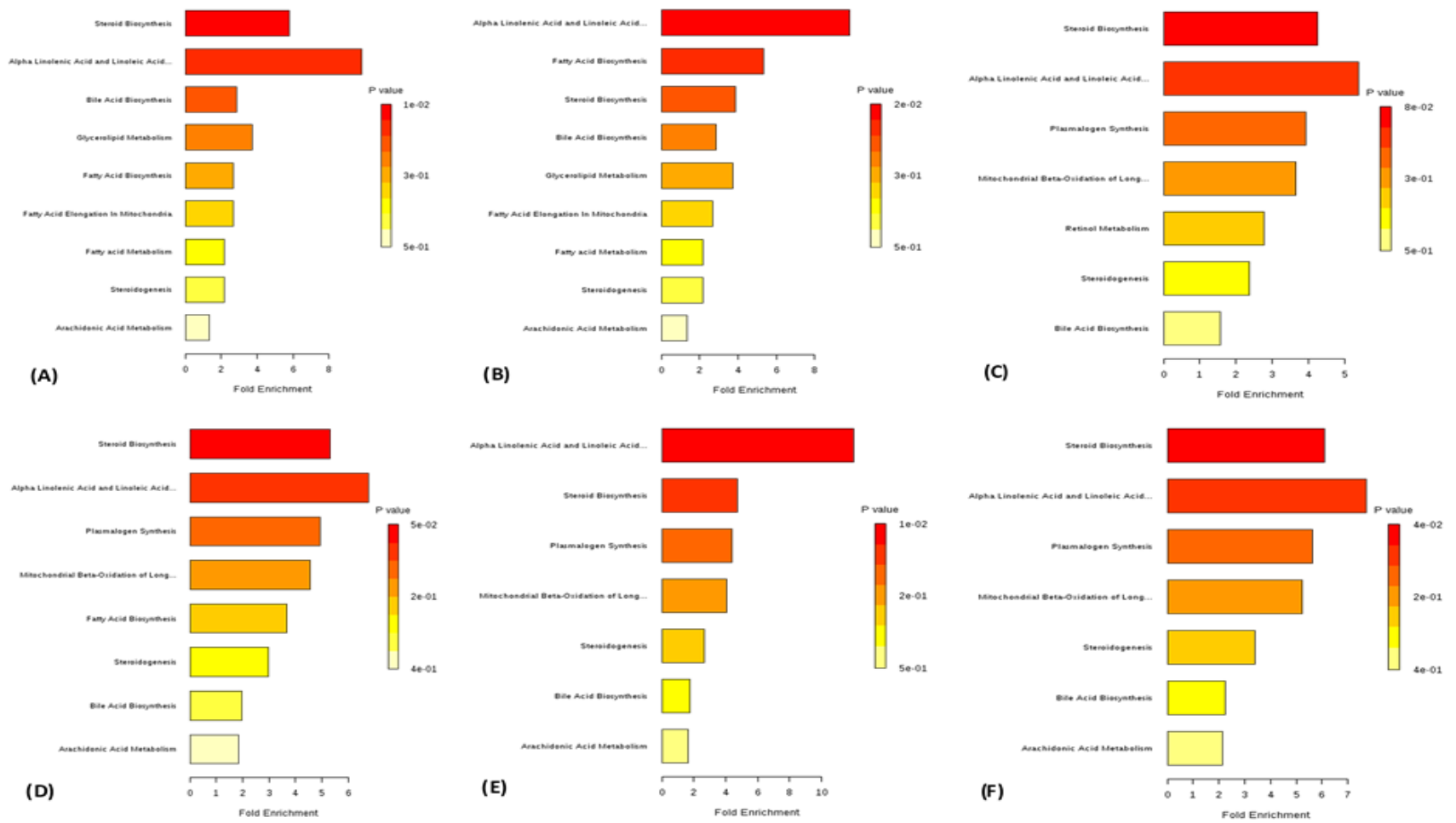

Figure 3.7.2: Pathway enrichment analysis of GC-MS identified testicular lipid metabolites of (A) NC; (B) DC; (C) DSG-LD; (D) DSG; (E) DSM; and (F) PHD. **NC** = normal rats, **DC** = diabetic control, **DSG-LD** = diabetic rats + Raffia palm wine (150 g/kg bw), **DSG** = diabetic rats + Raffia palm wine (300 g/kg bw), **DSM** = diabetic rats + metformin (200 g/kg bw), and **PHD** = Normal rats + Raffia palm wine (300 g/kg bw).

Higher dose of Raffia palm wine inactivated glycerolipid, and fatty acid metabolisms and fatty acid elongation in mitochondria pathways in T2D rats (**Table 3.7.2**), while concomitantly activating plasmalogen synthesis and mitochondrial beta-oxidation of long chain saturated fatty acids pathways. Based on the p-value (**Figure 3.7.2**), arachidonic acid was the least activated.

Treatment with metformin also activated plasmalogen synthesis and mitochondrial beta-oxidation of long chain saturated fatty acids pathways (**Table 3.7.2**), and inactivated the pathways for glycerolipid, fatty acid metabolisms, fatty acid biosynthesis, and elongation in mitochondria. This was also observed in normal rats administered Raffia palm wine (**Table 3.7.2**).

3.7.5 Discussion

The importance of testicular lipids has been demonstrated in their roles in spermatogenesis, sperm maturity, viability and capacitation, steroidogenesis, membrane remodeling of developing germ cells, and fertilization (Maqdasy et al. 2013; Pushpendra and Jain 2015; Shi et al. 2018). These lipids also increase the testes and sperm cells susceptibility to peroxidative damage inspite of the testicular oxygen torsion (Asadi et al. 2017; Erukainure et al. 2012), which alters the testicular functions. In this study, we investigated the effect of T2D on testicular lipid metabolites and their metabolic pathways.

Cholesterol has been recognized as a crucial lipid in the male reproductive system, as the testicular physiology is dependent on its homeostasis (Maqdasy et al. 2013; Pushpendra and Jain 2015). This is demonstrated by its role in endocytosis, intracellular signaling, membrane permeability, fluidity, and composition (Maqdasy et al. 2013). The high cholesterol level of the normal rats (NC) correlates with the identified metabolic pathways: steroid biosynthesis, bile acid biosynthesis, and steroidogenesis (**Table 3.7.2**). These pathways are involved in spermatogenesis and the generation of steroids important for fertility (Ajiboye et al. 2016; Maqdasy et al. 2013; Sèdes et al. 2017), with cholesterol as their major precursor (Maqdasy et al. 2013). Thus, their activation may indicate a functional reproductive system. The presence of the androgen derivative, androstane-3,11,17-triol may also attest to this. Palmitic acid is the most studied saturated fatty acid component of the testicular and sperm phospholipids (Štramová et al. 2015). Its presence in the NC group correlates with the steroid, bile acid, and fatty acid biosynthesis, glycerolipid, and fatty acid metabolisms, and fatty acid elongation metabolic pathways (**Table 3.7.2**). These pathways are also involved in

optimal testicular functions (Maqdasy et al. 2013; Sèdes et al. 2017), thus further demonstrating a functional reproductive system in the normal rats. Although both NC and DC groups had the same metabolic pathways, the high level of fatty acid biosynthesis in the latter can be attributed to the presence of tetradecanoic acid (myristic acid). The depleted cholesterol level coupled with the complete depletion of androstane-3,11,17-triol as well as the reduced level of steroid biosynthesis pathway in DC may suggest a suppressive effect on the reproductive system on induction of T2D. This corroborates previous reports on the suppressive effect of T2D on male fertility (Dinulovic and Radonjic 1990; Erukainure et al. 2012; Obode et al. 2015; Xu et al. 2014). The increased cholesterol level on treatment with Raffia palm wine, suggests a therapeutic potential as evident by the increased level of steroid biosynthesis and steroidogenesis (**Figure 3.7.2**). The reduced level of bile biosynthesis in the treated groups can be attributed to the complete depletion of palmitic acid, which is also evident in the inactivation of glycerolipid and fatty acid metabolisms, fatty acid biosynthesis and elongation pathways (**Figure 3.7.2** and **Table 3.7.2**).

There was activation of mitochondrial beta-oxidation of long chain saturated fatty acids in all the treated group (DSG-LD, DSG, DSM and PHD) (**Table 3.7.2**), which can be attributed to the presence of stearic acid (**Table 3.7.1**). Stearic acid, a non-essential fatty acid has been reported in the lipid composition of testicular tissues and sperm cells (Mateo and Roldan 2015) as well as a precursor and/or fuel for mitochondria beta-oxidation of long chain saturated fatty acids pathway (Singh et al. 1989). This pathway plays a major role in ATP production in tissues associated with high energy requirement (Adeva-Andany et al. 2018; Bastin 2014). It generates the electron carriers NADH and $FADH_2$, which is channeled to respiratory chain to produce ATPs. The acetyl-coA also produced, can either be channeled to the citric acid cycle to yield more electron carriers for ATP production or used as a precursor for the biosynthesis of other metabolites and/or hormones. The activation of this pathway also potentiates the therapeutic effects of the treatments on T2D-induced male reproductive toxicity, as spermatogenesis as well as sperm motility have been shown to be energy-demanding (Guraya 2012; Mukai and Travis 2012).

Similarly, the activation of plasmalogen synthesis (**Table 3.7.2**) in the treatment groups can also be attributed to the presence of stearic acid (**Table 3.7.1**). Plasmalogens have been recognized as one of the most predominant phospholipids commonly found in tissue membrane, with ethanolamine and choline-linked headgroups being the majors in animal cells (Gorgas et al. 2006; Nagan and Zoeller 2001). The activation of this pathway may be attributed to the development and

maintenance of testicular membrane integrity by Raffia palm wine and metformin, owing to the inactivation of glycerolipid metabolism. Plasmalogens have also been implicated in male fertility as they play a major role in spermatogonial amplification and differentiation during spermatogenesis (Gorgas et al. 2006), which is also evident on the arrest of spermatogenesis in plasmalogen-deficient mice (Gorgas et al. 2006; Zhang et al. 2005). They also makeup the plasma-membrane of spermatozoa (Gorgas et al. 2006).

The presence of the vitamin D metabolites, 24,25-dihydroxyvitamin D and 1,25-dihydroxyvitamin D3, TMS derivative in NC and PHD respectively (**Table 3.7.1**) connotes vitamin D metabolism in the normal rats. The physiological role of 24,25-dihydroxyvitamin D is still controversial, but it has been linked to calcium transportation and homeostasis (St-Arnaud and Glorieux 1998). Calcium plays an important role in cell signaling and spermatogenesis (Golpour et al. 2017; Lee et al. 2006), thus emphasizing the importance of this metabolite. While 1,25-dihydroxyvitamin D3 and its derivatives have been reported for their role in steroidogenesis and other testicular functions (Hofer et al. 2014; Lundqvist 2011; Lundqvist et al. 2010). The absence of these metabolites in T2D rats (untreated and treated) further denotes a suppressive effect on normal testicular functions.

The presence of the antioxidant metabolites, vitamin A aldehyde and ascorbic acid dipalmitate in DSG-LD and DSM respectively depicts an antioxidant activity. Oxidative stress has been recognized as a major mechanism by which DM induces testicular damage (Erukainure et al. 2012; Obode et al. 2015). It occurs when the hyperglycemia-generated free radicals surpasses the testicular antioxidant system (Obode et al. 2015). The antioxidant activities of both metabolites have been established (Mueller and Boehm 2011; Sirmali et al. 2014) as well as their therapeutic role in male reproductive dysfunction (Azari et al. 2014; Goralczyk et al. 1992; Hogarth et al. 2011; Vernet et al. 2006). The presence of vitamin A aldehyde correlates with the activation of retinal metabolism in DSG-LD group (**Table 3.7.2**) which plays a key role in spermatogenesis by regulating germ cell differentiation (Hogarth and Griswold 2010). The presence of vitamin A aldehyde can be attributed to the reported vitamin A content of Raffia palm wine (Chandrasekhar et al. 2012; Ogbonna et al. 2013).

Arachidonic, icosapentaenoic, docosahexaenoic, alpha- linolenic, and linoleic acids constitute the most studied polyunsaturated fatty acids (PUFA) in testicular and sperm phospholipids (Safarinejad et al. 2010; Štramová et al. 2015). Their roles in testicular – functions and antioxidant, and male fertility have been established (Safarinejad et al. 2010; Wathes et al. 2007). Due to their

unsaturation, they are susceptible to peroxidative attack (Štramová et al. 2015; Wathes et al. 2007) which also explains the susceptibility of the testicular tissue to oxidative damage. In this study, the presence of arachidonic, icosapentaenoic, and docosahexaenoic acids, as well as linolenic, and linoleic esters correlates with the activation of alpha linolenic acid and linoleic, and arachidonic acids metabolisms pathways (**Table 3.7.2**). Although induction of T2D had little or no effect on these pathways, the levels of the PUFAs were however altered with the complete depletion of docosahexaenoic acid. Docosahexaenoic acid has been reported for its diverse biological activities, particularly its role in steroidogenesis and male fertility (Maillard et al. 2018; Roqueta-Rivera et al. 2009). Thus, implying that the complete depletion in T2D rats may contribute to suppressed steroidogenesis.

3.7.6 Conclusion

These results potentiate the alterative effect of T2D on testicular lipids metabolites and their metabolic pathways which may have detrimental effect on steroidogenesis and spermatogenesis. The replenishment and generation of these metabolites and additional ones as well as activation of pathways involved in energy generation, phospholipids, antioxidant activity, steroidogenesis and spermatogenesis in the treated groups, suggests a therapeutic effect of Raffia palm wine against hyperglycemic – induced testicular lipid dysregulation.

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CHAPTER 4

THE ANTI-OXIDATIVE AND ANTI-DIABETIC ACTIVITIES OF *PHASEOLUS LUNATUS* Linn (LIMA BEANS) *IN VITRO* AND *EX VIVO*

4.1 Phaseolus lunatus L.

Figure 4.1: *Phaseolus lunatus* seeds; Common names*:* lima beans *(English);* akidi *(Igbo, Nigeria);* fève créole, haricot de lima *(French);* indische mondbohne, limabohne, mondbohne *(German);* sem phali *(Hindi);* alubia de lima *(Spanish). Photo: https://phytomaniac.com/high-protein-legumes/ (accessed on July 11, 2018 without permission).*

4.1.2 Background

Phaseolus lunatus L. is regarded globally as a minor crop and amongst the underutilized legumes despite its nutritional and medicinal properties, diversity, and yield potential (Nwokolo and Smartt 1996). It is an annual perennial crop cultivated in both temperate and sub-tropic regions of the world, with wide spread in Canada, Argentina, as well as in Asia, Africa, Europe and Australia (Adeparusi 2001; Baudoin et al. 2008; Nwokolo and Smartt 1996).

4.1.3 Ethnopharmacological uses

The seeds have been reported for their low glycaemic and insulinomic indices (Brand et al. 1990). Decoctions of the seeds are used in treating and managing diabetes (Ezuruike and Prieto 2014).

Macerations from the seeds are rubbed on small cuts, tumors and boils for pus discharge (Royal Botanic Gardens 2016). The seeds are employed in treating fever (Saleem et al. 2016).

4.1.4 Biological activities

The protein hydroxylates from *P. lunatus* have been demonstrated to inhibit Angiotensin converting enzyme-1 (ACE-I) activity (Betancur-Ancona et al. 2015; Chel-Guerrero et al. 2012). They have been reported for their potent free radical scavenging activities (Agostini-Costa et al. 2015; Sundaram et al. 2013). Its other reported biological activities include antidiabetic and hypocholesterolemic (Johnson et al. 2013; Oboh and Omofoma 2008), antifungal and antiproliferative (Saleem et al. 2016), cysteine proteinase, trypsin and chymotrypsin inhibition (Lawrence and Nielsen 2001; Saleem et al. 2016).

4.1.5 Nutritional properties

The seeds of *P. lunatus* are rich sources of the dietary minerals, calcium, magnesium, sodium, iron, zinc, potassium and phosphorus (Saleem et al. 2016). They are also rich in vitamins A, B2, B3, B6, C, E and K (Bolade et al. 2017; Saleem et al. 2016). The seed coats have been reported to be rich in threonine, valine, isoleucine, tryptophan, leucine, lysine and histidine (Seidu et al. 2015).

4.1.6 Phytochemistry

Phytochemical studies on *P. lunatus* revealed the presence of tannins, saponins and its derivatives, sapogenol and sojasapogenol (Oboh et al. 1998), robinin, cyanidin (Onyilagha and Islam 2009), lunatusin, kievitone and its derivatives, kievitol and cyclokievitone (Wong and Ng 2005), phaseollidin, coumestrol, lunatone, psoralidin (O'Neill et al. 1986), cyanogenic glycosides (Akande et al. 2010), cytokinin (Mok et al. 1982), linamarin (Frehner and Conn 1987), phaseolin (Moraes et al. 2000), and phytic acid (Adeparusi 2001).

3.1.7 Aims and objective

The aims of this study are to investigate the antioxidative and antidiabetic activities of the aqueous extract of *P. lunatus*, using *in vitro* and *ex vivo* models as well as its phytochemical and amino acid constituents.

4.2 *Phaseolus lunatus* (Lima Beans) Abates Fe^{2+}-induced hepatic redox imbalance; inhibits Intestinal Glucose Absorption and Major Carbohydrate and Lipids Catabolic Enzymes; and Modulates Muscle Glucose Uptake

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Preface: This article investigated the *in vitro* antioxidant and enzyme inhibitory activities, as well as the *ex vivo* antioxidative, and glucose absorption and uptake effects of *P. lunatus*. The amino acid and phytochemical constituents were also investigated. It has been published in Journal of Food Biochemistry (**Erukainure OL**, Mopuri R, Chukwuma CI, Koorbanally NA, Islam MS (2018) *Phaseolus lunatus* (Lima Beans) Abates Fe2+-induced hepatic redox imbalance; inhibits Intestinal Glucose Absorption and Major Carbohydrate Catabolic Enzymes; and Modulates Muscle Glucose Uptake. Journal of Food Biochemistry. DOI: 10.1111/jfbc.12655).

4.2.1 Abstract

The antioxidative and antidiabetic effects of the aqueous extract of *Phaseolus lunatus* were investigated *in vitro* and *ex vivo*. The seeds were subjected to aqueous extraction, after defatting with n-hexane. The extract (15, 30, 60, 120, 240 µg/mL) showed significant ($p<0.05$) free radical scavenging and enzyme inhibitory activities *in vitro*. It significantly ($p<0.05$) elevated GSH level with concomitant depletion of malondialdehyde (MDA) level as well as increased superoxide dismutase (SOD) and catalase activities in rat liver. Fe^{2+}-induced hepatic DNA fragmentation was also significantly ($p<0.05$) suppressed. The extract significantly inhibited intestinal glucose absorption and increased muscle glucose uptake with and without insulin. The inhibitory activities

and reversion of hepatic redox imbalance, as well as inhibition of intestinal glucose absorption and increased muscle glucose uptake by *P. lunatus* suggest its anti-oxidative and anti-hyperglycemic effects. These can be ascribed to the synergistic effect of the phytochemicals and amino acids identified in the extract.

KEYWORDS: Anti-hyperglycemia; Antioxidative; Enzymes inhibition; Legumes; and *Phaseolus lunatus*

4.2.2. Introduction

Oxidative stress in type 2 diabetes (T2D) has been attributed to hyperglycemia–induced elevated reactive oxygen species (ROS), which causes an imbalance in the body's antioxidant system (Tiwari et al. 2013). It has been implicated in the progression of T2D, which could lead to chronic pathogenic micro- and macro-vascular complications such as nephropathy, microangiopathy, retinopathy and death (Barar 2000; Constantino et al. 2013). Thus, making T2D one of the major contributors to global mortality and morbidity (IDF 2016). Unlike type 1 diabetes, T2D has been described as a metabolic disorder, which affects carbohydrate, protein and lipid metabolism and characterized by insulin resistance and pancreatic β-cell dysfunction (Maritim et al. 2003).

In most regions of the world, legumes have been recognized as an important source of dietary protein especially in developing countries where animal proteins are not affordable by most human beings. Legumes are also good source of carbohydrates, dietary fibres, bioactive peptides, essential amino acids and vitamins (Yellavila et al. 2015). In addition to the nutritional benefits, their medicinal properties have also been reported in many previous studies. Protective effects against cardiovascular disease and diabetes have also been correlated with the consumption of legumes (Bahadoran and Mirmiran 2014). The abilities of legume to inhibit α-amylase and β-glucosidase activities have also been reported previously (Yao et al. 2012). Their attenuation of postprandial glycemic response and suppression of dyslipidemia has also been reported previously (Preuss 2009; Thompson et al. 2012). These activities can be linked to the reported presence of amino acids, isoflavones, phenolic acids, saponins, and phytic acid in legumes (Bahadoran and Mirmiran 2014).

However, there are little or no scientific evidences to ascertain the antidiabetic claims of *P. lunatus*. Thus, this paper aimed to report the antioxidative activities of the aqueous extract of *P. lunatus*

seeds on oxidative imbalance in hepatic tissues; its inhibitory effect on key carbohydrate and lipids digestive enzymes linked to T2D; its effect on intestinal glucose absorption and muscle glucose uptake; as well as its amino acid and phytochemical compositions.

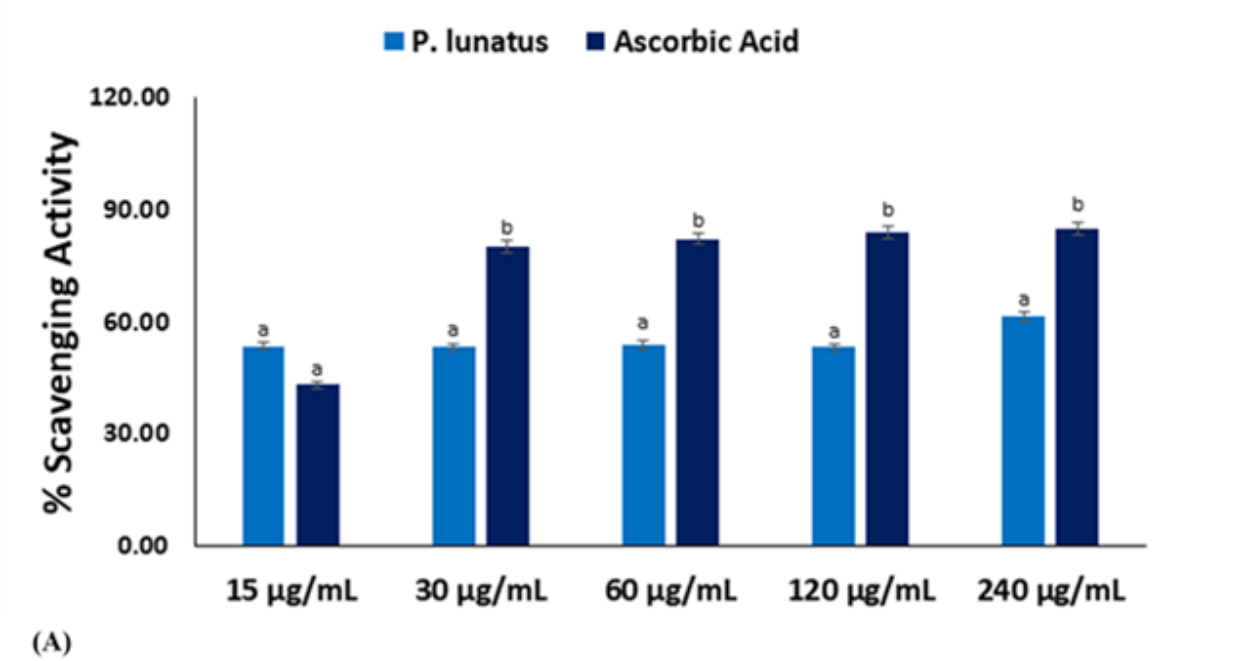
4.2.3 Materials and Methods

Kindly refer to chapter 2; sub sections 2.1 – 2.1.2; 2.1.5; 2.6 – 2.7.2; 2.10.1; 2.10.6, 2.11.1 and 2.13 for details.

4.2.4 Results

Total phenol analysis revealed a rather very low phenolic content (2.89 ± 0.006 µg/g Gallic Acid Equivalent) in the *P. lunatus* extract. However, the extract showed significant ($p<0.05$) antioxidant activities compared to ascorbic acid measured by DPPH and FRAP methods as depicted in **Figures 4.2.1A and 4.2.1B**, respectively. Their lower IC_{50} values indicate potent antioxidant activities of *P. lunatus* compared to ascorbic acid.

IC_{50} (µg/mL): *P. lunatus* = 6.60; Ascorbic Acid = 8.31



(A)

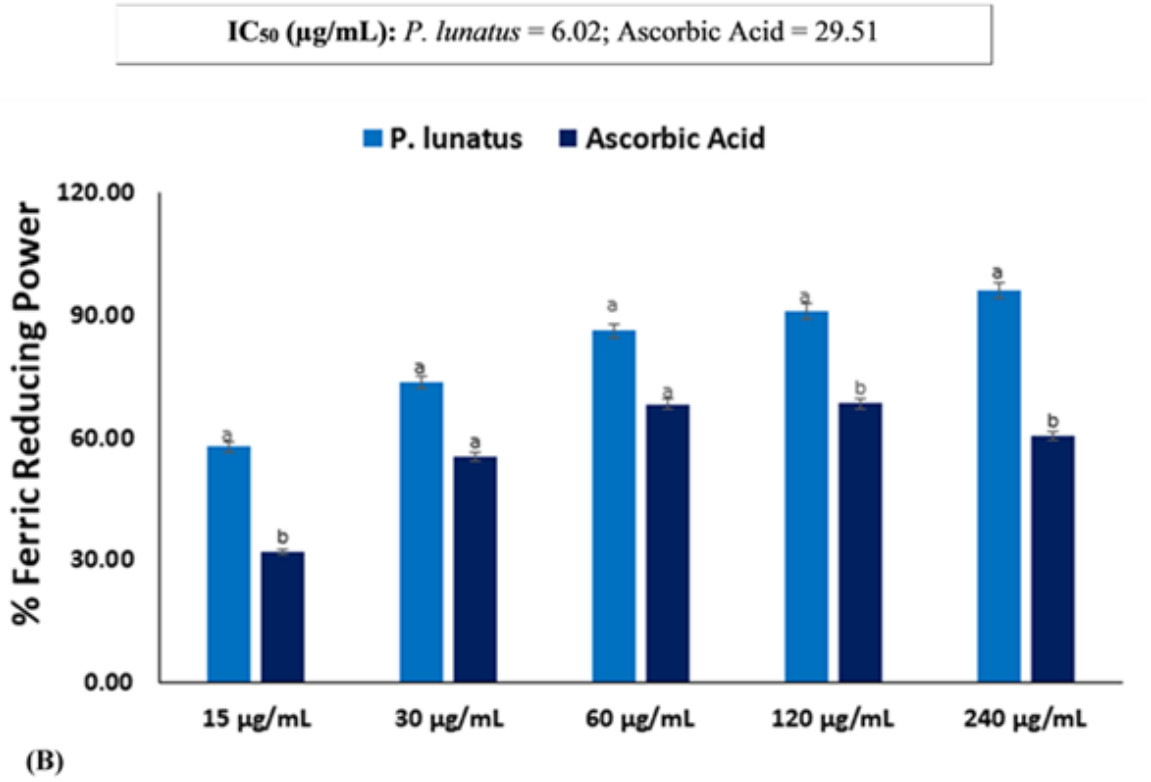


Figure 4.2.1: (A) DPPH scavenging activity; and (B) ferric reducing power of *P. lunatus* aqueous extract. Data are presented as mean ± SEM. [ab]Values with different letters above the bars for a given concentration are significantly different from each other ($p < 0.05$, Tukey's HSD-multiple range post-hoc test, IBM SPSS for Windows).

Incubation of hepatic tissues with $FeSO_4$ caused a significant ($p<0.05$) reduction in GSH level indicating an incidence of oxidative injury as depicted in **Figure 4.2.2A**. Treatment with the extract significantly ($p<0.05$) elevated the GSH levels as compared to gallic acid, with the highest concentration of *P. lunatus* showing an 83.39% increase which portrays a potent antioxidant activity.

IC_{50} (µg/mL): *P. lunatus* = 52.48; Gallic Acid = 50.56

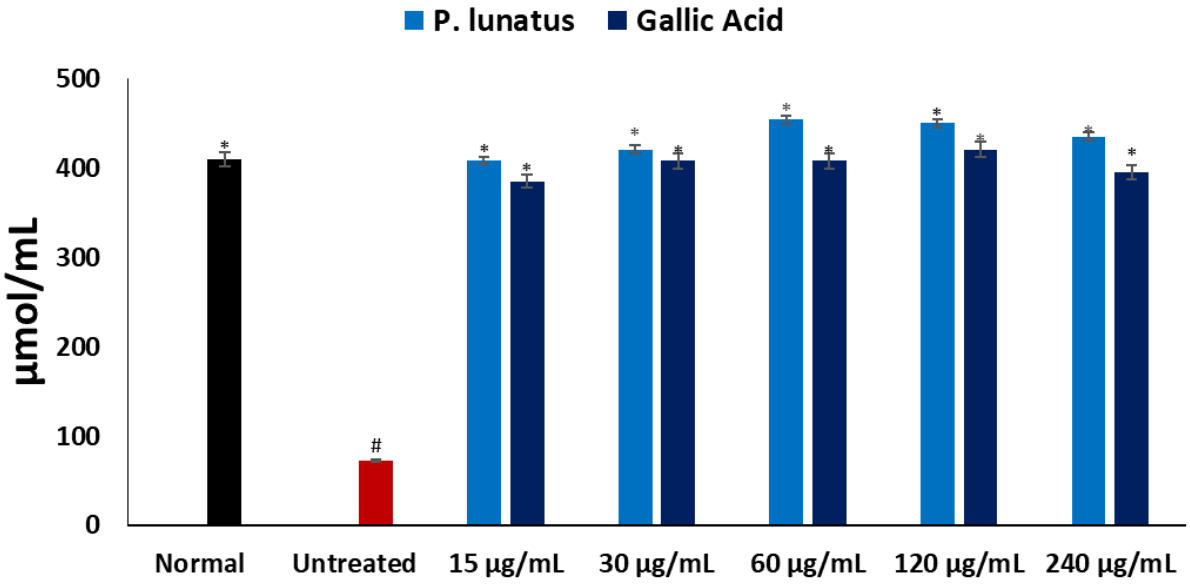


Figure 4.2.2A: Effects of *P. lunatus* aqueous extract on GSH level in oxidative hepatic injury. Data are presented as mean ± SEM. *Significantly different from untreated sample and #Significantly different from normal sample ($p < 0.05$, Tukey's HSD-multiple range post-hoc test, IBM SPSS for Windows).

The reduced SOD and catalase activities in hepatic tissues further indicate an occurrence of oxidative injury on incubation with $FeSO_4$ as depicted in **Figures 4.2.2B and 4.2.2C** respectively. Treatment with the extract caused an increase in their activities, with their highest concentration causing 77.91 and 44.87% increases respectively. *P. lunatus* showed a more potent SOD activity (**Figure 4.2.2B**) than gallic acid as indicated by its lower IC_{50} value.

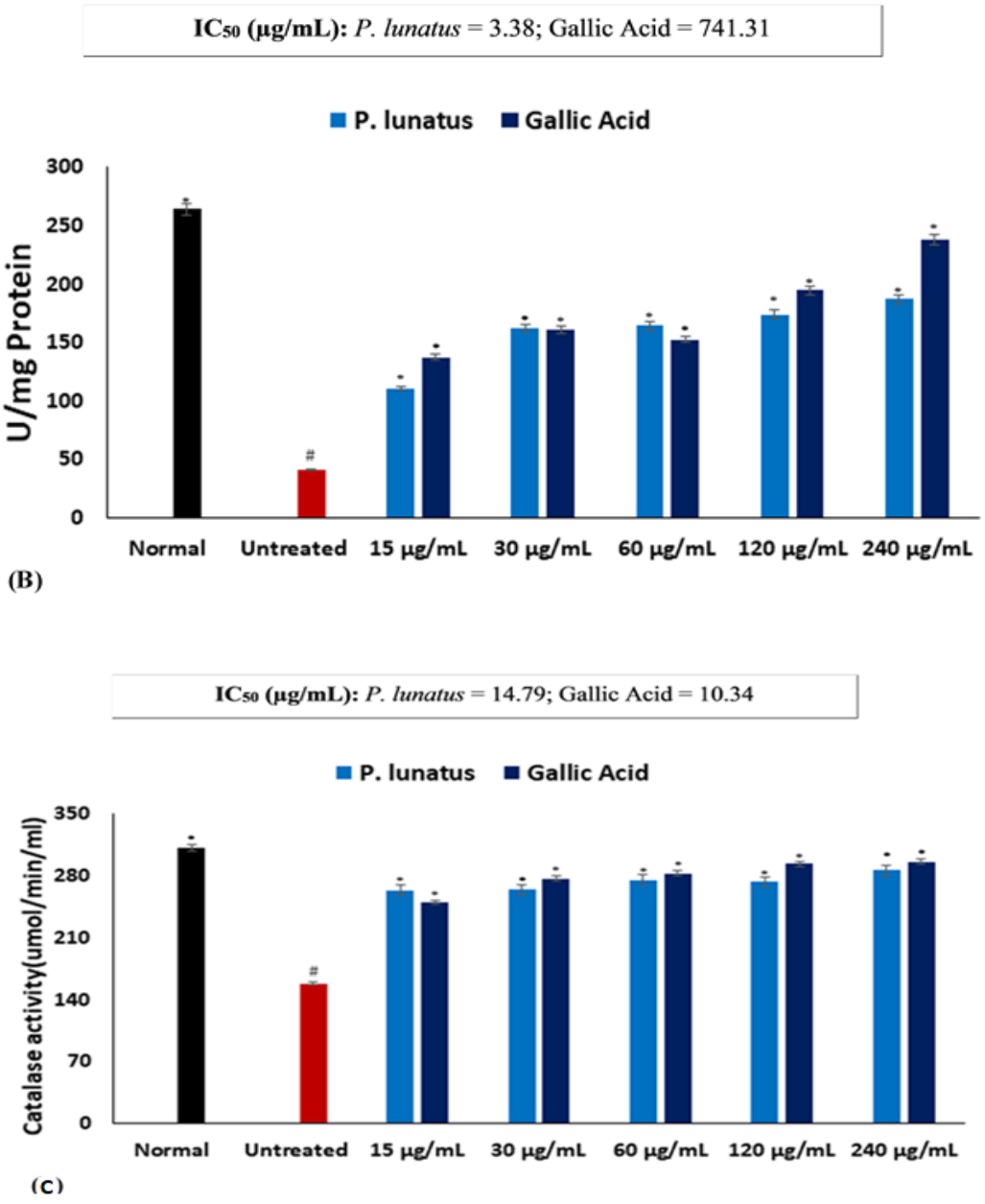


Figure 4.2.2: Effect of *P. lunatus* aqueous extract on (B) SOD and (C) catalase activities in oxidative hepatic injury. Data are presented as mean ± SEM; U = µmol/mL. #Significantly different from normal sample and *Significantly different from untreated sample ($p < 0.05$, Tukey's HSD-multiple range post-hoc test, IBM SPSS for Windows).

The increased hepatic MDA level on incubation with $FeSO_4$ indicates a manifestation of lipid peroxidation as shown in **Figure 4.2.2D**. Treatment with the extract caused a dose–dependent reduction, with the highest concentration causing a 40.82% decrease.

IC_{50} (µg/mL): *P. lunatus* = 58.88; Gallic Acid = 17.37

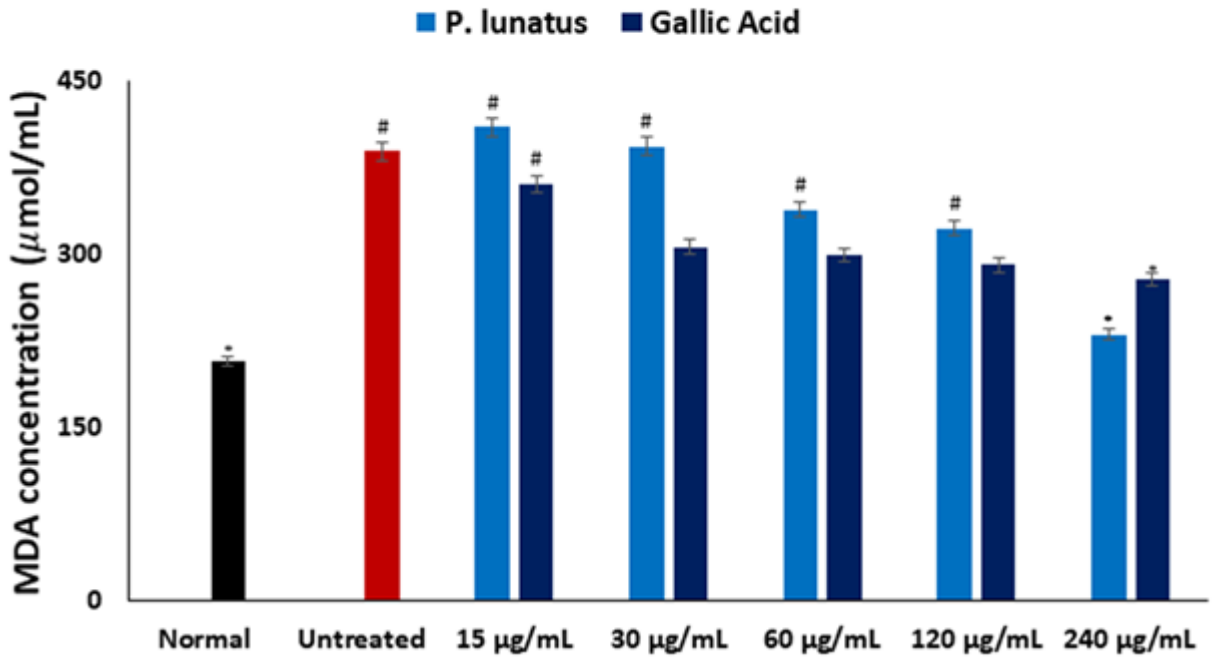


Figure 4.2.2D: Effect of *P. lunatus* aqueous extract on lipid peroxidation in oxidative hepatic injury. Data are presented as mean ± SEM. *Significantly different from untreated sample and #Significantly different from normal sample ($p < 0.05$, Tukey's HSD-multiple range post-hoc test, IBM SPSS for Windows).

The extract significantly inhibited α–glucosidase activity compared to acarbose as depicted in **Figures 4.2.3A**. The extract showed little or no significant inhibitory activity on pancreatic lipase as depicted in **Figure 4.2.3B**. The extract also showed significant inhibition of α-amylase compared to acarbose particularly at 15 µg/mL as depicted in **Figure 4.2.3C.**

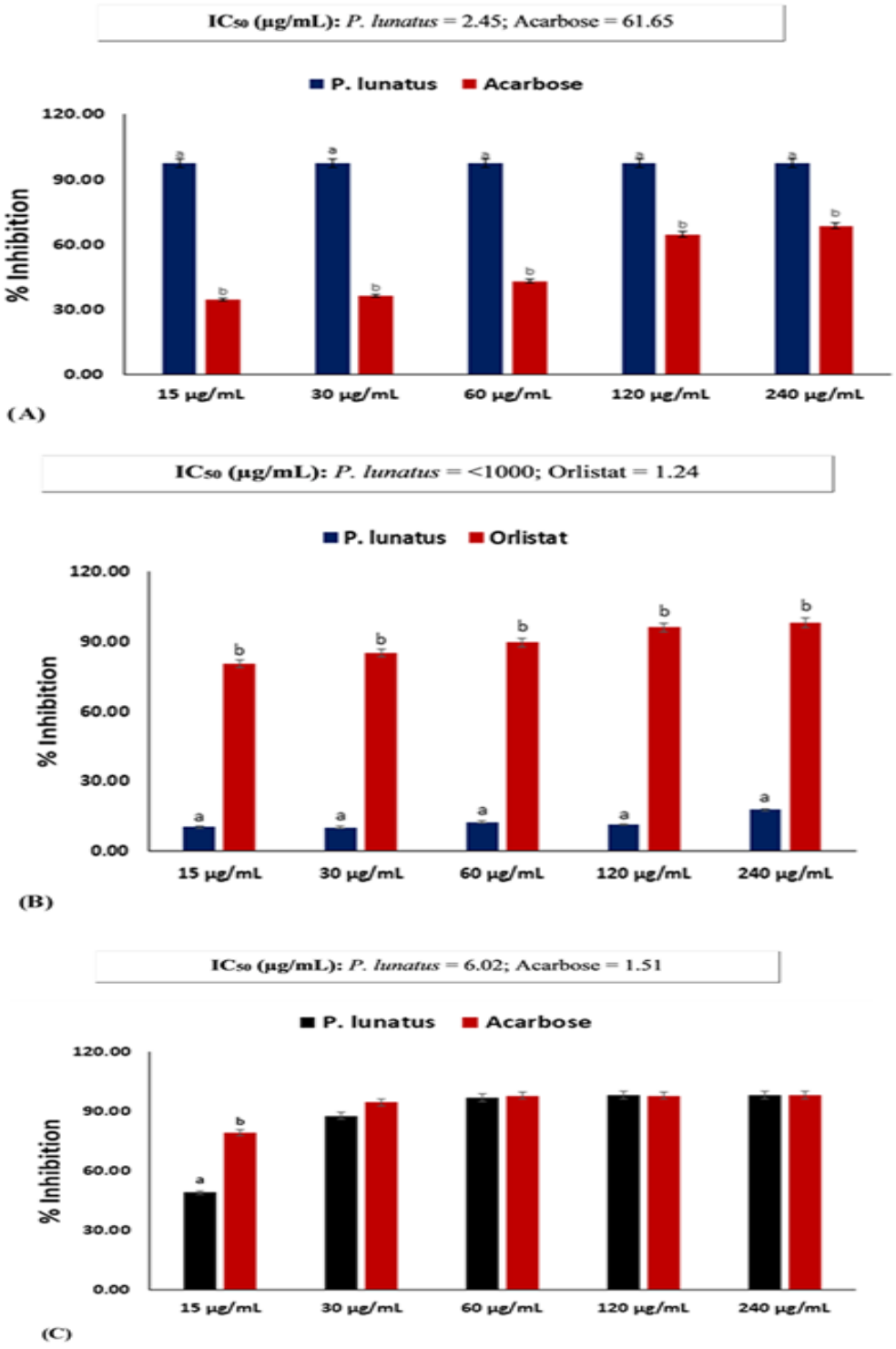


Figure 4.2.3: (A) α-glucosidase, (B) pancreatic lipase, and (C) α-amylase inhibitory activities of *P. lunatus* aqueous extract. Data are presented as mean ± SEM. [ab]Values with different letter above the bars for a given concentration are significantly different from each other ($p < 0.05$, Tukey's HSD-multiple range post-hoc test, IBM SPSS for Windows).

Induction of oxidative injury on incubation with $FeSO_4$ led to significant ($p<0.05$) increase in glucose-6-phosphatase activity as depicted in **Figure 4.2.4**. Treatment with the extract significantly inhibited the activity, when a dose dependent effect was observed as the concentration increased from 60 – 240 µg/mL. The highest concentration caused a 59.52% inhibitory effect, thus portraying a hypoglycemic potential.

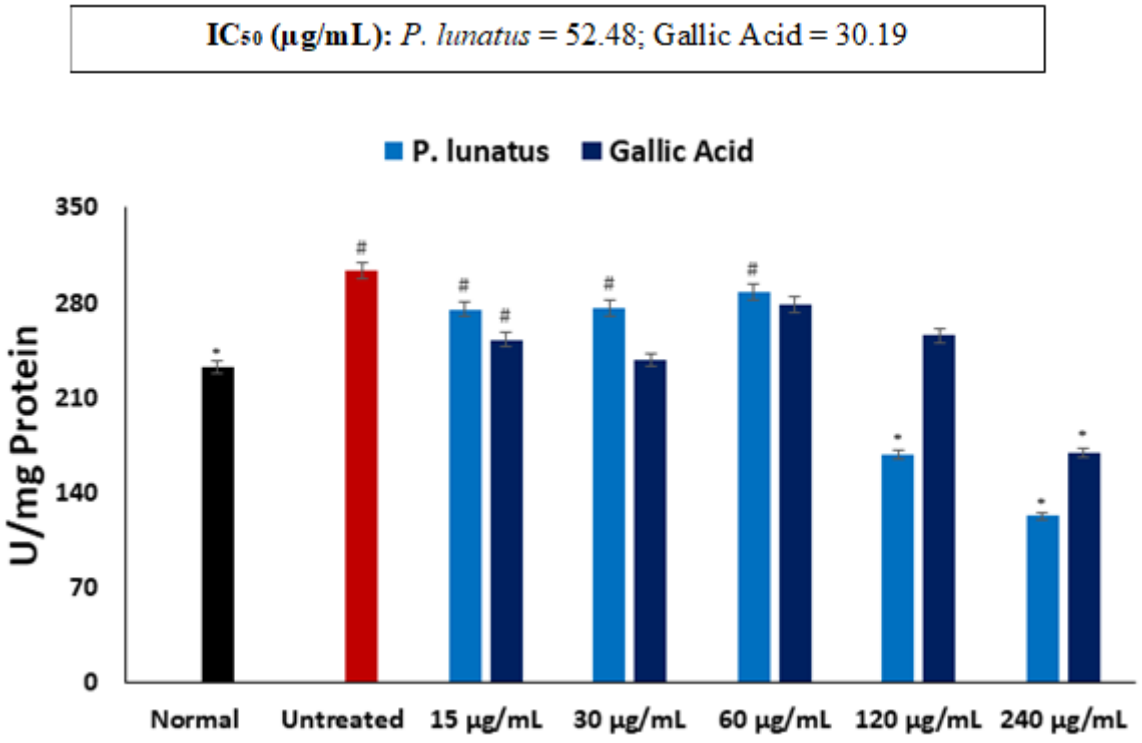


Figure 4.2.4: Effect of *P. lunatus* aqueous extract on Glucose-6-Phosphatase activity in oxidative hepatic injury. Data are presented as mean ± SEM; U = µmol/mL. *Significantly different from untreated sample and #Significantly different from normal sample ($p< 0.05$, Tukey's HSD-multiple range post-hoc test, IBM SPSS for Windows).

Induction of oxidative hepatic injury led to significant ($p<0.05$) increase in DNA fragmentation as shown in **Figure 4.2.5**. This was significantly ($p<0.05$) and dose dependently reduced after the treatment with extract, when highest concentration caused a 39.12% reduction.

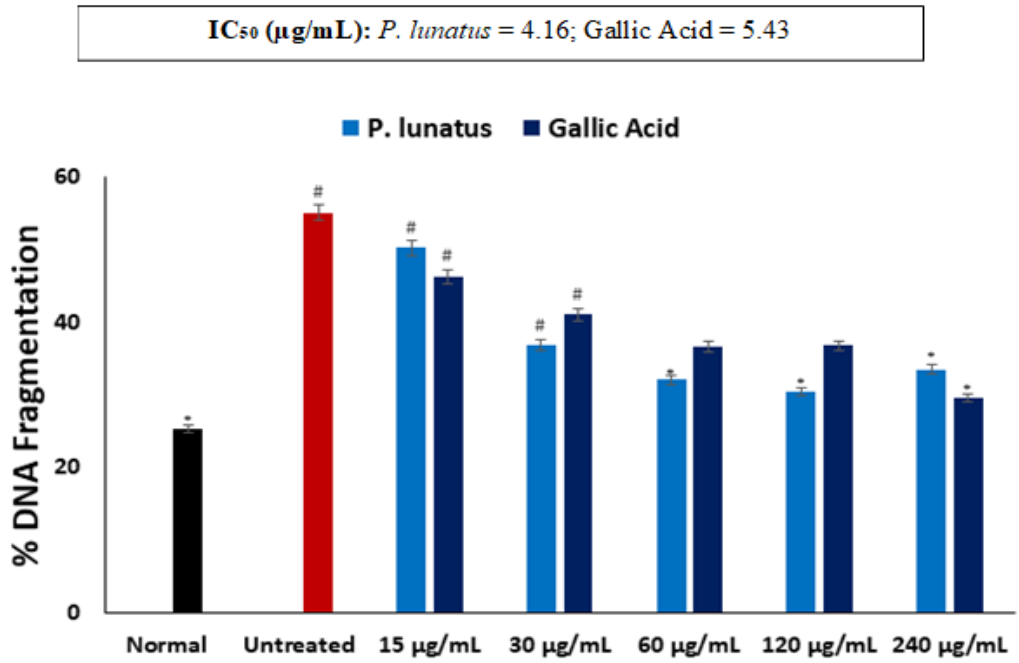


Figure 4.2.5: Effect of *P. lunatus* aqueous extract on DNA fragmentation in oxidative hepatic injury. Data are presented as mean ± SEM. *Significantly different from untreated sample and #Significantly different from normal sample ($p < 0.05$, Tukey's HSD-multiple range post-hoc test, IBM SPSS for Windows).

Incubation of isolated rat jejunal tissue with *P. lunatus* extract led to significant ($p<0.05$) inhibition of glucose absorption dose-dependently with an IC_{50} of 0.53% and the highest concentration causing an 81.25% inhibition as depicted in **Figure 4.2.6A**. Incubation of isolated rat psoas muscle with *P. lunatus* extract without insulin exhibited a significant ($p<0.05$) and dose-dependent increase in glucose uptake, 94.44% increase was observed for the highest concentration as depicted in **Figure 4.2.6B**. This effect was significantly ($p<0.05$) enhanced by *P. lunatus* extract on incubation with insulin as the highest concentration caused a 92.31% increase depicting a synergetic effect.

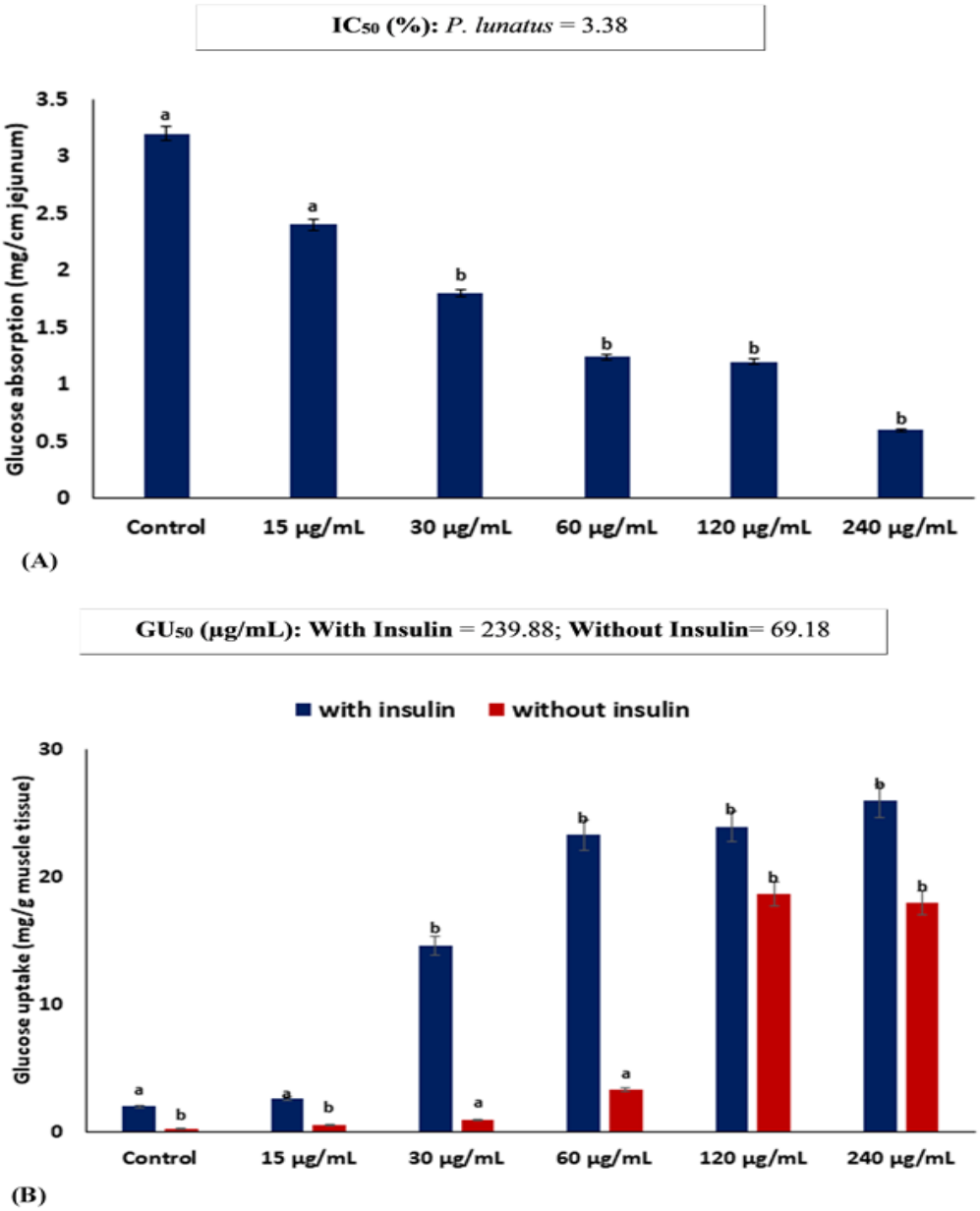


Figure 4.2.6: Effects of *P. lunatus* aqueous extract on (A) glucose absorption in isolated rat jejunum and (B) glucose uptake with or without insulin in isolated rat psoas muscle. Data are presented as mean ± SEM. [ab]Values with different letter above the bars for a given concentration are significantly different from each other ($p < 0.05$, Tukey's HSD-multiple range post-hoc test, IBM SPSS for Windows).

GC-MS analysis of *P. lunatus* aqueous extract showed the presence of 6,7-pentadecadien-1-ol; phytol acetate; hexadecanoic acid methyl ester; 9,12-octadecadienoic acid methyl ester;

Octadecanoic acid methyl ester; N-Acetyl-l-alanine ethylamide; 2-Acetylpyridine; beta.-stigmasterol; and stigmast-5-en-3-yl (9Z)-9-octadecenoate as shown in **Figure 4.2.7** and **Table 4.2.1**.

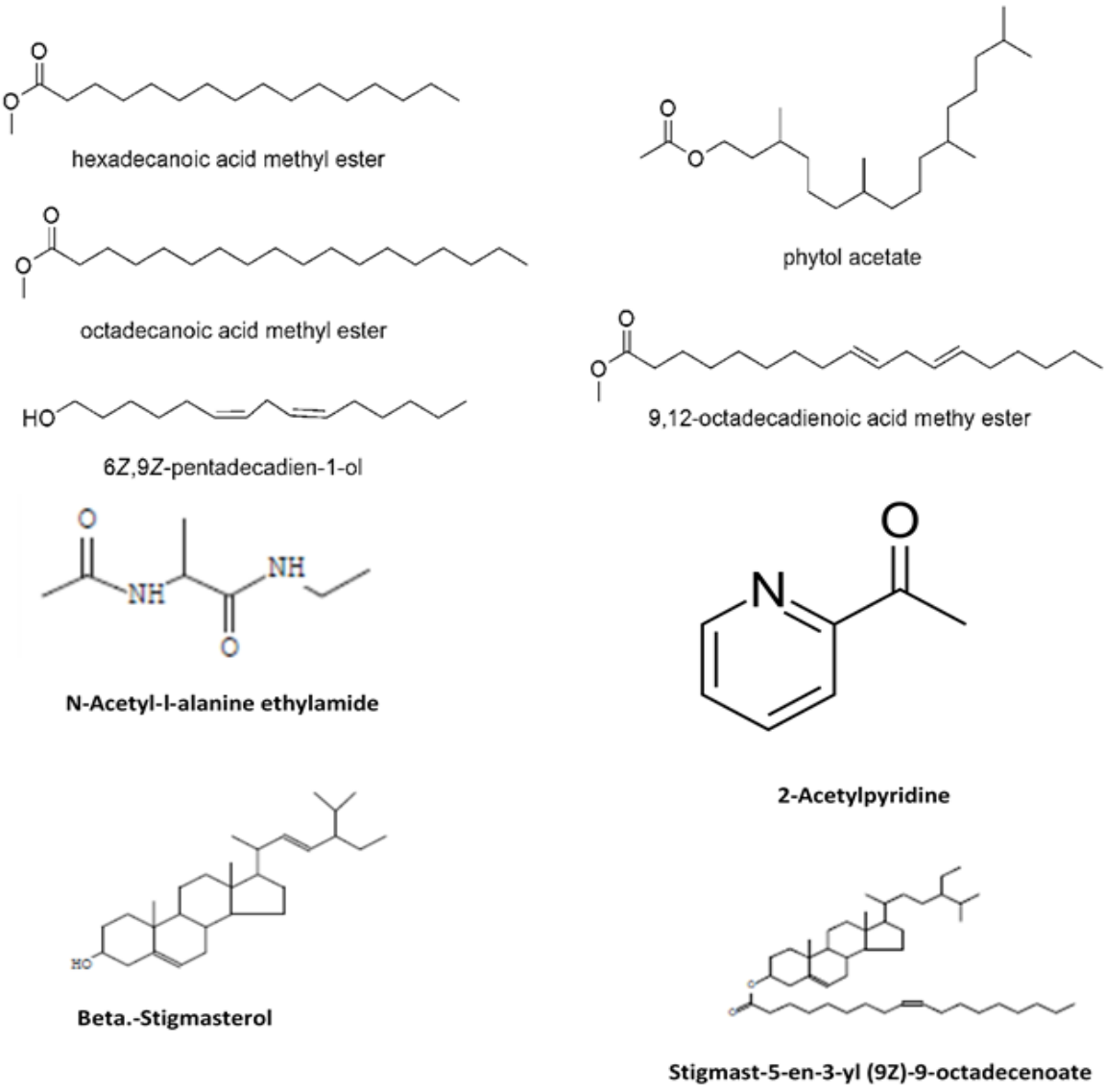


Figure 4.2.7: Structures of the compounds identified in the aqueous extract of *P. lunatus*

Table 4.2.1: Identified compounds of the aqueous extract of the seeds of *P. lunatus* by GC-MS

Compounds	RT (min)	Molecular mass (g/mol)	Relative abundance (%)
N-Acetyl-l-alanine ethylamide	5.39	131	4.68
2-Acetylpyridine	6.29	121	7.56
Phytol acetate	16.36	338	3.16
Hexadecanoic acid methyl ester	17.26	270	8.23
9,12-octadecadienoic acid methyl ester	18.90	294	2.10
Octadecanoic acid methyl ester	19.20	298	3.48
6,7-pentadecadien-1-ol	19.37	224	10.70
Beta.-Stigmasterol	26.42	413	6.75
Stigmast-5-en-3-yl (9Z)-9-octadecenoate	26.84	679	4.90

Amino acid analysis of *P. lunatus* revealed the concentration of glutamic acid to be the highest (**Table 4.2.2**). This was followed by aspartic acid and leucine, while cysteine was the least. The essential amino acids constituted 39.28% of the total amino acids concentration, of which the branched chain amino acids were 17.46% with leucine having the highest in concentration.

Table 4.2.2: Amino acids composition of the aqueous extract of the seeds of *P. lunatus*

Amino Acids	**Concentration (g/100g protein)**
Leucine	7.70 ± 0.50
Lysine	5.54 ± 0.09
Isoleucine	2.29 ± 0.01
Phenylalanine	3.55 ± 0.10
Tryptophan	0.81 ± 0.02
Valine	4.00 ± 0.08
Methionine	1.01 ± 0.11
Proline	3.25 ± 0.03
Arginine	6.53 ± 0.05
Tyrosine	2.75 ± 0.29
Histidine	3.19 ± 0.07
Cystine	0.84 ± 0.02
Alanine	4.28 ± 0.44
Glutamic acid	12.11 ± 0.59
Glycine	4.20 ± 0.13
Threonine	3.80 ± 0.40
Serine	4.00 ± 0.26
Aspartic acid	10.29 ± 0.91

Values = mean ± SEM; n = 3.

Prediction of oral toxicity of the identified compounds on ProTox (tox.charite.de) web-based server showed that all the identified compounds are in class 6, except hexadecanoic acid methyl, octadecanoic acid methyl esters, N-Acetyl-l-alanine ethylamide, 2-Acetylpyridine, beta.-stigmasterol, and stigmast-5-en-3-yl (9Z)-9-octadecenoate. Hexadecanoic acid methyl, octadecanoic acid methyl esters, 2-Acetylpyridine and stigmast-5-en-3-yl (9Z)-9-octadecenoate were in class 5. While N-Acetyl-l-alanine ethylamide and beta.-stigmasterol were class 4 as depicted in **Table 4.2.3**.

Table 4.2.3: Oral Toxicity prediction of identified compounds of the aqueous extract of *P. lunatus*

Ligands	Predicted LD_{50} (mg/kg)	Toxicity Class	Prediction Accuracy (%)
6,7-pentadecadien-1-ol	5600	6	70.97
Phytol acetate	8000	6	72.9
Hexadecanoic acid methyl ester	5000	5	100
9,12-octadecadienoic acid methyl ester	20000	6	72.9
Octadecanoic acid methyl ester	5000	5	100
N-Acetyl-l-alanine ethylamide	1750	4	69.26
2-Acetylpyridine	2280	5	100
Beta.-Stigmasterol	890	4	72.9
Stigmast-5-en-3-yl (9Z)-9-octadecenoate	3000	5	70.97

4.2.5. Discussion

The increasing prevalence of diabetes with concomitant high cost of treatment particularly in developing countries has led to the search for novel affordable therapies to combat the disease. The antioxidative and anti-hyperglycemia properties of pulses particularly legumes are well documented (Pari and Venkateswaran 2003; Singhal et al. 2014). This has been attributed to the synergetic effect of their phytochemicals as well as amino acid constituents (Grover et al. 2002; Newsholme et al. 2007). In this study, the aqueous extract of *P. lunatus* was investigated for its antioxidative vis-à-vis anti-diabetic potentials *in vitro* and *ex vivo*.

The dose-dependent DPPH scavenging activity of the extract suggests a free radical mopping potential of *P. lunatus* (**Figure 4.2.1A**). This is further evident by the potent reducing power displayed by the extract (**Figure 4.2.1B**). Several studies have documented the *in vitro* free radical scavenging properties of legumes (Sowndhararajan et al. 2011, Zia-Ul-Haq et al. 2013, Adefegha et al. 2017). The abilities of *Vigna vexillata*, *Vigna unguiculata* and M*ucuna pruriens* to scavenge free radicals *in vitro* have been reported as well (Adefegha et al. 2017; Sowndhararajan et al. 2011; Zia-Ul-Haq et al. 2013). The antioxidant activities were linked to their total phenolic contents. The total phenolic content of *P. lunatus* in this study was rather very low, thus suggesting synergistic effect of the amino acids, especially the hydrophobics, which have been reported in peptides with high antioxidant activity (Zou et al. 2016). The antioxidant activity of phytol derivatives have also been studied (Santos et al. 2013), thus suggesting the presence of phytol acetate (**Table 4.2.1** and **Figure 4.2.7**) may also contributed to the free radical scavenging activity.

The depleted level of GSH on incubation of hepatic tissue with $FeSO_4$ indicates an occurrence of oxidative damage, as depleted levels have been regarded as a marker for oxidative stress (**Figure 4.2.2A**). It is the body's first line of endogenous antioxidant defense system and abundantly found in hepatic tissues (Mohamed et al. 2016; Tiwari et al. 2013). The observed depletion can be attributed to the oxidation of Fe^{2+} to Fe^{3+} leading to the production of ROS. The increased level on treatment with the *P. lunatus* extract indicates an anti-oxidative therapeutic activity against oxidative hepatic injury.

Abundant levels of SOD and catalase levels have been reported in hepatic tissues (Davari et al. 2013). SOD catalyzes the dismutation of $O_2^{\cdot -}$ to H_2O_2, which is then converted to O_2 and H_2O

(Erukainure et al. 2017). Thus, their depleted activities in the hepatic tissue on incubation with $FeSO_4$ depict an incidence of oxidative hepatic injury (**Figures 4.2.2B and 4.2.2C**). Their significant ($p<0.05$) increase on treatment with *P. lunatus* extract also substantiates its antioxidative potentials.

Depletion of cellular GSH level and concomitant decreased catalase activity leads to an accumulation of H_2O_2. In Fe^{2+} toxicity, there is a continuous reaction of the accumulating H_2O_2 with the unstable cation yielding hydroxyl radicals ($^{\bullet}OH$) (Aslan et al. 2000). The generated hydroxyl radicals then attack the membrane lipid, leading to production of highly reactive aldehydes, including MDA, acrolein, 4-hydroxynonenal (HNE), 4-oxononenal (ONE), and isolevuglandins (IsoLGs) (Guo et al. 2012). This causes a disturbance in the membrane assembly and normal metabolic processes. The increased MDA level in the untreated tissues indicates an occurrence of lipid peroxidation. This also corroborates with the depleted GSH level and decreased catalase activity, thus further depicting an incidence of oxidative damage on incubation with $FeSO_4$. This was significantly ($p<0.05$) reversed by *P. lunatus* extract, indicating an anti-peroxidative effect (**Figure 4.2.2D**).

Inhibition of key carbohydrate catabolic enzymes has been demonstrated in several studies as the mode of action of most antidiabetic drugs and herbs (Ademiluyi and Oboh 2012; Ademiluyi and Oboh 2013). Their inhibition translates to a lower postprandial blood glucose level (Chukwuma and Islam 2015). The dose–dependent inhibition of α–glucosidase (**Figure 4.2.3A**) by the extract portrays the ability of *P. lunatus* to cause a delay in carbohydrate digestion, depicting an anti-hyperglycemic effect. Furthermore, the inhibition of α-amylase (**Figures 4.2.3C**) by the extract also indicates the anti-hyperglycemia activity of *P. lunatus*. Similar activities reported by Ademiluyi & Oboh (2012, 2013) for soybean, African locust bean, bambara groundnut, African yam bean, and melon seed corroborates the inhibitory effect of legumes on α–glucosidase. The little or no inhibitory effect by *P. lunatus* on pancreatic lipase suggests a weak anti-obesogenic activity.

The increased glucose-6-phosphatase activity in the untreated tissue indicates an incidence of increased gluconeogenesis and glycogenolysis (**Figure 4.2.4**). This reflects an occurrence of hyperglycemia, which has been linked to the progression and pathogenesis of T2D (Ghosh et al.

2004). The reduced activity on treatment with the extract, thus suggests an anti-hyperglycemia activity by *P. lunatus,* which corroborates with its inhibitory activities (**Figures 4.2.3A – 4.2.3C**).

Hepatocellular apoptosis, characterized by DNA fragmentation and cellular shrinkage in T2D has been reported to be majorly influenced by hyperglycemia – induced oxidative stress (Donath et al. 1999). The increased DNA fragmentation in the untreated tissue, may indicate an incidence of apoptosis (**Figure 4.2.5**). This can be attributed to the Fe^{2+} - induced oxidative injury via the Fenton pathway which corresponds to previous the reports on iron induced apoptotic cell death in isolated rat hepatocytes (Allameh et al. 2010). The decreased fragmentation on treatment with the extract indicates an anti-apoptotic activity by *P. lunatus*.

The dose – dependent inhibition of intestinal glucose absorption (**Figure 4.2.6A**) by the extract correlates with its inhibitory effects on the studied carbohydrate catabolic enzymes particularly intestinal alpha – glucosidase activity (**Figure 4.2.3A**), further depicting an anti-hyperglycemia effect of *P. lunatus*. This can be attributed the amino acid constituents particularly L- and D-alanine, which has been demonstrated to inhibit D-glucose uptake by intestinal membrane (Murer et al. 1975). The increased muscle glucose uptake by the extract with insulin (**Figure 4.2.6B**) demonstrates the ability of *P. lunatus* to improve insulin sensitivity. Insulin resistance or insensitivity has been recognized as a major metabolic abnormality in T2D and has been implicated in most diabetic complications such as dyslipidemia, hypertension and high coronary and cerebrovascular mortality (Monzillo and Hamdy 2003). The observed muscle glucose uptake effect of *P. lunatus* aqueous extract can also be attributed to its amino acid composition (**Table 4.2.2**), as their impact on muscle glucose uptake is well known (Kleinert et al. 2011). Kleinert et al. (2011) demonstrated the ability of an amino acid mixture of isoleucine, cysteine, methionine, valine, and leucine to improve insulin-stimulated glucose uptake in isolated rat epitrochlearis muscle.

The increasing paradigm shift to medicinal plants in the treatment and management of several ailments has led to increased concerns of their safety and toxic effects. Their various extracts elicit different effects in the body, which could be toxic (Ezuruike and Prieto 2014). Analysis of the identified compounds (**Table 4.2.1** and **Figure 4.2.7**) on the ProTox server predicted them to be safe when swallowed as they fell between classes 5 – 6 (**Table 4.2.3**). Computational prediction

of drugs and small molecules toxicity has been gaining much ground due to increasing ethical questions and animal rights in most developed countries.

4.2.6. Conclusion

The inhibitory activities and reversion of hepatic redox imbalance by *P. lunatus* suggests anti-hyperglycemia and anti-oxidative effects. This is further portrayed by its ability to inhibit intestinal glucose absorption and increase muscle glucose uptake with and without insulin. This can be attributed to the synergistic effect of the identified phytochemicals and amino acids. These findings also validate the folkloric use of *P. lunatus* in the treatment and management of diabetes.

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CHAPTER 5

THE ANTI-OXIDATIVE AND ANTI-DIABETIC ACTIVITIES OF *VERNONIA AMYGDALINA* (BITTER LEAF) *IN VITRO* AND *EX VIVO*

5.1 Vernonia amygdalina

Figure 5.1: *Vernonia amygdalina* leaves. Common names: bitter leaf (English); *Ewuro, Onugbu, Oriwo, Etidot, Ityuna, Chusar doki, Fatefata* (Nigeria); *Omubirizi, Mululuza* (Uganda); *Awonwono* (Ghana); *Aluma, Gbondutsi* (Togo); *Omubilizi* (Tanzania); *Umubilizi* (Rwanda); and *Ndoleh, Ying* (Cameroon). Photo: (Udochukwu et al. 2015).

5.1.2 Background

Vernonia amygdalina is a is a perennial shrub belonging to the *Asteraceae* family, with petiolate leaf of about 6 mm in diameter and elliptic shape (Ijeh and Ejike 2011). It is commonly known as bitter leaf owing to its bitter taste. It is amongst the common leafy vegetable in West Africa utilized for its nutritional and medicinal properties.

5.1.3 Ethnopharmacology

The leaves are consumed usually after washing to remove the bitter taste. They are employed as digestive stimulants. The leaf concoctions are used in the treatment of fever, diabetes, and hypertension well to reduce fever. The leaves are squeezed and placed on skin and tissue burns and cuts.

5.1.4 Biological activities

The leaves have been widely studied for their medicinal properties which include antidiabetes (Ong et al. 2011; Saliu et al. 2012), antioxidant (Adesanoye and Farombi 2010; Iwalokun et al. 2006), antihypertensive (Ajibola et al. 2011; Saliu et al. 2012), anti-sickling (Afolabi et al. 2012; Chikezie 2006), and anti-obesogenic (Adaramoye et al. 2008) activities. It is also used in the treatment and management of gastrointestinal disorders (Akah and Ekekwe 1995), dysentery (Moundipa et al. 2005), as well as antimicrobial and antiparasitic activities (Oyeyemi et al. 2018; Udochukwu et al. 2015).

5.1.5 Phytochemistry

The leaves of *V. amygdalina* have been studied for their phytochemical properties. This includes **(1) sesquiterpene lactones** which consists of vernolide, vernodalol, vernodalin, hydroxyvernolide, vernodalinol, vernomygdin, epivernodalol, 11,13-dihydrovernodalin, and 3′deoxyvernodalol; **(2) flavonoids**: luteolin, luteolin 7-O-β-glucuronoside, and lluteolin 7-O-β-glucoside; **(3) steroidal saponins**: vernoamyosides A, vernoamyosides B, vernoamyosides C, and vernoamyosides D; **(4) steroidal glucosides**: vemonioside B_1 (Farombi and Owoeye 2011; Ijeh and Ejike 2011; Oyeyemi et al. 2018; Saliu et al. 2012; Yeap et al. 2010).

5.1.6 Aim and objectives

The aim of this study was to investigate the *in vitro* and *ex vivo* antioxidative, and antidiabetic activities of the extracts and hot water infusion of *V. amygdalina*, and to identify the metabolic pathways that may be involved in this process. The histochemical and phytochemical properties of the leaves were also investigated in this study.

5.2 Phytol – Rich Extracts of *Vernonia Amygdalina* Stimulates Muscle Glucose Uptake, and Modulates Redox Activities and Functional Chemistry in Oxidative Hepatic Injury

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Preface: This article investigated the antioxidant, antidiabetic, and chemical properties of the ethyl acetate, ethanol and aqueous extracts of *V. amygdalina* leaves *in vitro* and *ex vivo*.

5.2.1 Abstract

The ethyl acetate, ethanol and aqueous extracts were obtained from the leaves of *Vernonia amygdalina* and investigated for their antidiabetic and antioxidant protective in oxidative hepatic injury. The 2,2'-diphenyl-1-picrylhydrazyl (DPPH) scavenging and Ferric reducing antioxidant power (FRAP) protocols were utilized in determining the *in vitro* antioxidant activities of the extracts. Their antidiabetic properties were determined via their inhibitory effect on α-glucosidase and α-amylase activities, and stimulation of muscle glucose uptake. Their mitigative effects on antioxidative biomarkers in Fe^{2+}-induced oxidative hepatic injury were also investigated *ex vivo*. The chemical functional chemistry of the tissues were investigated using FTIR spectroscopy. The extracts showed significant ($p<0.05$) free radical scavenging and reducing activities. They significantly ($p<0.05$) elevated the level of reduced glutathione (GSH) as well as increased the activities of superoxide dismutase and catalase, with concomitant depletion of malondialdehyde level. Treatment with the ethanol and aqueous extracts led to removal of oxidative chemical functional group at 1500-1200 (amide II) cm^{-1} region, while there was an inclusion of functional group at 3000-2800 (carboxylic acid) cm^{-1} region in tissues treated with the aqueous extract. The extracts significantly ($p<0.05$) inhibited the activities of α-glucosidase and α-amylase, and stimulated glucose uptake in rat muscles. GC-MS analysis of the ethyl acetate and ethanol extracts

revealed phytol as the predominant compound, with ethanol having the highest concentration. These results suggest the antioxidative and antidiabetic properties of *V. amygdalina* as evident by their ability to scavenge free radicals, modulate antioxidant biomarkers and oxidative chemistry changes, and stimulate muscle glucose uptake.

Keywords: Functional chemistry; Glucose uptake; Oxidative stress; and Vernonia amygdalina

5.2.2 Introduction

Diabetes mellitus (DM) has been recognized as the most prevalent of endocrine pathology affecting carbohydrate, protein and lipid metabolism (Saltiel and Kahn 2001; Volek et al. 2009). These metabolic alterations have been associated with inability of the pancreas to secrete insulin and/or inability of the body to utilize the insulin secreted, thereby leading to elevated blood glucose level also known as hyperglycemia (Erukainure et al. 2017a). Occurrence of hyperglycemia is often referred to as type 2 diabetes (T2D) and accounts for more than 90% of all types of DM, making it the major contributor of diabetic morbidity and mortality (I.D.F. 2016). In T2D, excessive hyperglycemia can be attributed to insulin insensitivity and pancreatic β-cell dysfunction (Erukainure et al. 2017a). Excessive hyperglycemia stimulates the production of ROS, leading to oxidative stress when it surpasses the body's endogenous antioxidant defense system (Saeed et al. 2012; Tiwari et al. 2013). Oxidative stress has implicated in the pathogenesis and progression of T2D complications.

Several studies have shown the therapeutic effect of antioxidants against hyperglycemia induced oxidative stress (Coskun et al. 2005; Rahimi et al. 2005). With natural products from medicinal plants showing better activities owing to their antioxidative-phytochemical constituents (Erukainure et al. 2013; Ezuruike and Prieto 2014; Saeed et al. 2012). Common amongst such plants is *Vernonia amygdalina*.

Despite the numerous reports on its medicinal properties, there are however a dearth on the functional chemistry of targeted tissues treated with *V. amygdalina*. Thus, this study aims to report the modulatory effect of *V. amygdalina* extracts on the functional chemistry and antioxidant activities in oxidative hepatic injury. The ability of the extracts to stimulate muscle glucose uptake and to inhibit major carbohydrate catabolic enzymes was also investigated.

5.2.3 Materials and Methods

Kindly refer to chapter 2, sections 2.1 – 2.4; 2.6.1 – 2.6.2; 2.6.6; 2.7.2; 2.10.1 and 2.10.4

5.2.4 Results

The total phenolic contents of the ethanol and aqueous extracts were significantly ($p<0.05$) higher than that of the ethyl acetate as depicted in **Figure 5.2.1**, with the ethanol extract having the highest content of phenolics.

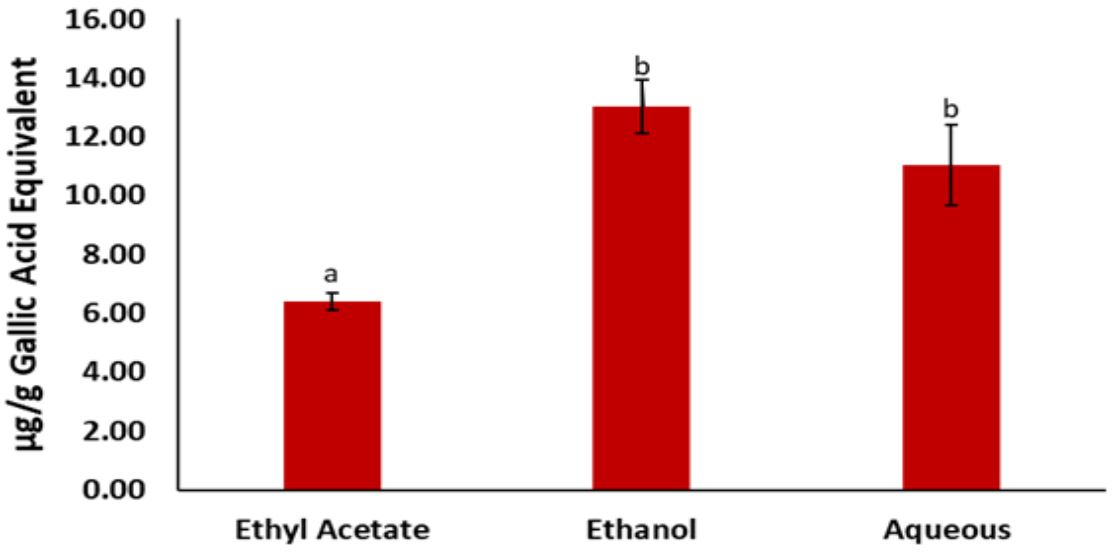


Figure 5.2.1: Total phenolic contents of *V. amygdalina* extracts. Data = mean ± SD; n = 3. [abc]Values with different letter above the bars for a given extract are significantly ($p < 0.05$) different from each other.

All extracts significantly scavenged DPPH radicals, indicating a potent free radical scavenging activity as compared to the standard drug, ascorbic acid (**Figure 5.2.2A**). The low IC_{50} value of the ethanol extract, suggests it as the most active as shown in **Table 5.2.1**.

There was a dose dependent increase in FRAP activity by the extracts which was significantly ($p<0.05$) lower than the standard drug as shown in **Figure 5.2.2B**. The ethanol extract showed the best activity with an IC_{50} value of 638.86 µg/mL (**Table 5.2.1**).

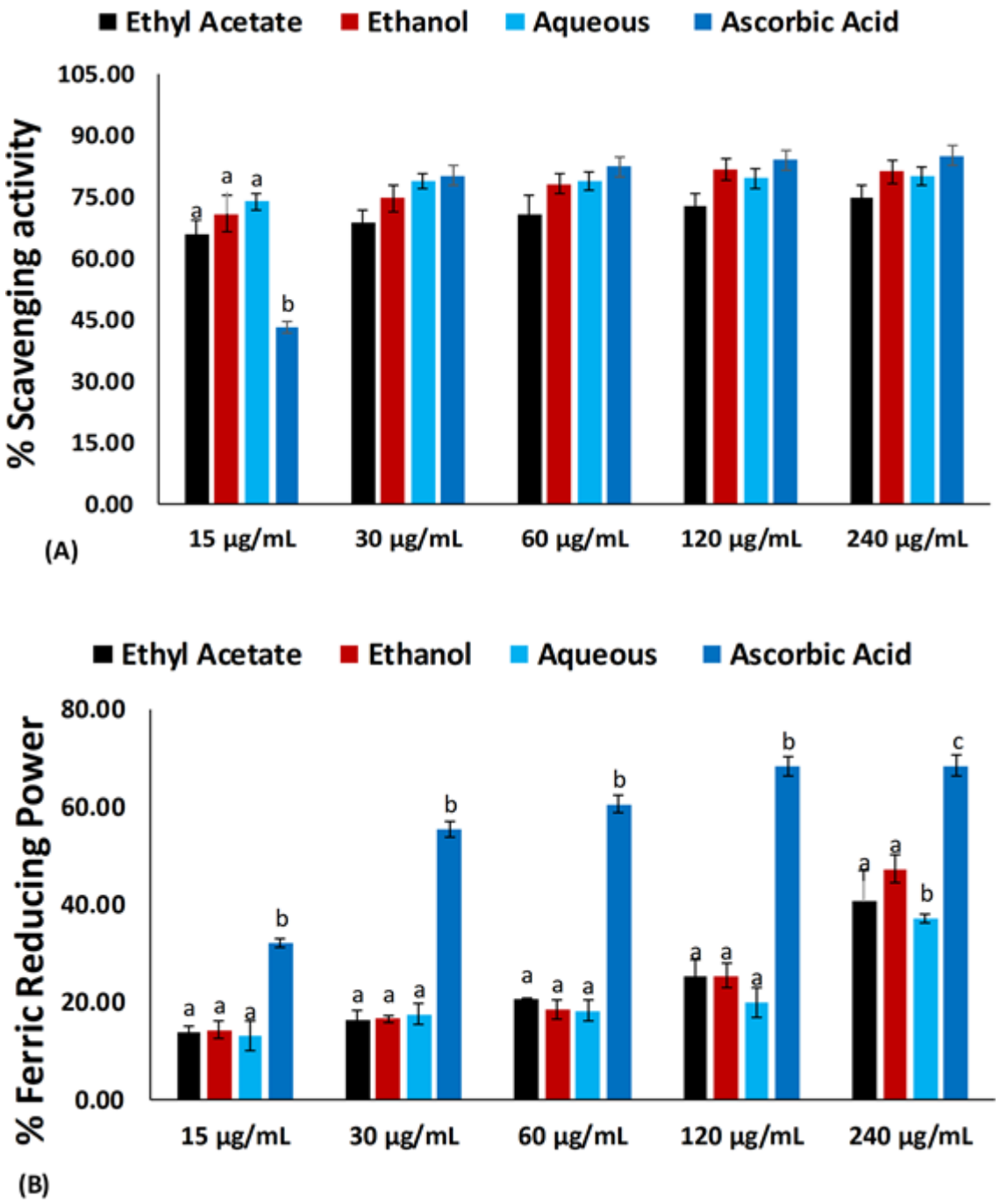


Figure 5.2.2: (A) DPPH scavenging and (B) FRAP activities of V. amygdalina extracts. Data = mean ± SD; n = 3. [abc]Values with different letter above the bars for a given extract are significantly ($p < 0.05$) different from each other.

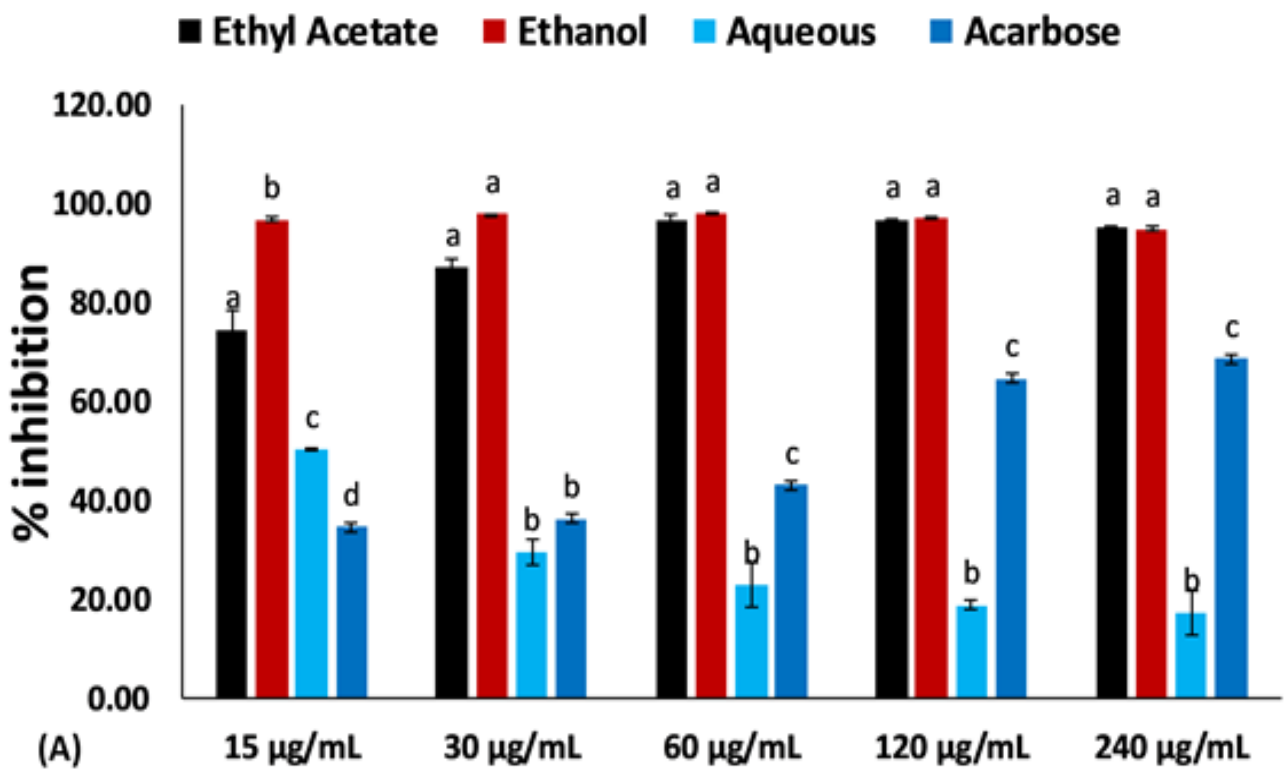
Table 5.2.1: IC_{50} values of studied biological activities

Activities	Ethyl Acetate	Ethanol	Aqueous	Ascorbic acid	Acarbose	Gallic acid
DPPH	0.08	0.07	0.10	8.32	–	–
FRAP	>1000	638.86	>1000	34.04	–	–
α-glucosidase	0.27	0.01	13.52	–	62.73	–
α-amylase	289.95	102.51	32.08	–	0.04	–
GSH	392.86	120.01	314.93	–	–	3.80
SOD	25.67	17.60	41.97	–	–	84.42
Catalase	178.09	178.11	171.67	–	–	533.41
Lipid peroxidation	3.65	14.12	7.05	–	–	137.56
Glucose uptake	48.84	41.11	160.61	–	–	–

Values are expressed as µg/mL. Key: – = not applicable

The ethyl acetate and ethanol extracts showed significant ($p<0.05$) inhibitory effect on α-glucosidase activities (**Figure 5.2.3A**), with IC_{50} values of 0.27 and 0.01 µg/mL respectively (**Table 5.2.1**). This was much higher than the standard drug, acarbose (IC_{50} = 62.73 µg/mL). The inhibitory activity of the aqueous extract was observed to reduce with increasing concentrations.

The aqueous extract had the highest inhibitory effect on α-amylase activity amongst the extracts, with an IC_{50} value of 32.08 µg/mL as depicted in **Figure 5.2.3B** and **Table 5.2.1**. This was followed by the ethanol extract (IC_{50} = 102.51 µg/mL), with the ethyl acetate having the least (IC_{50} = 289.95 µg/mL).



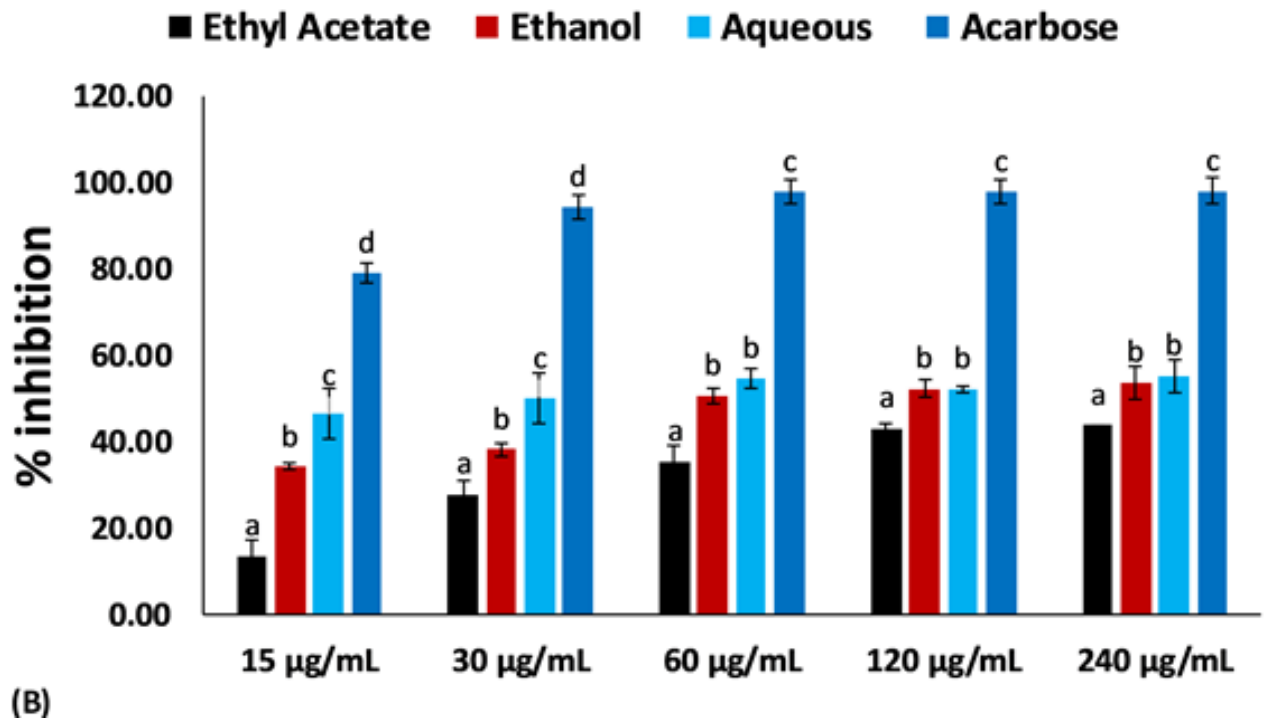


Figure 5.2.3: (A) α-glucosidase and (B) α-amylase inhibitory activities of *V. amygdalina*. Data = mean ± SD; n = 3. [abcd]Values with different letter above the bars for a given extract are significantly ($p < 0.05$) different from each other.

Incubation of hepatic tissues with $FeSO_4$ led to depleted GSH levels as depicted in **Figure 5.2.4A**. This was significantly increased on incubation with the extracts to near normal, with the ethanol extract showing the best activity (IC_{50} = 120.01 µg/mL).

Similarly, the extracts significantly ($p<0.05$) increased the activities of SOD and catalase in oxidative hepatic tissues as depicted in **Figures 5.2.4B** and **4C**, with the ethanol (IC_{50} = 17.60 µg/mL) and aqueous (IC_{50} = 171.67 µg/mL) extracts showing the best activities for SOD and catalase respectively. The increased hepatic MDA level on incubation with $FeSO_4$ was significantly ($p<0.05$) reduced by the extracts as shown in Fig. 4D, with the ethyl acetate (IC_{50} = 3.65 µg/mL) and aqueous (IC_{50} = 7.05 µg/mL) extracts showing the best activities.

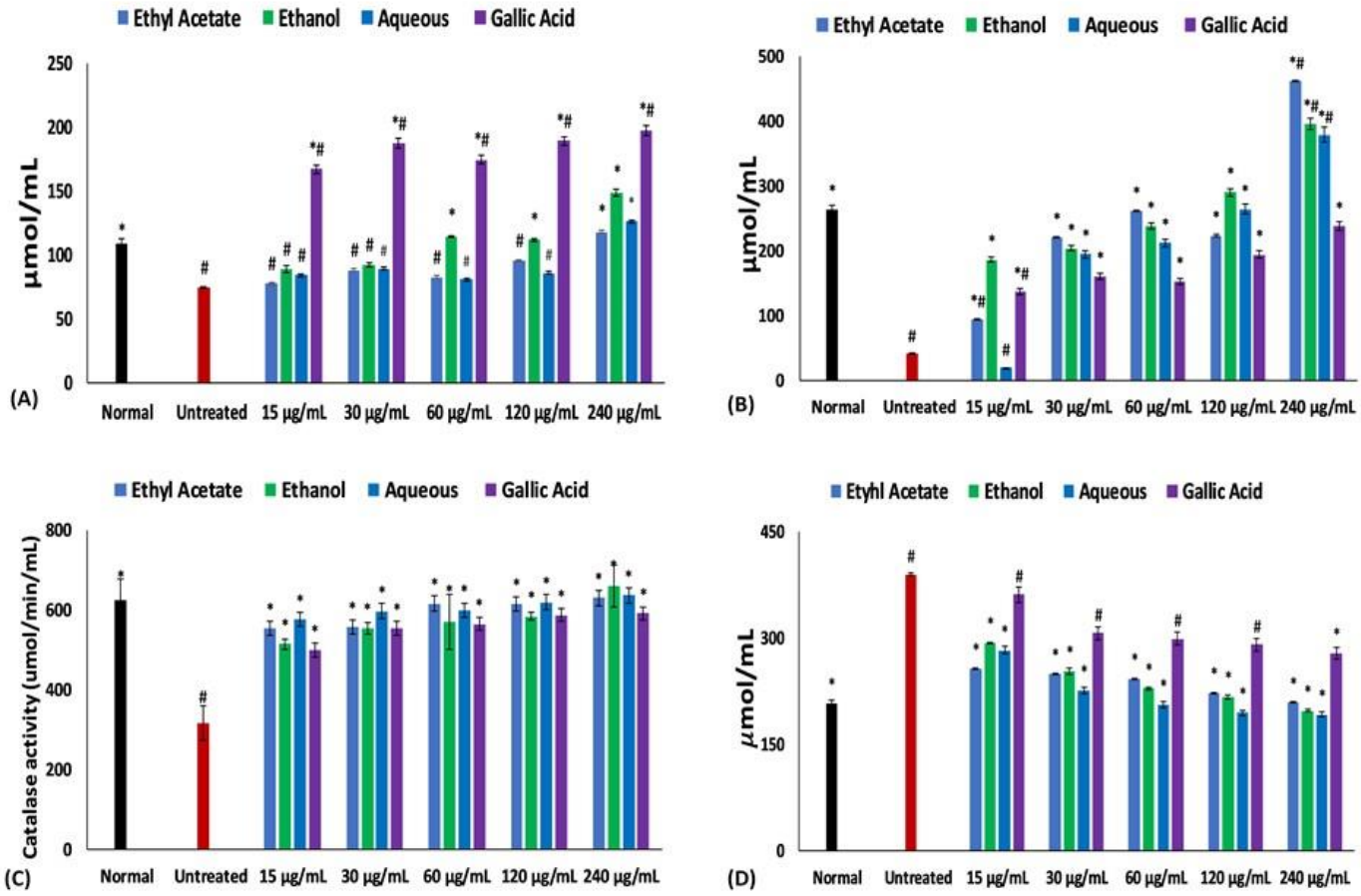


Figure 5.2.4: Effect of *V. amygdalina* extracts on (A) GSH level, (B) SOD activity, (C) catalase activity, and (D) MDA level in oxidative pancreatic injury. Values = mean ± SD; n = 3. *Significantly different from untreated sample and #Significantly different from normal sample ($p < 0.05$, Tukey's HSD-multiple range post-hoc test, IBM SPSS for Windows).

FTIR spectroscopy of the hepatic metabolite revealed that oxidative injury led to loss of chemical functional groups at 2175 – 2140 (thiocyanates) cm^{-1} regions, as well as addition of a chemical functional group at 1500 – 1200 (amide II) cm^{-1} region as shown in **Figure 5.2.5** and **Table 5.2.2**. Treatment with the ethanol and aqueous extracts led to removal of the added chemical functional group as well as amide I and aromatic amines (1500 – 1200 cm^{-1} region). The aqueous extract also led to the addition of another functional group at 3000 – 2800 (carboxylic acid) cm^{-1} region.

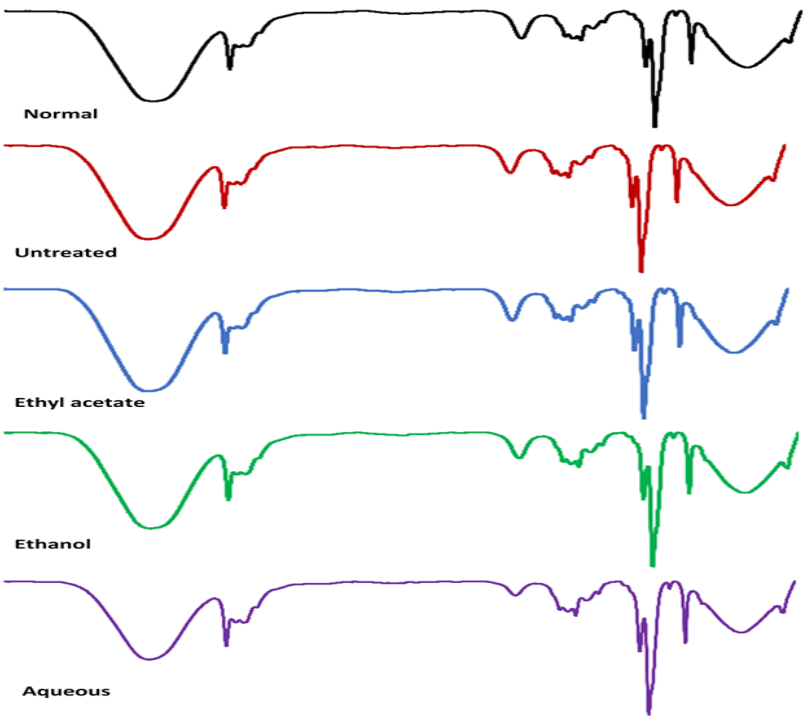


Figure 5.2.5: FTIR spectroscopy of oxidative hepatic tissues treated with *V. amygdalina* extracts

Incubation of psoas muscle with extracts of *V. amygdalina* in the presence of glucose led to significant ($p<0.05$) glucose uptake in a dose dependent manner, with the ethyl acetate (IC_{50} = 48.84 µg/mL) and ethanol (IC_{50} = 41.11 µg/mL) extracts showing the best activities as depicted in **Figure 5.2.6** and **Table 5.2.1**.

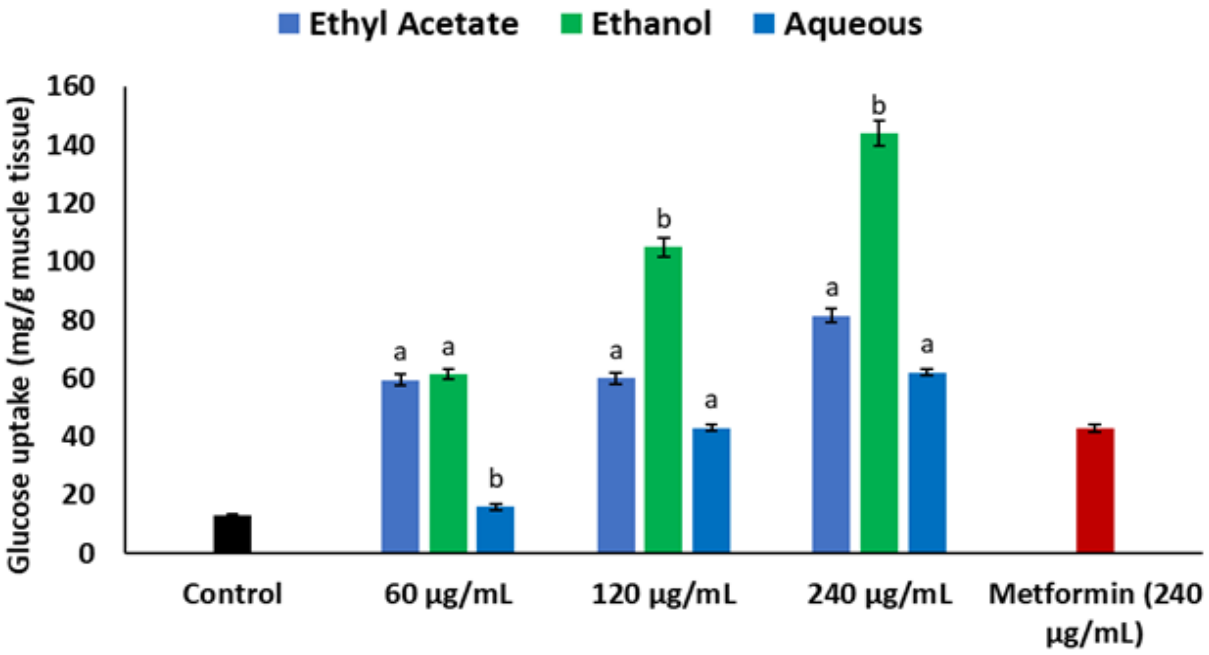


Figure 5.2.6: Effects of *V. amygdalina* extracts on glucose uptake in isolated rat psoas. Data = mean ± SD; n = 3. [ab]Values with different letter above the bas for a given extract are significantly ($p < 0.05$, Tukey's HSD-multiple range post-hoc test, IBM SPSS for Windows) different from each other

Table 5.2.2: Qualitative FTIR table of oxidative hepatic tissues treated with *V. amygdalina* extracts

Regions (cm^{-1})	**Normal**	**Untreated**	**Ethyl Acetate**	**Ethanol**	**Aqueous**	**Assignments**
900 – 700	878.92	879.07	878.99	879.15	879.65	1^o, 2^o amines
1200 – 900	950.67	950.67	950.24	950.91	951.84	Nucleic acid
	1043.09	1045.16	1045.07	1045.28	1045.78	Nucleic acid
	1086.92	1087.05	1086.97	1087.12	1087.57	Nucleic acid
1500 – 1200	1326.20	1328.41	1326.91	–	–	Amide I; Aromatic amines
	1381.91	1381.89	1382.29	1381.41	1380.89	Phenol –O–H
	1414.52	1417.24	1414.94	1416.40	1416.26	Nitramines
	–	1450.58	1450.24	–	–	Amide II
1800 – 1500	1650.82	1649.36	1651.07	1651.40	1655.20	β-ketoaldehyde in enol form; ketones
2175 – 2140	2169.64	–	–	–	–	Thiocyanates
3000 – 2800	–	–	–	–	2897.88	Carboxylic acids
	2975.82	2975.32	2975.64	2975.25	2974.09	Carboxylic acids
3500 – 3300	3327.89	3328.18	3329.20	3330.18	3328.41	Amine and imine

GC-MS analysis of the extract revealed phytol to be the predominant compound in the analyzed extracts, with the ethanol having the highest concentration as depicted in **Figure 5.2.7A** and **B**, and **Table 5.2.3**).

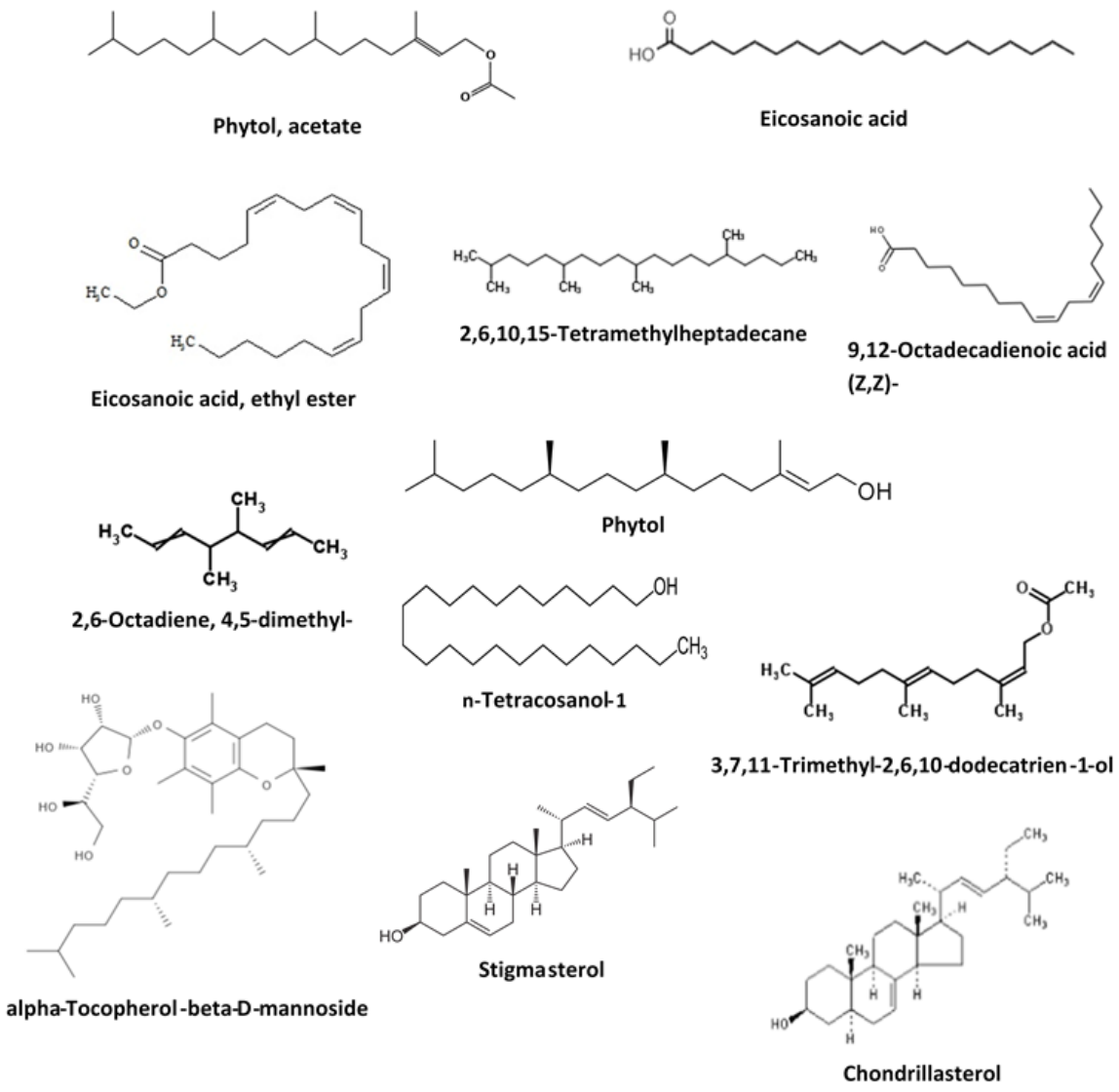


Figure 5.2.7A: GC-MS identified compounds in *V. amygdalina* ethyl acetate extract

Other compounds common in both extracts are: phytol, acetate; eicosanoic acid, ethyl ester; and chondrillasterol. Eicosanoic acid; 2,6,10,15-Tetramethylheptadecane; 9,12-Octadecadienoic acid (Z,Z)-; 2,6-Octadiene, 4,5-dimethyl-; 3,7,11-Trimethyl-2,6,10-dodecatrien-1-ol; n-Tetracosanol-

1; alpha.-Tocopherol-.beta.-D-mannoside; and stigmasterol were identified in the ethyl acetate extract (Fig. 6A and table 3). While 3,7,11,15-tetramethyl-2-hexadecen-1-ol; 9-eicosyne; tridecanoic acid; alpha-linolenic acid; vitamin E; and ethyl iso-allocholate were identified in the ethanol extract (**Figures 5.2.7B** and **Table 5.2.3**).

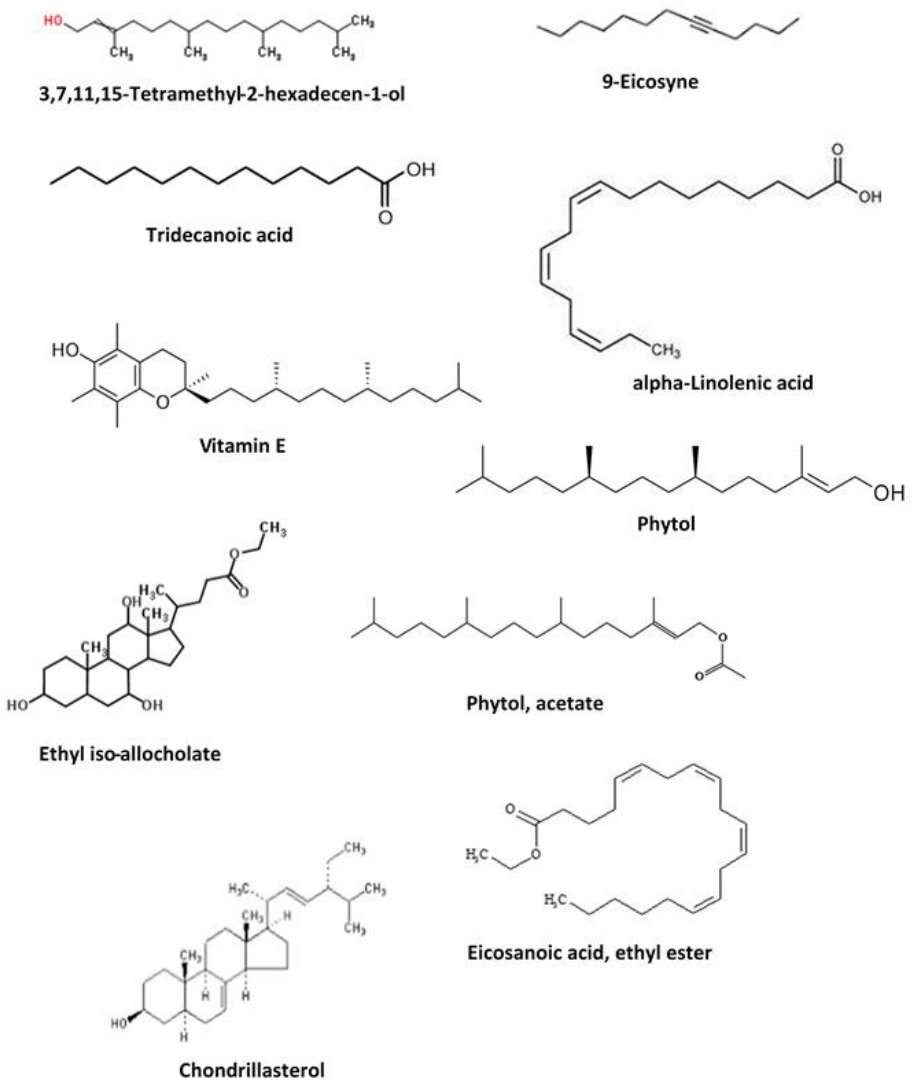


Figure 5.2.7B: GC-MS identified compounds in *V. amygdalina* ethyl acetate extract

Table 5.2.3: GC-MS identified compounds in ethyl acetate and ethanol extracts of *V. amygdalina*

Compounds	Ethyl Acetate Extract (%)	Ethanol Extract (%)
Phytol, acetate	1.03	8.23
Eicosanoic acid	5.49	–
Eicosanoic acid, ethyl ester	1.69	1.00
2,6,10,15-Tetramethylheptadecane	2.54	–
Phytol	15.68	21.69
9,12-Octadecadienoic acid (Z,Z)-	2.34	–
2,6-Octadiene, 4,5-dimethyl-	1.33	–
3,7,11-Trimethyl-2,6,10-dodecatrien-1-ol	2.07	–
n-Tetracosanol-1	0.82	–
alpha.-Tocopherol-.beta.-D-mannoside	0.82	–
Stigmasterol	0.72	–
Chondrillasterol	1.71	2.40
3,7,11,15-Tetramethyl-2-hexadecen-1-ol	–	1.51
9-Eicosyne	–	2.52
Tridecanoic acid	–	3.30
alpha-Linolenic acid	–	2.82
Vitamin E	–	1.50
Ethyl iso-allocholate	–	1.32

Key: – = Absent

5.2.5 Discussion

The role of oxidative stress in the progression and pathogenesis of complications associated with T2D have been widely studied (Robertson et al. 2004; Wellen and Hotamisligil 2005). It arises owing to hyperglycemia as well as uncontrolled production of free radicals on the onset of hyperglycemia. The excess free radicals react with cell biomolecules, causing an alteration in their biochemistry and function (Toyokuni 1999). In this study, the effect of oxidative stress on the functional chemistry of hepatic tissues was investigated as well as the oxidant mitigative effect of *V. amygdalina* and its antidiabetic activity.

Plants have been demonstrated to be potent antioxidants (Dudonné et al. 2009), which is evident by the potent free radical scavenging activities as well as the reducing power of the *V. amygdalina* extracts (**Figure 5.2.1**). This can be attributed to their total phenol constituents (**Figure 5.2.1**). Polyphenols scavenge free radicals by donating of H-atom from their OH group/s (Papuc et al. 2017) and/or delocalization of the double bonds in the benzene ring (Dreosti 2000). The vitamin E content and its glycoside, alpha-tocopherol-beta-D-mannoside as well as phytol in the ethanol and ethyl acetate extracts (**Figure 5.2.7** and **Table 5.2.3**) may also play a synergetic role, as they have been reported for their potent free radical scavenging activities (Kamal- Eldin and Appelqvist 1996; Santos et al. 2013).

Iron toxicity has been attributed to the pro-oxidant activity of its ferrous (Fe^{2+}) state via the Fenton's reaction which distorts the cell's redox balance (Andrews 1999; Jiang et al. 2004; Rajpathak et al. 2009). This may be responsible for the depleted GSH level with concomitant increased MDA level (lipid peroxidation) as well as decreased SOD and catalase activities in hepatic tissues incubated with $FeSO_4$ (**Figure 5.2.3A – 3D**), signifying oxidative injury. Depleted level of GSH is the marker of oxidative stress as it is regarded as the first line of the endogenous antioxidant system (Tiwari et al. 2013). SOD catalyzes the dismutation of $O_2^{\cdot -}$ to H_2O_2, which if not broken down by catalase to O_2 and H_2O would further react with Fe^{3+} to generate $^{\bullet}OH$ (Aslan et al. 2000). Hydroxyl radicals ($^{\bullet}OH$) have been reported as potent initiators of membrane lipid peroxidation, as they attack the membrane lipids (Dinis et al. 1994; Gutteridge 1995). The reversion of these levels and enzymatic activities by the extracts (**Figure 5.2.3A – 3D**), further portrays the antioxidative activities of *V. amygdalina* which also correlates with its reported hepatotoxic protective activities (Adesanoye and Farombi 2010; Arhoghro et al. 2009; Atangwho et al. 2007). These activities can also be attributed to the identified phytochemicals, particularly phytol, vitamin E and its glycoside, unsaturated fatty acids and esters (**Figure 5.2.7** and **Table 5.2.3**). The antioxidative protective effects of these phytochemicals have been demonstrated, with vitamin E regarded as the most potent (Kamal- Eldin and Appelqvist 1996; Richard et al. 2008; Santos et al. 2013).

Some antidiabetic drugs such as acarbose exhibit their actions by inhibiting major carbohydrate digestive enzymes implicated in T2D (Scheen 1997; Yee and Fong 1996). These enzymes digest dietary carbohydrate, leading to the increase of postprandial glucose level. Thus, the inhibition of

α-glucosidase and α-amylase by the extracts (**Figure 5.2.4**) indicates an antidiabetic potential which correlates with the reported antidiabetic properties of *V. amygdalina* (Atangwho et al. 2009; Michael et al. 2010; Okolie et al. 2008; Ong et al. 2011). This can be attributed to the synergetic effect of the polyphenol constituents and the identified phytochemicals, as polyphenols are reported as potent inhibitors of the studied enzymes as well as modulator of glucose homeostasis (Ong et al. 2011; Saliu et al. 2012).

Fourier Transform Infra-Red spectroscopy (FT-IR) techniques was utilized in this study to characterize the oxidative hepatic metabolites. The respective altered and added functional groups in the untreated tissues (**Figure 5.2.5** and **Table 5.2.2**) can be attributed to the induction of oxidative stress, causing amino acid residues fragmentation and functional loss (Berlett and Stadtman 1997; Dalle-Donne et al. 2003). The removal of the added oxidative – functional group by the ethanol and ethyl acetate extracts may demonstrate a therapeutic effect. However, the removal of amide I and aromatic amines may portray toxicity. Similarly, the inability of the ethyl acetate to remove the generated functional group may also portray toxicity.

Stimulation of skeletal muscle glucose uptake have been demonstrated as an effective mechanism employed by some antidiabetic drugs such as metformin in regulation of blood glucose homeostasis (Pereira et al. 2017a). This has been attributed to the activation of GLUT-4 translocation, which transports glucose to the muscle (Satoh 2014). The stimulated glucose uptake by the extracts (**Figure 5.2.6**) thus indicates an antidiabetic therapeutic effect of *V. amygdalina*. This corresponds with previous studies which reported the ability of the *V. amygdalina* to stimulate glucose uptake and utilization in Chang-liver, C2C12 muscle and 3T3-L1 cells (Erasto et al. 2009). This also corroborates previous report on the ability of the leaf extract to down regulate the expressions of gluconeogenic enzymes consisting of glucose 6-phosphatase, fructose 1,6-bisphosphatase and phosphoenol pyruvate carboxykinase in diabetic rats' muscles (Atangwho et al. 2014).

5.2.6 Conclusion

These results suggest *V. amygdalina* leaves may bring about its antioxidative and antidiabetic properties via free radical scavenging, modulation of antioxidant biomarkers and oxidative-induced chemistry changes, and stimulation of muscle glucose uptake. These can be attributed to

the synergetic effects of the identified phytochemicals particularly phytol, and vitamin E and its glycoside. This further corroborates and validates previous reports on its antidiabetic properties.

Acknowledgments

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5.3 Histochemistry, phenolic content, antioxidant, and anti-diabetic activities of Vernonia amygdalina leaf extract

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Preface: This article investigated the phytochemical distribution, antioxidant, and antidiabetic properties of the hot water infusion of the leaves of *V.* amygdalina *in vitro* and *ex vivo*. The manuscript has been published in the Journal of Food Biochemistry (**Erukainure OL**, Chukwuma CI, Sanni O, Matsabisa MG, Islam MS. (2018). Histochemistry, phenolic content, antioxidant, and anti- diabetic activities of *Vernonia amygdalina* leaf extract. J Food Biochem., e12737. https://doi.org/10.1111/jfbc.12737)

5.3.1 Abstract

Vernonia amygdalina leaves were investigated for their histochemical properties and antidiabetic activities. Histochemical analysis of the leaf revealed distributions of acidic lipid, mucilage and pectin, lipids, polyphenols and alkaloids at the mid rib, glandular trichome and epidermis. HPLC analysis of the leaves hot-water infusion revealed the presence of quercetin and (-)-epi-catechin. The infusion had significant ($p<0.05$) 2,2'-diphenyl-1-picrylhydrazyl (DPPH) scavenging and ferric reducing antioxidant power (FRAP) activities. Ex vivo antioxidative analysis revealed the ability of the infusion to increase GSH level, SOD and catalase activities, while concomitantly depleting MDA level and DNA fragmentation in Fe2+-induced hepatic injury. The infusion showed significant ($p<0.05$) inhibitory activity against α-glucosidase and pancreatic lipase. It also inhibited intestinal glucose absorption and enhanced muscle glucose uptake respectively. The ability of the infusion to inhibit oxidative stress, DNA fragmentation and stimulate muscle glucose

uptake may suggest an antioxidative, anti-apoptotic and insulin-sensitivity activity of *V. amygdalina*.

Keywords: Antioxidative; Anti-hyperglycemia; Histochemistry; and *Vernonia amygdalina*

5.3.2 Introduction

The use of medicinal plants in the treatment and management of several ailments and diseases dates to time immemorial, making these plants and their uses an incumbent part of most cultures. About 80% of the third world population are estimated to still rely on medicinal plants as their main healthcare source (Ekor 2014), which indicates the relevance of these plants in modern days. Several studies have attributed the medicinal properties of these plants to their phytochemical constituents (Bruneton 1993; Van Wyk et al. 1997). The efficacies of these plant and phytochemicals constituents have been authenticated in several studies to be potent in the treatment of several disease types (Chevallier 1996; Ghani 1998; Hutchings 1996). Amongst the many diseases treated with medicinal plants with proven efficacy, is diabetes mellitus (DM).

Diabetes mellitus remains the one of the fastest rising global epidemics, with 425 million people globally reported to be living with the disease in 2017 (I.D.F. 2018). This figure has been postulated to increase to 625 million in 2045, when Africa to account for 6.5% of the increase (I.D.F. 2018). It is a metabolic disease which is resulting from insulin deficiency (type 1 diabetes) and/or inability of the body to utilize secreted insulin (type 2 diabetes), thus leading to increased blood glucose level (Erukainure et al. 2013; Vinayagam et al. 2016).

T2D accounts for 90% of all diabetes types, thus a major contributor to diabetic morbidity and mortality (Erukainure et al. 2017a; Giugliano et al. 1996; I.D.F. 2018; Lauritzen et al. 2000). It arises from insulin resistant and pancreatic β cell dysfunction, leading to hyperglycemia (Erukainure et al. 2017a). Chronic hyperglycemia then causes an increased production of free radicals which overwhelms the body's endogenous antioxidant, thereby leading to oxidative stress (Giugliano et al. 1996; Saeed et al. 2012; Tiwari et al. 2013). Oxidative stress has been recognized as the major underlying factor behind T2D's micro and macro – vascular complications such as retinopathy, neuropathy, nephropathy, and hypertension (Baynes and Thorpe 1999; Stadler 2013).

Antioxidative and inhibition of carbohydrate metabolizing enzymes are amongst the most studied mechanism of antidiabetic drugs and medicinal plants (Coskun et al. 2005; Rahimi et al. 2005). This has been attributed to the ability of these drugs and plants' constituents to scavenge free radicals and inhibit key carbohydrate digestive enzymes particularly the α-glucosidase and α-amylase. Several studies have demonstrated better carbohydrate digesting enzymes activities by medicinal plants due to their phytochemical constituents (Erukainure et al. 2013; Ezuruike and Prieto 2014; Saeed et al. 2012).

In this study, we aimed at investigating the histochemistry of the leaves of *V. amygdalina* which describes the distribution of its phytochemical for the first time. The effect of its hot infusion on key carbohydrate digestive enzymes linked to T2D as well as glucose absorption and uptake were also investigated to further ascertain its antidiabetic properties.

5.3.3 Materials and Methods

Kindly refer to chapter 2, sections 2.1 – 2.4; 2.6.1 – 2.6.2; 2.7.1; and 2.7.2

5.3.4 Results

Histochemical analysis of *V. amygdalina* revelead the presence of acidic lipid as portrayed by the blue colour (**Figure 5.3.1A**). Mucilage and pectin were also identified by the red/rose pink colours (**Figure 5.3.1B**), with much concentration at the mid rib (**Figure 5.3.1C**). Lipids were identified by the orange/red colouration of the glandular trichome (**Figure 5.3.1D**). The blue coloration of the epidermis and mid rib depicts the presence of polyphenols (**Figure 5.3.1E** and **1F**). While the brown coloration of the mid rib indicates the presence of alkaloids (**Figure 5.3.1G**).
Total phenolic analysis of *V. amygdalina* infusion revealed a total phenolic concentration of 13.96 ± 3.10 GAE/mg.

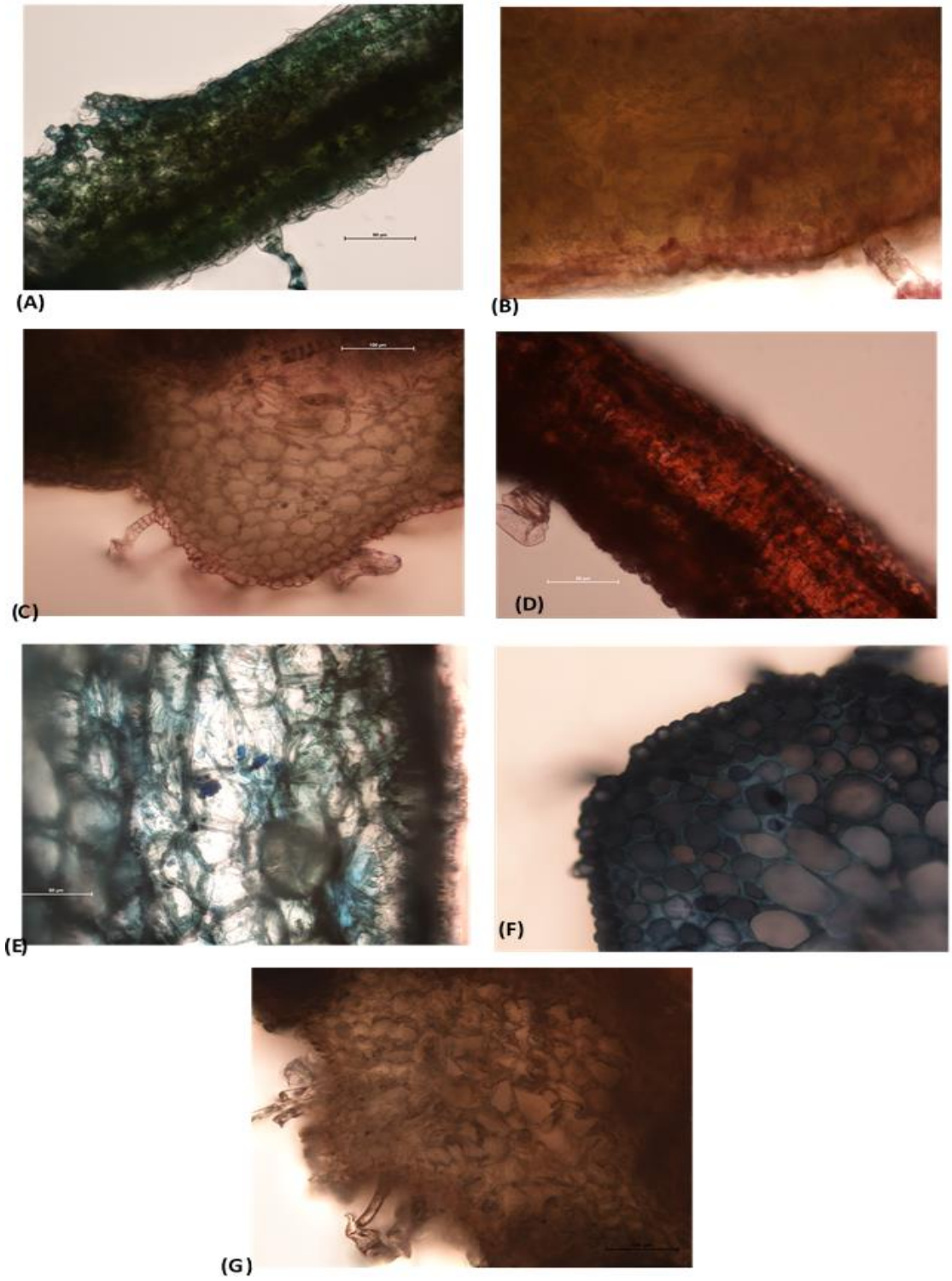

Figure 5.3.1: Histochemical properties of *V. amygdalina* leaves

According to HPLC chromatogram, quercetin and (-) epi-catechin were identified in the infusion at retention times 25.04 and 16.08 min, respectively. The percentage peak areas were 6.25% and 1.91%, respectively (**Figure 5.3.2**). HPLC retention time of standard quercetin and (-) epi-catechin were 25.21 and 16.8 min, respectively.

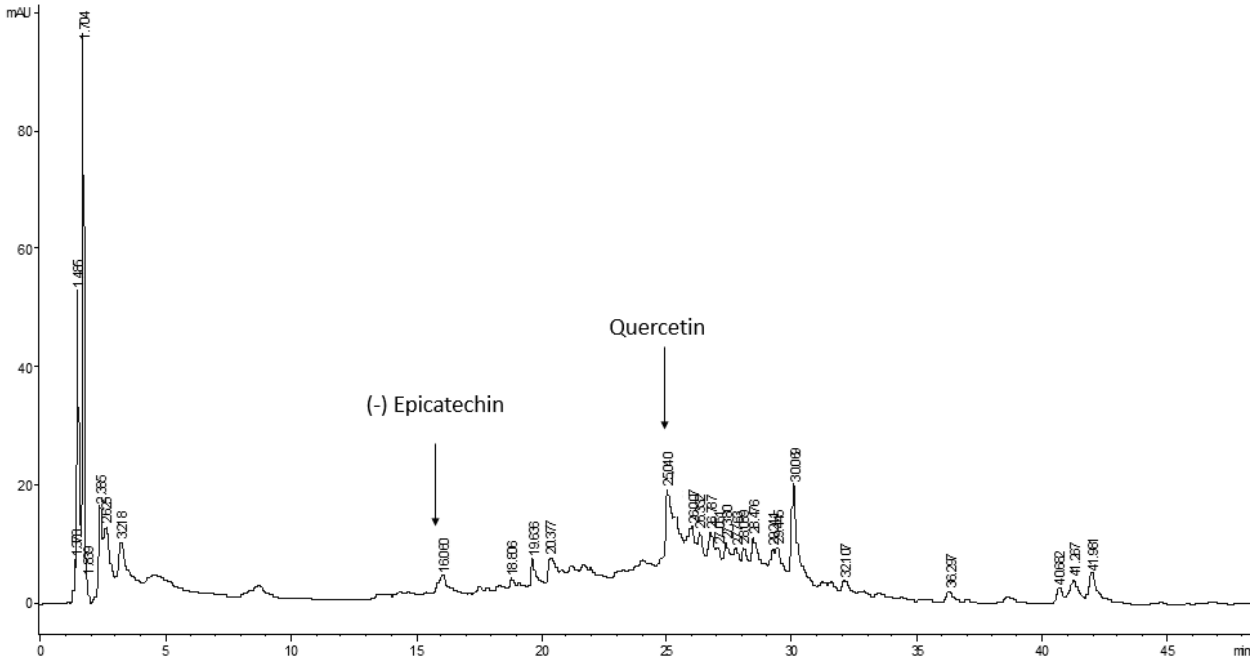

Figure 5.3.2: HPLC chromatogram of *V. amygdalina* infusion

The infusion significantly ($p<0.05$) scavenged DPPH radical and significantly ($p<0.05$) increased FRAP activity, portraying a potent antioxidant activity as depicted in **Figures 5.3.3A** and **3B**. This is further portrayed by their low IC_{50} values (7.03 and 58.64 µg/mL respectively) as shown in **Table 5.3.1**.

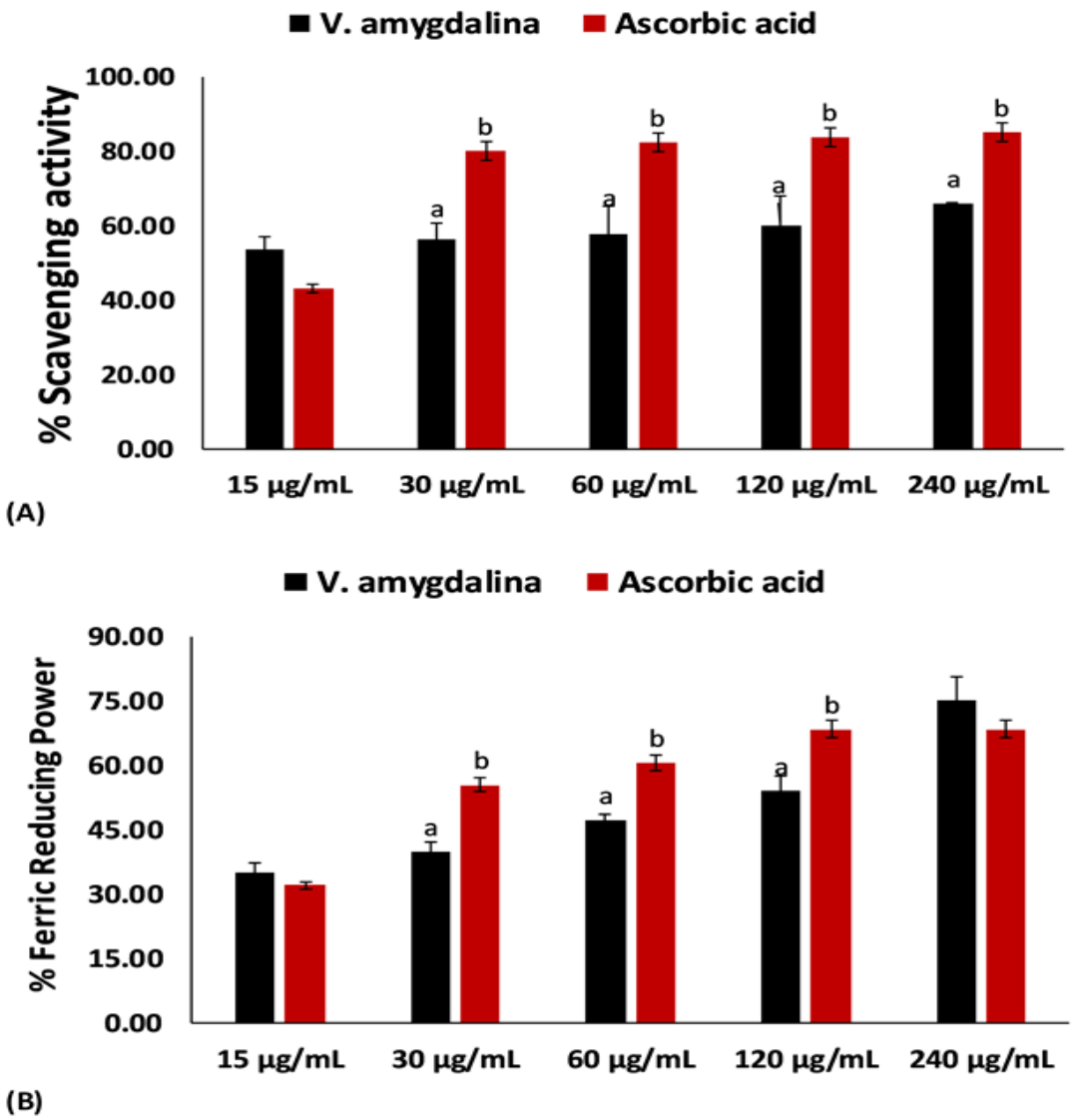

Figure 5.3.3: (A) DPPH scavenging and (B) FRAP activities of *V. amygdalina* infusion. Data = mean ± SD; n = 3. [ab]Values with different letter above the bars for a given concentration are significantly ($p < 0.05$) different from each other.

Table 5.3.1: IC_{50} values of studied biological activities

Activities	Infusion	Ascorbic acid	Acarbose	Gallic acid	Orlistat
DPPH	7.03	8.32	–	–	–
FRAP	58.64	34.04	–	–	–
α-glucosidase	124.72	–	62.73	–	–
Lipase	>1000	–	0.04	–	0.15
GSH	5.24	–	–	3.80	–
SOD	26.32	–	–	84.42	–
Catalase	1.08	–	–	533.41	–
Lipid peroxidation	20.25	–	–	137.56	–
DNA Fragmentation	33.11	–	–	27.13	–

Values are expressed as µg/mL. Key: – = not applicable

Induction of oxidative stress in hepatic tissues led to significant ($p<0.05$) depletion of GSH level, SOD and catalase activities, with concomitant increase in MDA level as depicted in **Figures 5.3.4A – 4D**. These activities and levels were significantly ($p<0.05$) reversed on treatment with the infusion as portrayed by the increased GSH level (**Figure 5.3.4A**), SOD and catalase (**Figure 5.3.4B** and **4C**), and reduced MDA level (**Figure 5.3.4A**). Their respective IC_{50} values of 5.24, 26.32, 1.08, and 20.25 µg/mL (**Table 5.3.1**) indicate a potent antioxidative activity.

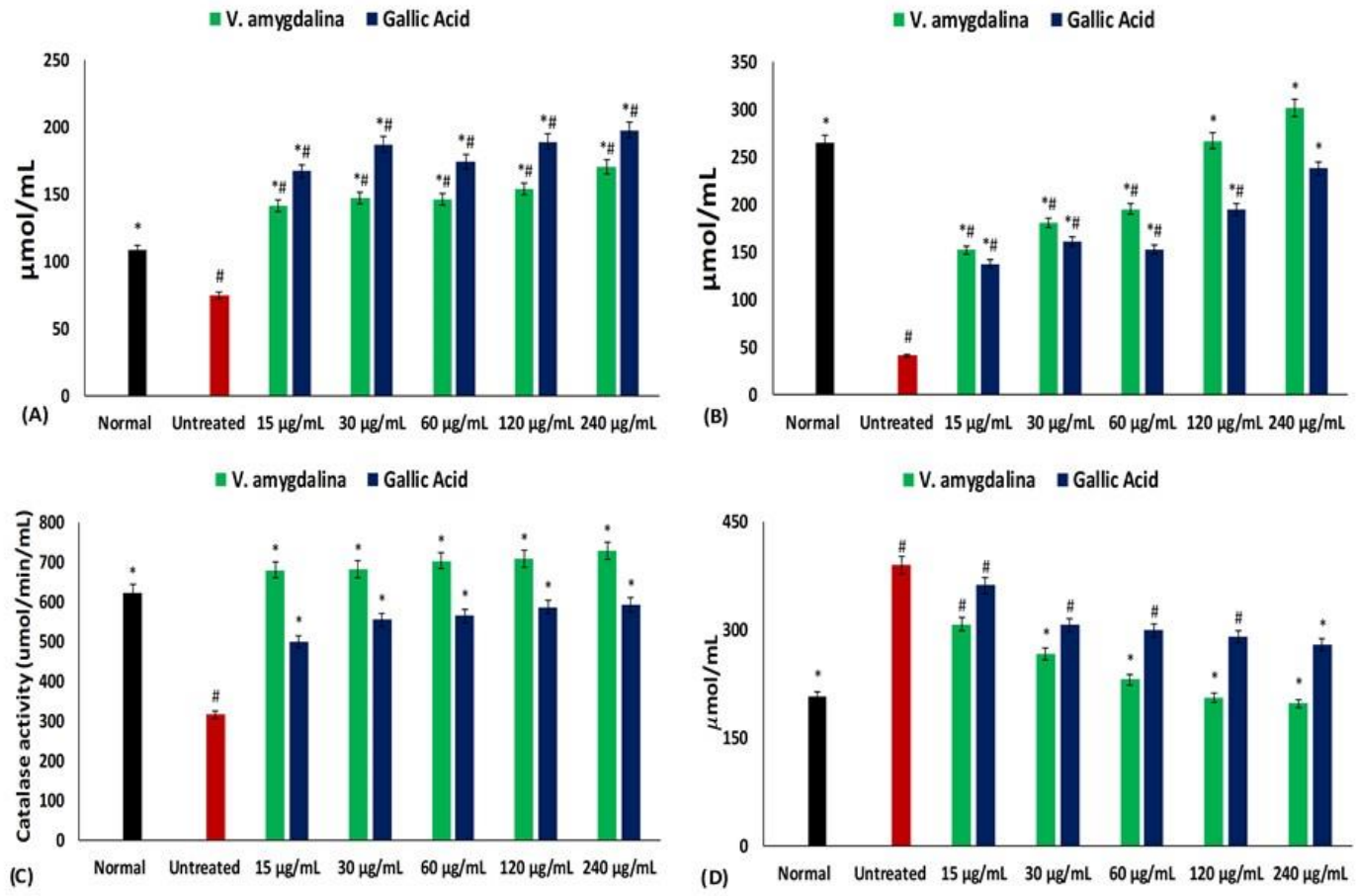

Figure 5.3.4: Effect of *V. amygdalina* infusion on (A) GSH level, (B) SOD activity, (C) catalase activity, and (D) MDA level in oxidative pancreatic injury. Values = mean ± SD; n = 3. *Significantly different from untreated sample and #Significantly different from normal sample ($p < 0.05$).

There was a significant ($p<0.05$) inhibition of α-glucosidase activity by the infusion in a dose – dependent manner as shown in Fig. 4A. While the infusion caused a slight inhibition of pancreatic lipase activity (**Figure 5.3.5B**).

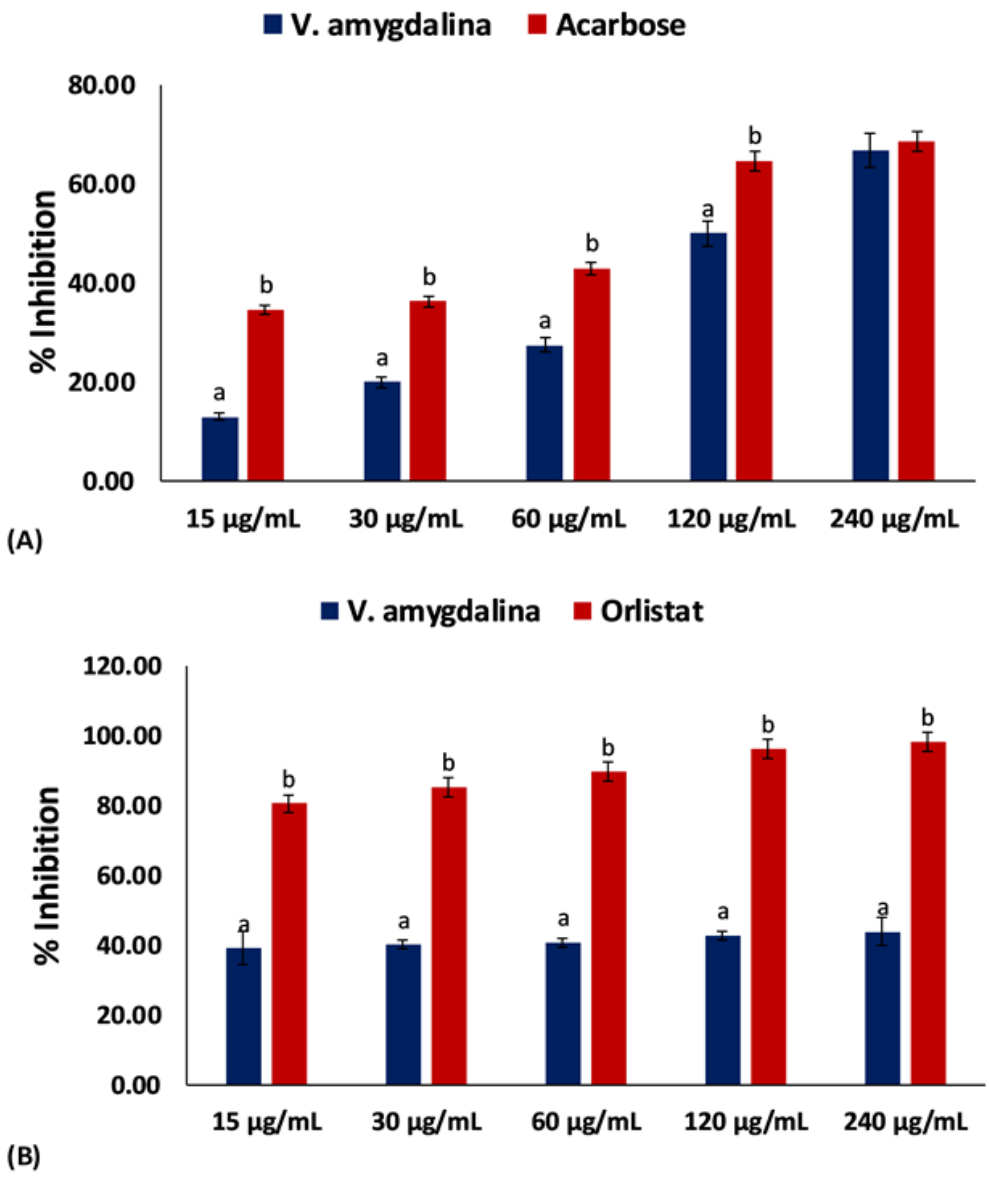

Figure 5.3.5: (A) α-glucosidase and (B) pancreatic lipase inhibitory properties of V. amygdalina infusion. Data = mean ± SD; n = 3. [ab]Values with different letter above the bars for a given concentration are significantly ($p < 0.05$) different from each other.

Incubation of isolated rat jejunum with the infusion in the presence of glucose led to significant ($p<0.05$) reduction in glucose absorption with increasing concentrations, indicating a dose-dependent inhibitory effect as shown in **Figure 3.6.6A**. There was a dose-dependent increased glucose uptake in isolated psoas muscles incubated with the infusion (with and without insulin) in

the presence of glucose (**Figure 5.3.6B**). Incubation with insulin had little or no effect on the glucose-uptake enhancing activity of the infusion.

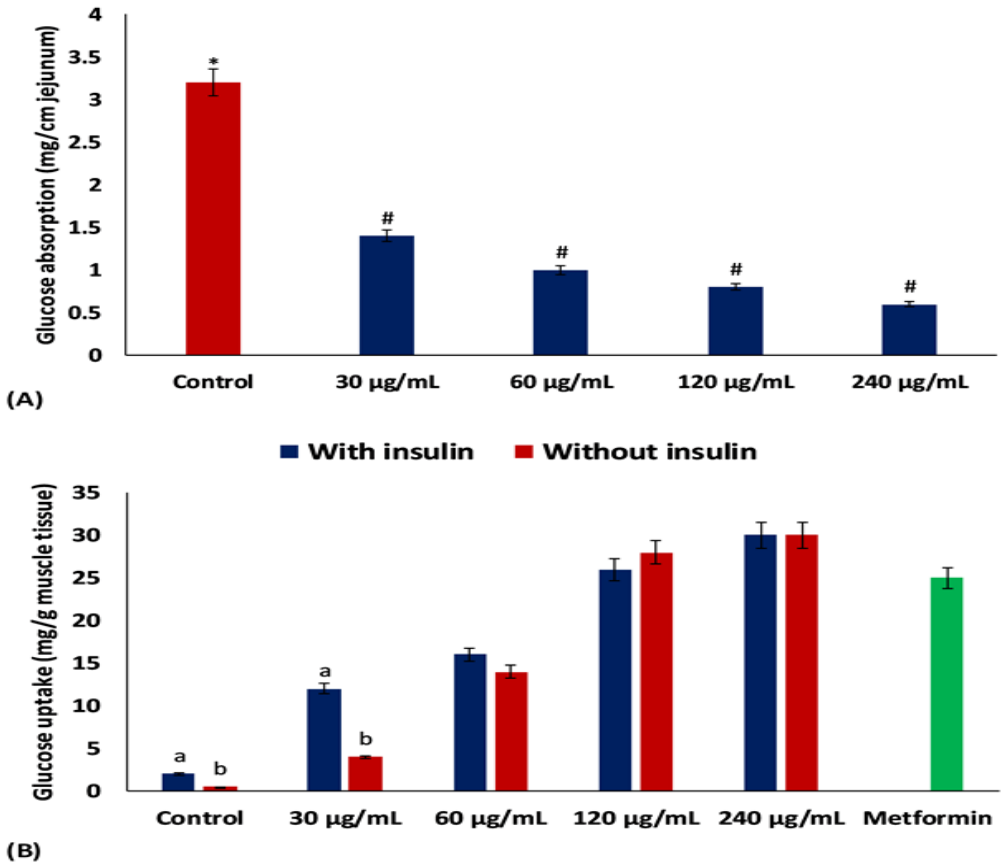

Figure 5.3.6: Effect of *V. amygdalina* infusion on (A) intestinal glucose absorption and (B) muscle glucose uptake. Data = mean ± SD; n = 3. *Significantly different from untreated sample (Control) and #Significantly different from treated samples; abValues with different letter above the bars for a given extract are significantly ($p < 0.05$) different from each other.

There was a significant increase in DNA fragmentation on incubation with hepatic tissue with $FeSO_4$ as depicted in **Figure 5.3.7**. Treatment with the infusion led to significant ($p<0.05$) reduction of the fragmentation in a dose dependent manner, with an IC_{50} value of 33.11 µg/mL (**Table 5.3.1**).

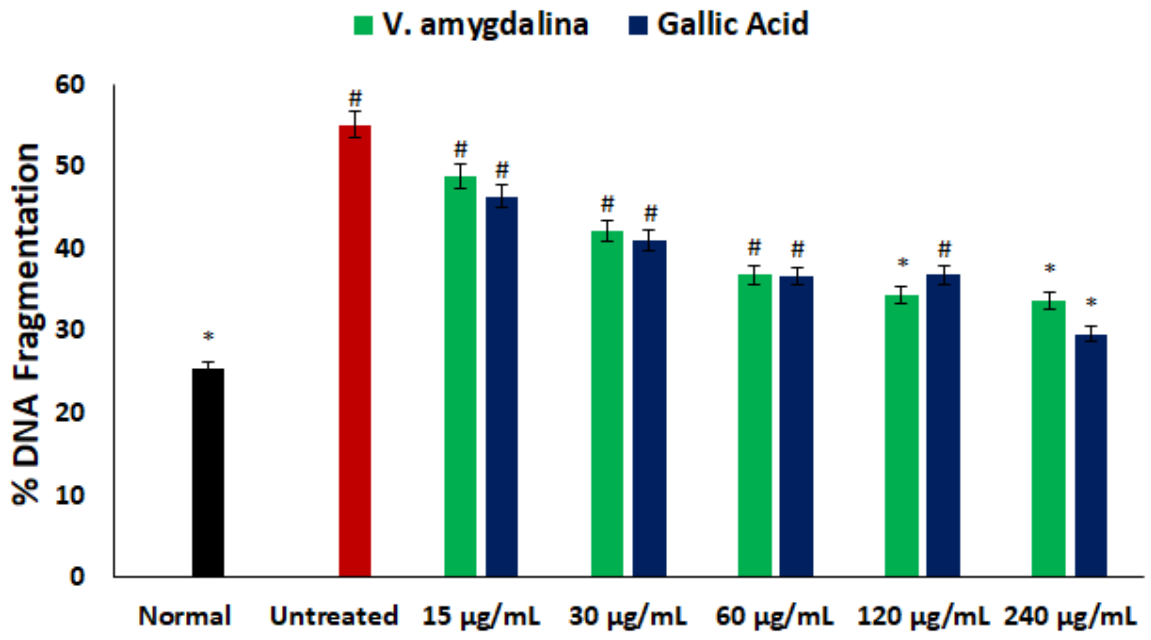

Figure 5.3.7: Effect of *V. amygdalina* infusion on hepatic DNA fragmentation. Significantly different from untreated sample and #Significantly different ($p < 0.05$) from normal sample.

5.3.5 Discussion

There has been an increased trend on the use medicinal plants in the treatment and management of DM and its complications (Ezuruike and Prieto 2014; Mohammed et al. 2014). This can be attributed to its reduced side effects coupled to its affordability compared to synthesized commercial drugs. In this study, we investigated the distribution of phytochemicals in *V. amygdalina* leaves and its ability modulate enzymes and activities involved in glucose homeostasis.

The total phenolic content, and the identified flavonoids, quercetin and (-) epi-catechin (**Figure 5.3.2**) correlates with the phenolic distribution of *V. amygdalina* leaves (**Figures 5.3.1E and 5.3.1F**). These properties also portray potent biological activities, as phenols have been recognized as the most influential phytochemical responsible for the medicinal properties of most plants (Lin et al. 2016; Rasouli et al. 2017; Selamoglu 2017).

The role of oxidative stress in the etiogenesis, pathogenesis and progression of T2D complications makes it an important aspect in the treatment and management of T2D. Antioxidants particularly,

the polyphenols have been demonstrated to be beneficial in the management of T2D and its complication (Hanhineva et al. 2010; Kim et al. 2016). The ability of the infusion to scavenge DPPH and reduce Fe^{3+} (**Figure 5.3.2**), indicates an antioxidant potential and corroborates previous reports on the free radical mopping activities of *V. amygdalina* leaves (Ho et al. 2012; Saliu et al. 2012). The depleted GSH level, SOD and catalase activities, as well as elevated MDA level on incubation of hepatic tissue with $FeSO_4$ (**Figure 5.3.3**) indicates an onset of oxidative stress. This can be attributed to the pro-oxidant activity of $FeSO_4$ owing to oxidation of Fe^{2+} to Fe^{3+} via the Fenton's reaction, thereby initiating iron toxicity (Andrews 1999; Jiang et al. 2004; Rajpathak et al. 2009). The ability of infusion to reverse these activities further portrays its antioxidative potentials, which correlates with its free radical scavenging activities (**Figure 5.3.3A**). These activities can be attributed to the total phenol, quercetin and (-) epi-catechin contents of the infusion (**Figure 5.3.2**), as well as the other identified phytochemicals distributed in the leaf, particularly the phenolic distribution on the epidermis and mid rib (**Figure 5.3.1**). Polyphenols are the most studied plant antioxidant phytochemicals and have been shown to exhibit their antioxidant activities by reducing free radicals via donation of H-atom from their OH group/s (Papuc et al. 2017). (-) epi-catechin has been recognized as the most common flavonoids of green teas (Forester and Lambert 2011; Kim et al. 2014). Identification of (-) epi-catechin in medicinal plants have also been correlated with their antioxidant activities (Chen et al. 2018; Grzesik et al. 2018). Quercetin is one of the most studied flavonoids and has been reported for its potent antioxidant activities (Bentz 2009; Ozgen et al. 2016). Several studies of reported have also reported the antioxidant activities of alkaloids (Erukainure et al. 2017b; Tiong et al. 2013), thereby indicating an influential role of the identified alkaloids on the mid rib of *V. amygdalina* leaf (**Figure 5.3.1G**).

The inhibitory effect of the infusion on α-glucosidase and pancreatic lipase activities (**Figure 5.3.5**) indicates an antidiabetic activity. These enzymes catalyze the breakdown of dietary carbohydrate and fats respectively. Inhibition of these enzymes reduces the amount of dietary glucose and fatty acids absorbed via the intestinal villi, thereby delaying postprandial rise in blood glucose and fatty acid levels (Erukainure et al. 2017a; Erukainure et al. 2018b). Several studies have reported the inhibition of these enzymes by phytochemicals (Ranilla et al. 2010), which portrays that the identified phytochemicals (**Figure 5.3.1**) and total phenolic content may be responsible for the inhibitory activity. Quercetin and (-) epi-catechin have been reported as major constituents of plant extracts with enzyme inhibitory activities (Li et al. 2009; Liu et al. 2016;

Proença et al. 2017; Yilmazer-Musa et al. 2012), and may also contribute to the enzyme inhibiting properties of *V. amygdalina* infusion.

The dose-dependent inhibition of intestinal glucose absorption (**Figure 5.3.6A**) indicates a potential of the infusion to inhibit postprandial rise in blood glucose level. This may be attributed to its ability to inhibit intestinal α-glucosidase activity (**Figure 5.3.5A**), thus delaying the breakdown of dietary carbohydrate to glucose while concomitantly inhibiting intestinal absorption of the glucose. Most antidiabetic drugs such as metformin act by stimulating muscle glucose uptake. Thus, the stimulation of muscle glucose uptake by the infusion with and without insulin (**Figure 5.3.6B**) further indicates its anti-hyperglycemia activity. Glucose uptake in skeletal muscle under resting conditions have been shown to be insulin dependent (Pereira et al. 2017b; Sinacore and Gulve 1993). However, this is impaired in T2D patients owing to insulin resistant (Alvim et al. 2015). The increased glucose uptake on incubation with insulin may therefore indicate improved insulin-sensitivity by *V. amygdalina*.

The increased DNA fragmentation on induction of oxidative hepatic injury (**Figure 5.3.7**) may reflect an occurrence of apoptosis (Erukainure et al. 2017a). Induction of apoptosis have been reported in rat hepatocytes subjected to iron overload induced oxidative stress (Allameh et al. 2010). Thus, implying that the induced DNA fragmentation in this study may be attributed to the pro-oxidant activity of the ferrous moiety of $FeSO_4$ via the Fenton reaction (Andrews 1999; Jiang et al. 2004; Rajpathak et al. 2009). Reduction in DNA fragmentation by the infusion may indicate an anti-apoptotic activity in oxidative hepatic injury, which correlates with its anti-oxidative activities (**Figure 5.3.4**) (Erukainure et al. 2017a).

5.3.6 Conclusion

This study portrays the distribution of phytochemicals in various parts of *V. amygdalina* leaf, which may be responsible for the antioxidative and antidiabetic activity of the infusion. The ability of the infusion to inhibit DNA fragmentation and stimulate muscle glucose uptake may suggest an anti-apoptotic and insulin-sensitivity activity of *V. amygdalina*. Thereby, indicating its anti-hepatotoxic and anti-hyperglycemia potentials.

5.4 *Vernonia Amygdalina* Stimulated Glucose Uptake in Brain Tissues Exacerbates Antioxidative and Anti-Proinflammatory Activities; and Modulates Functional Chemistry and Dysregulated Metabolic Pathways

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Preface: This article investigated the ability of the hot water infusion of *V.* amygdalina leaves to stimulate glucose uptake in brain tissues *ex vivo*. It also investigated the effect of stimulated glucose uptake on the antioxidant, and metabolic pathways of brain tissues. The manuscript has been accepted for publication in Metabolic Brain Disease (MEBR-D-18-00320).

5.4.1 Abstract

Brain glucose uptake is usually reduced in type 2 diabetes owing to downregulation of brain glucose transporters. The ability of *Vernonia amygdalina* to stimulate glucose uptake as well as ameliorate glucose-induced oxidative stress and proinflammation were investigated in rat brains. Hot infusion of *V. amygdalina* leaves was incubated with rat brain tissues for 2 hours in the presence of glucose. Another incubation with glucose only, served as negative control while metformin served as the standard drug. Incubation of brain tissues with *V. amygdalina* led to significant ($p<0.05$) increased glucose uptake, reduced glutathione, nitric oxide and non-thiol proteins levels, superoxide dismutase, catalase and ATPase activities, while concomitantly decreasing myeloperoxidase activity and malondialdehyde level compared to the control.

Incubation with glucose only, led to the development of nitrate, amide II and amide I functional groups which were removed on incubation with the infusion. LC-MS analysis revealed depletion of oxidative-induced generated 2-keto-glutaramic acid and cysteinyl-tyrosine metabolites in brain tissues, with concomitant generation of S-formylglutathione and adenosine tetraphosphate by the infusion. Pathway analysis of the metabolites revealed an activation of the pyruvate metabolism pathway in the negative control, with the infusion reducing the intensity fold. LC-MS analysis of the infusion revealed the presence of l–serine, l-cysteine, l-proline, nicotinic acid, cumidine, salicylic acid, isoquinoline, 3-methyl-, and γ-octalactone. Except for l–serine, l-cysteine and l-proline, the other compounds were predicted to be permeable across the blood brain barrier. These results indicate the brain glucose uptake stimulatory and neuroprotective effect of *V. amygdalina*.

Keywords: Antioxidative; Diabetic Brain; Glucose uptake; Proinflammatory; and *Vernonia amygdalina*

5.4.2 Introduction

Diabetes mellitus (DM) has been recognized as a global epidemic affecting over 425 million people in 2017, with an estimated 48% increase postulated for 2045 (I.D.F. 2018). It is metabolic disorder characterized by high blood glucose (hyperglycemia) owing to failure of the pancreatic β cell to secrete insulin and/or inability of the cells to utilize secreted insulin as seen in type 1 diabetes (T1D) and type 2 diabetes (T2D) respectively (Erukainure et al. 2017a). T2D accounts for more than 90% of all diabetes types, thus making it a major contributor to diabetic mobility and mortality (I.D.F. 2018). Increased production of reactive oxygen species (ROS) have been reported in extreme hyperglycemia in T2D (Erukainure et al. 2018a; Maritim et al. 2003). Inability of the tissues' endogenous system to mop these free radicals, results to oxidative stress which has been implicated in the pathogenesis and progression of microvascular and macrovascular complications in T2D (Whitlow et al. 2015). Studies have implicated T2D in microvascular complications in the brain (Vagelatos and Eslick 2013; Whitlow et al. 2015; Wrighten et al. 2009). This has been attributed to alteration in insulin signaling and glucose homeostasis in the CNS (Wrighten et al. 2009). Glucose is the predominant source of energy in the brain and is transported across the blood brain barrier (BBB) by glucose transporters (McEwen and Reagan 2004; Reagan et al. 2008; Wrighten et al. 2009). These transporters are often down regulated in T2D, thus impairing glucose uptake in the brain (Gejl et al. 2017; Hwang et al. 2017; Pardridge et al. 1990).

The neuroprotective effects of medicinal plants have been established (Aslam and Sial 2014; Mohebbatia et al. 2017; Pandit 2011; Uddin et al. 2013). These effects have been attributed to the phytochemical constituents of the plants, particularly the phenolics with reported antioxidant, antidiabetic and ability to transverse the BBB (Rice-Evans et al. 1997; Saravanan and Parimelazhagan 2014; Youdim et al. 2003). Some of these plants have also been reported for their antidiabetic properties, indicating their protective potential against diabetic brain degeneration. Amongst such plants is *Vernonia amygdalina*.

Although the neuroprotective effect of *V. amygdalina* has been reported, there is however a dearth in its ability to stimulate glucose uptake in brain tissues and the metabolic pathways that may be involved. Hence, this study was undertaken to investigate the glucose uptake enhancing properties of *V. amygdalina* hot infusion, and its antioxidative and anti-proinflammatory effects in brain tissues, as well as the metabolic pathways and metabolites that may be involved.

5.4.3 Materials and Methods

Kindly refer to chapter 2, sections 2.1, 2.7.3 – 2.8

5.4.4 Results

Incubation of brain tissues in *V. amygdalina* infusion in the presence of d-glucose, led to significant ($p<0.05$) increase in glucose uptake compared to the control (glucose only) and metformin as depicted in **Figure 5.4.1**. The uptake was dose dependent, with the highest concentration stimulating the highest uptake. Metformin showed little or no significant effect on glucose uptake.

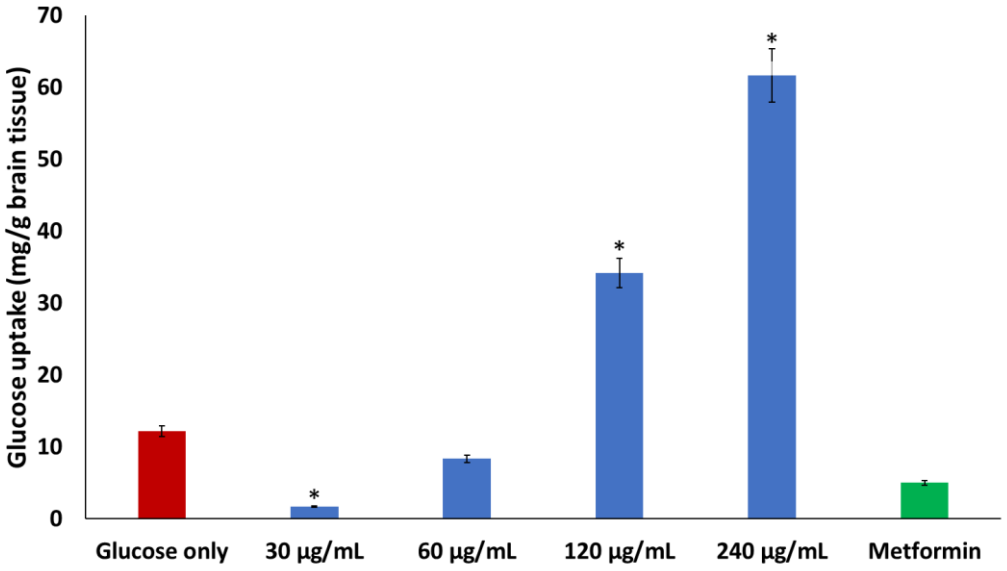
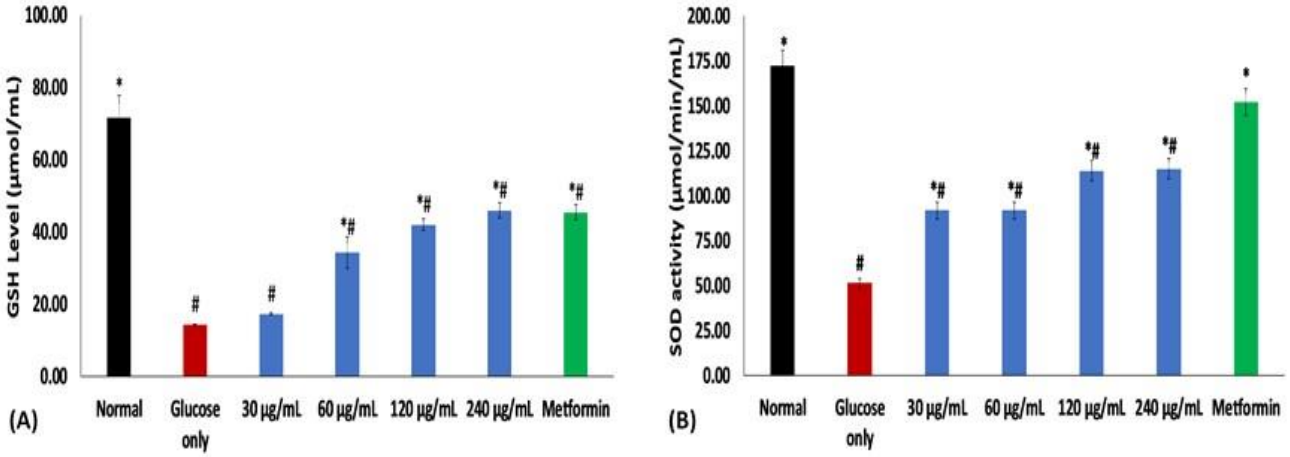


Figure 5.4.1: Effect of V. amygdalina infusion on brain glucose uptake. Data = mean ± SD; n = 3. *Statistically significant compared to Glucose-only treated tissues

Incubation of brain tissues with d-glucose significantly ($p<0.05$) depleted GSH and NTP level, SOD and catalase activities, with concomitant increased levels of MDA as depicted in **Figures 5.4.2A – 2E**. These were significantly ($p<0.05$) reversed on incubation with *V. amygdalina* and metformin respectively, depicting an antioxidative effect.



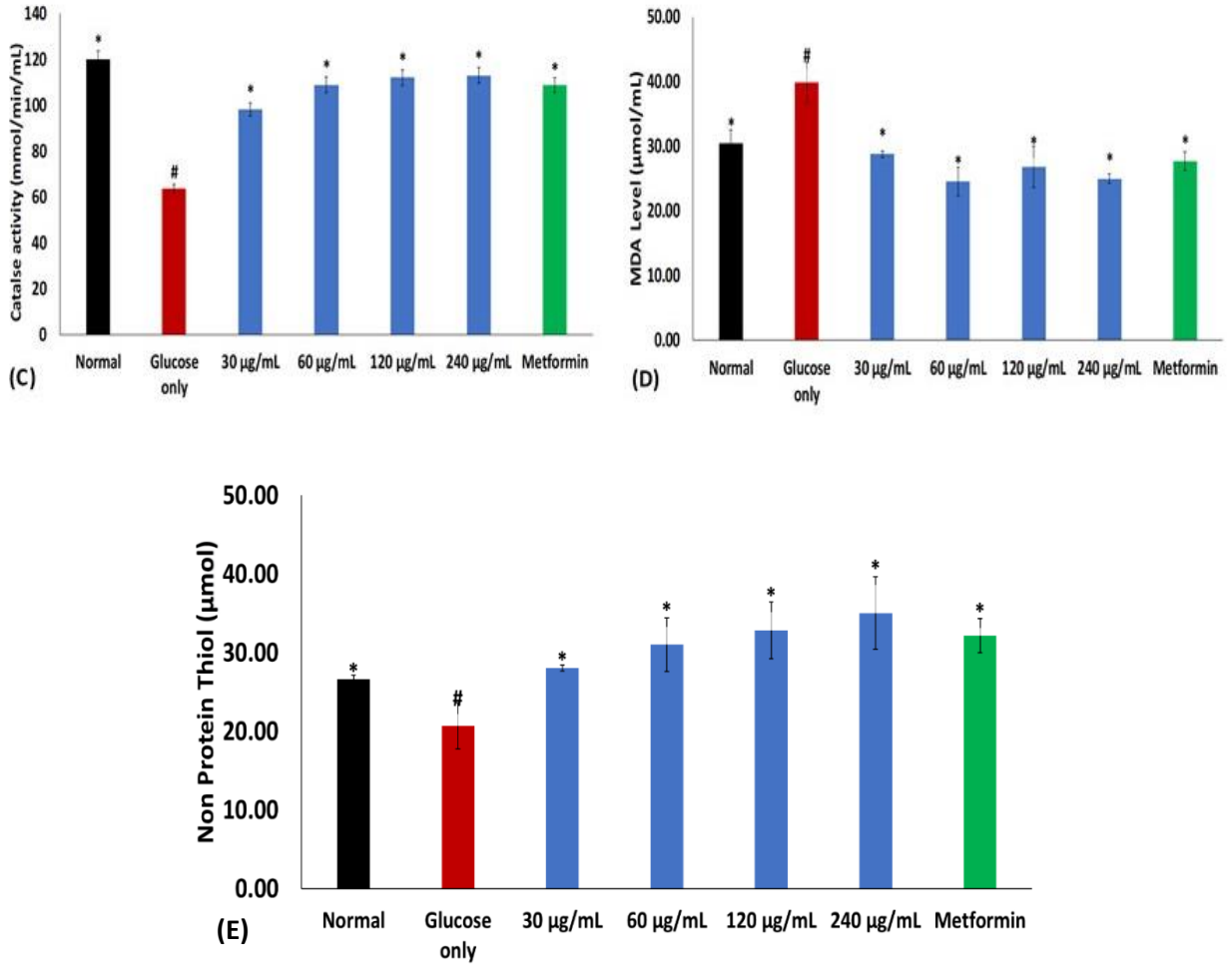


Figure 5.4.2: Effect of *V. amygdalina* infusion on (A) GSH level; (B) SOD and (C) catalase activities; (D) MDA; and (E) non-thiol proteins levels in glucose treated brain. Data = mean ± SD; n = 3. *Statistically significant compared to Glucose-only treated tissues. #Statistically significant compared to normal tissues

There was an increased NO level and myeloperoxidase activity on incubation of brain tissues with d-glucose only as shown in **Figures 5.4.3A and 3B**, portraying an occurrence of proinflammation. Incubation with the infusion and metformin led to significant ($p<0.05$) depleted level and activity.

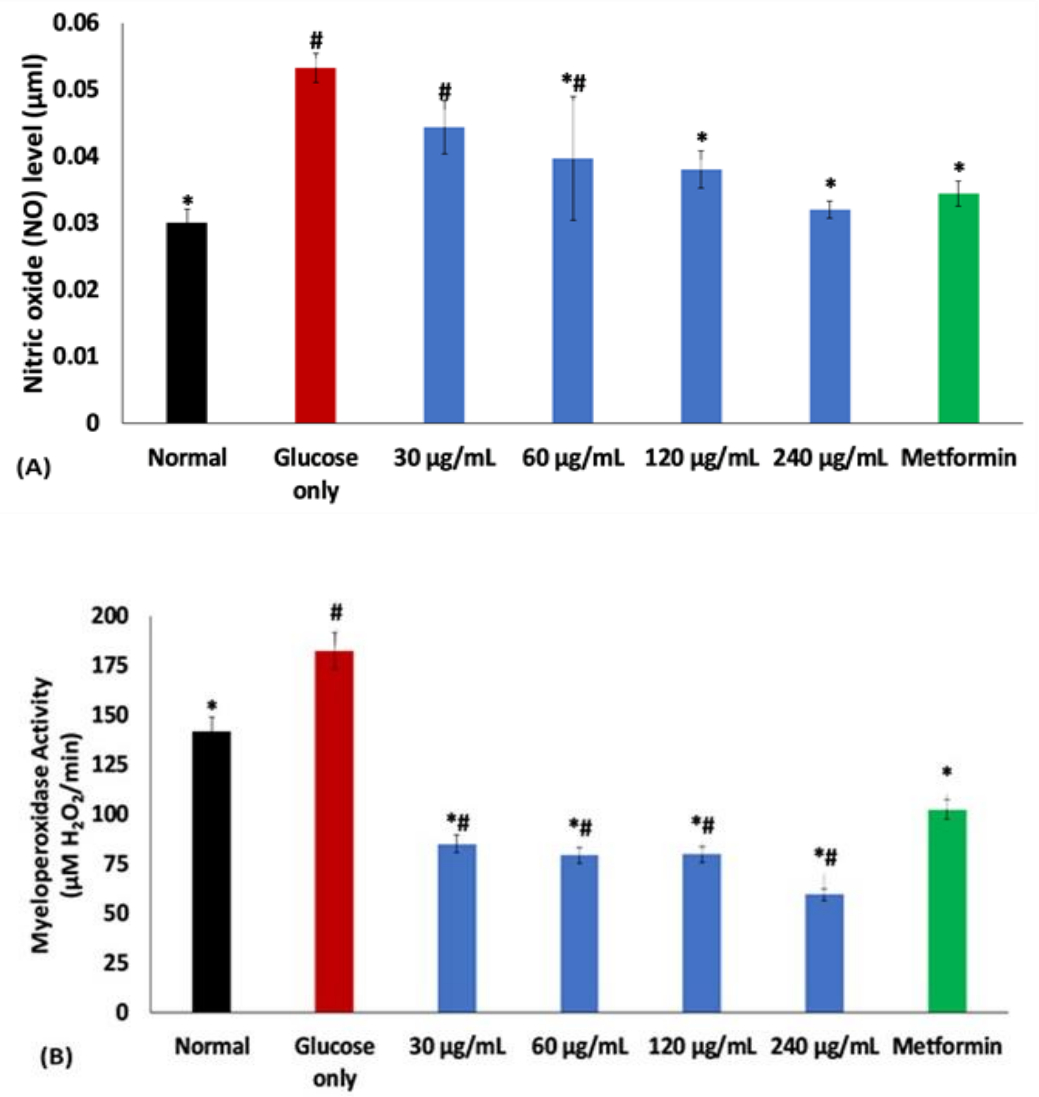


Figure 5.4.3: Effect of *V. amygdalina* infusion on (A) NO level and (B) myeloperoxidase activity in glucose treated brain. Data = mean ± SD; n = 3. *Statistically significant compared to Glucose-only treated tissues; #Statistically significant compared to normal tissues

Incubation of brain tissues with d-glucose only, caused a significant ($p<0.05$) increase in ATPase activity as depicted in **Figure 5.4.4**. This was significantly reduced dose – dependently on incubation with the infusion, with metformin showing little or no effect.

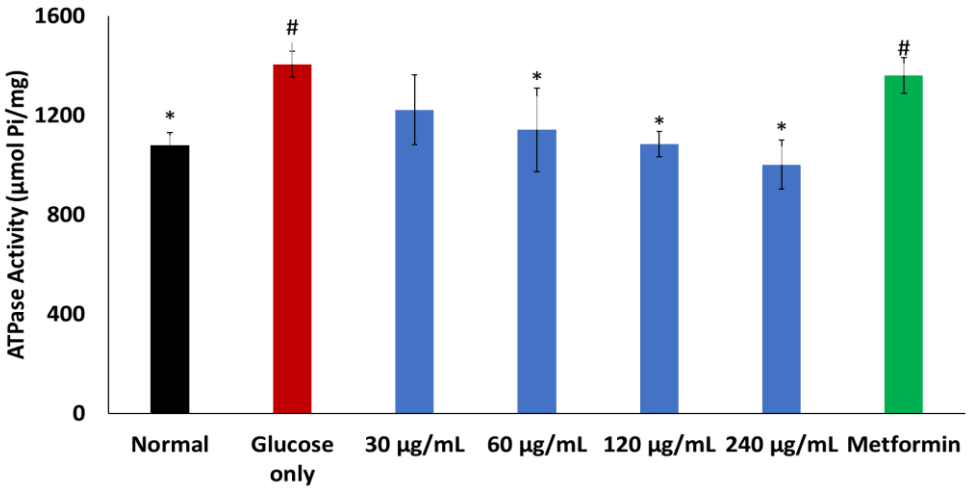
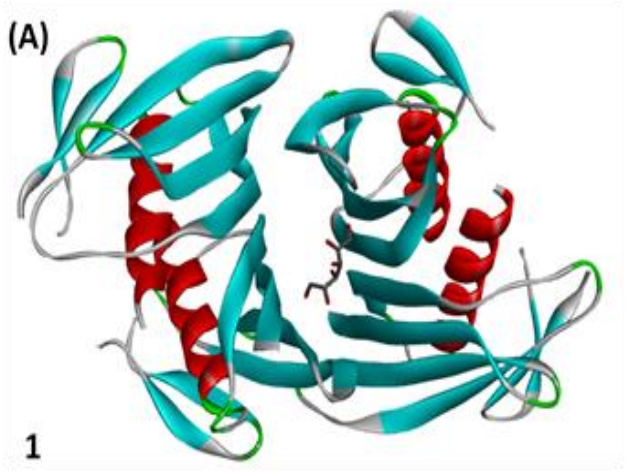
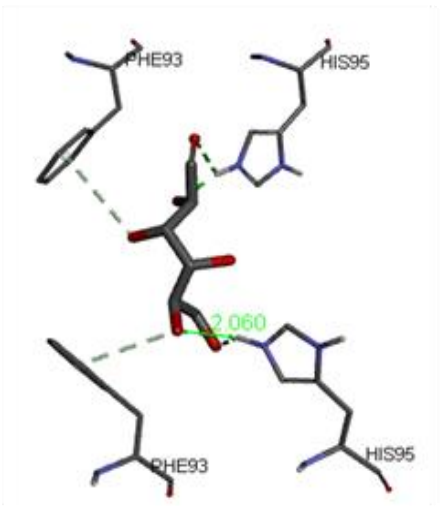
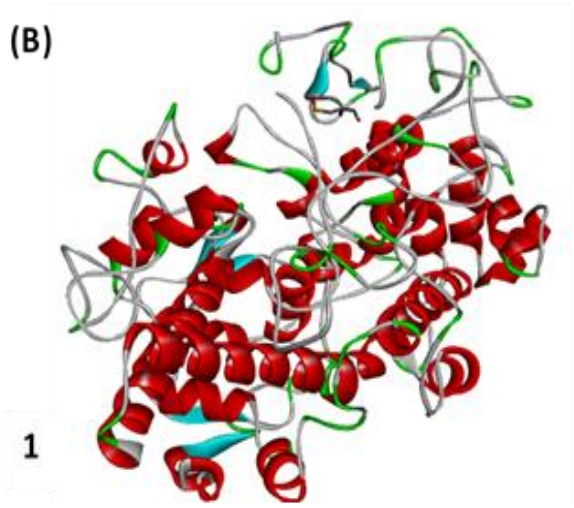


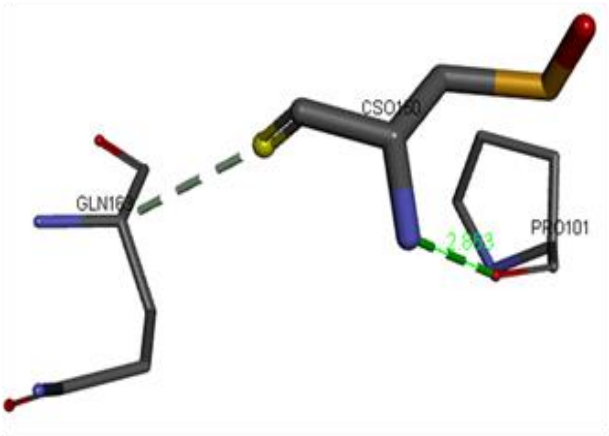
Figure 5.4.4: Inhibitory activities of *V. amygdalina* infusion on ATPase activity in glucose – treated brain. Data = mean ± SD; n = 3. *Statistically significant compared to Glucose-only treated tissues; #Statistically significant compared to normal tissues

Molecular docking of d-glucose with catalase, myeloperoxidase, and ATPase revealed significant interactions, with ATPase showing the highest interaction (-5.45 kcal/mol) as depicted in **Figures 5.4.5A – 5C** and **Table 5.4.1**. The core amino acid residues and hydrogen bond distance between the residues and ligand are shown in **Table 5.4.1**, with ATPase interaction having the highest number of residues (threonine (THRE), asparagine (ASN), leucine (LEU), and glycine (GLY)) and atoms of residues (oxygen (O), nitrogen (N) and hydrogen (H)).


(A)
1


PHE93
HIS95
2.060
PHE93
HIS95
2


(B)
1


CSO150
GLN16
PRO101
2.883
2

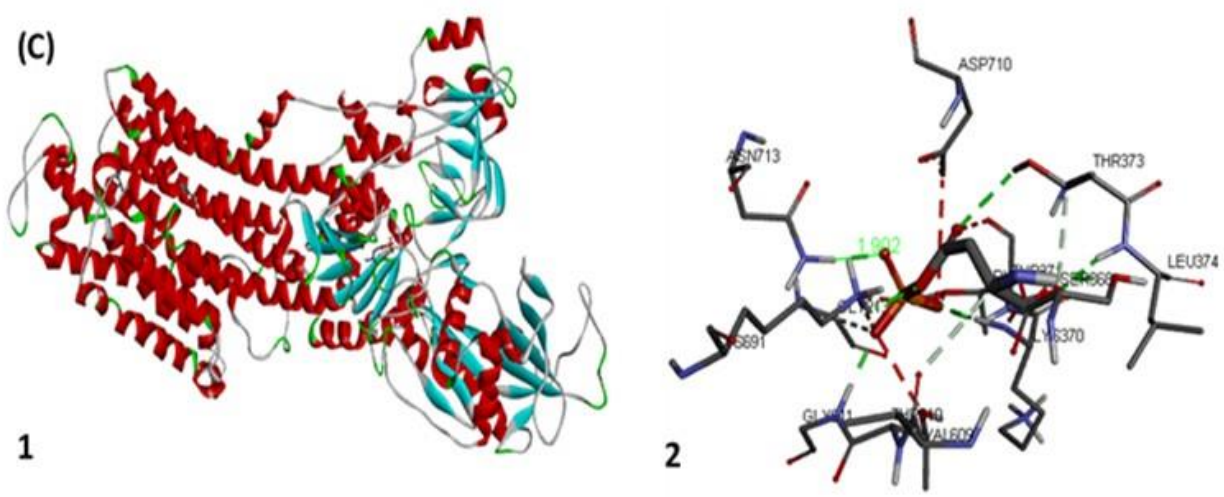


Figure 5.4.5: Molecular interactions of D-glucose with (A) myeloperoxidase; (B) catalase; and (C) ATPase activities.

Table 5.4.1: Calculated binding energies, core amino acid residue and hydrogen bond distance between residues and ligand from molecular docking

Enzymes	**Binding energies (kcal/mol)**	**Residues**	**Atoms of the residues**	**Distance (Å)**
ATPase	-5.45	THRE, ASN, LEU, GLY	O, N, H	2.56
Catalase	-4.20	HIS, PHE	H, N	2.06
myeloperoxidase	1.49	PRO, GLN	C, N	2.86

Incubation of brain tissues with glucose only, led to the development of nitrate, amide II and amide I functional groups as shown in **Figure 5.4.6** and **Table 5.4.2**. Incubation with the infusion and metformin led to removal of these functional groups.

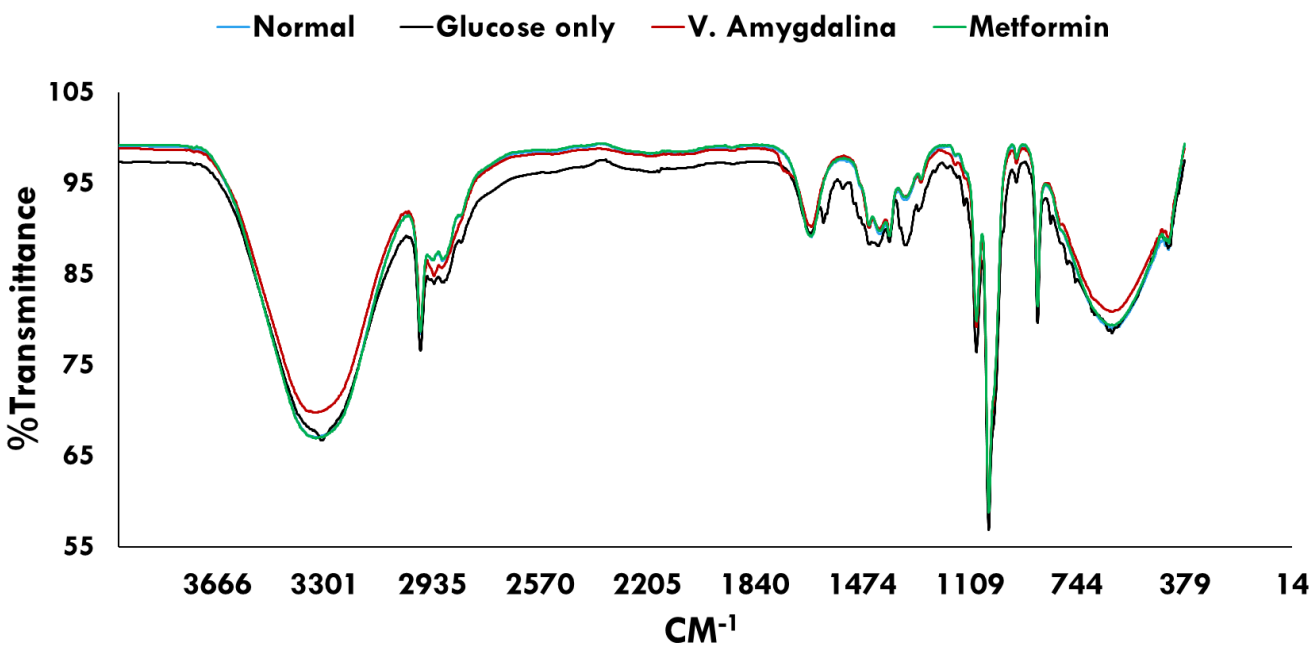


Figure 5.4.6: FTIR Spectroscopy of the effect of *V. amygdalina* infusion on glucose – oxidative brain metabolites

Table 5.4.2: Quantitative Analysis of FTIR Spectra of Glucose Brain Metabolites

Regions (cm^{-1})	**Normal (cm^{-1})**	**Glucose only (cm^{-1})**	***V. amygdalina* (cm^{-1})**	**Metformin (cm^{-1})**	**Assignment**
900 - 680	–	834.85	–	–	Nitrate
	878.92	879.09	879.09	878.93	1°, 2° amines
1200 - 900	951.14	951.52	951.34	950.88	Nucleic acid
	1045.07	1045.34	1045.26	1045.15	Nucleic acid
	1086.92	1087.36	1086.99	1086.95	Phosphates
1500 – 1200	1275.56	1284.50	1275.63	1275.68	Amide 1
	1324.42	1326.02	1331.77	1325.42	Aromatic amines
	1382.87	1382.82	1381.95	1382.53	Phenol –O–H
	1416.85	1420.77	1416.70	1413.51	Nitramines
	1450.26	–	1451.16	1451.38	Amide II
1800 – 1500	–	1540.09	–	–	Amide II
	–	1606.51	–	–	Amide I
	1648.08	1647.32	1647.63	1643.61	Alkenes
3000 – 2800	2938.70	2928.95	2975.30	2931.57	Lipid (CH_2)
	2901.91	2896.84	–	2975.91	Lipid (CH_2
	2975.59	2975.52	2975.30	2975.91	Lipid (CH_2)
3180 – 3030	3327.11	3311.62	3333.92	3331.03	1°, 2° amines, amides

Key: – = absent

LC-MS characterization of *V. amygdalina* infusion revealed an alkaloid rich extract consisting of nicotinic acid, cumidine, and isoquinoline, 3-methyl- (**Figure 5.4.7**). Amino acids consisting of l-serine, l-cysteine and l-proline were also identified. Other compounds identified were salicylic acid and γ-octalactone.

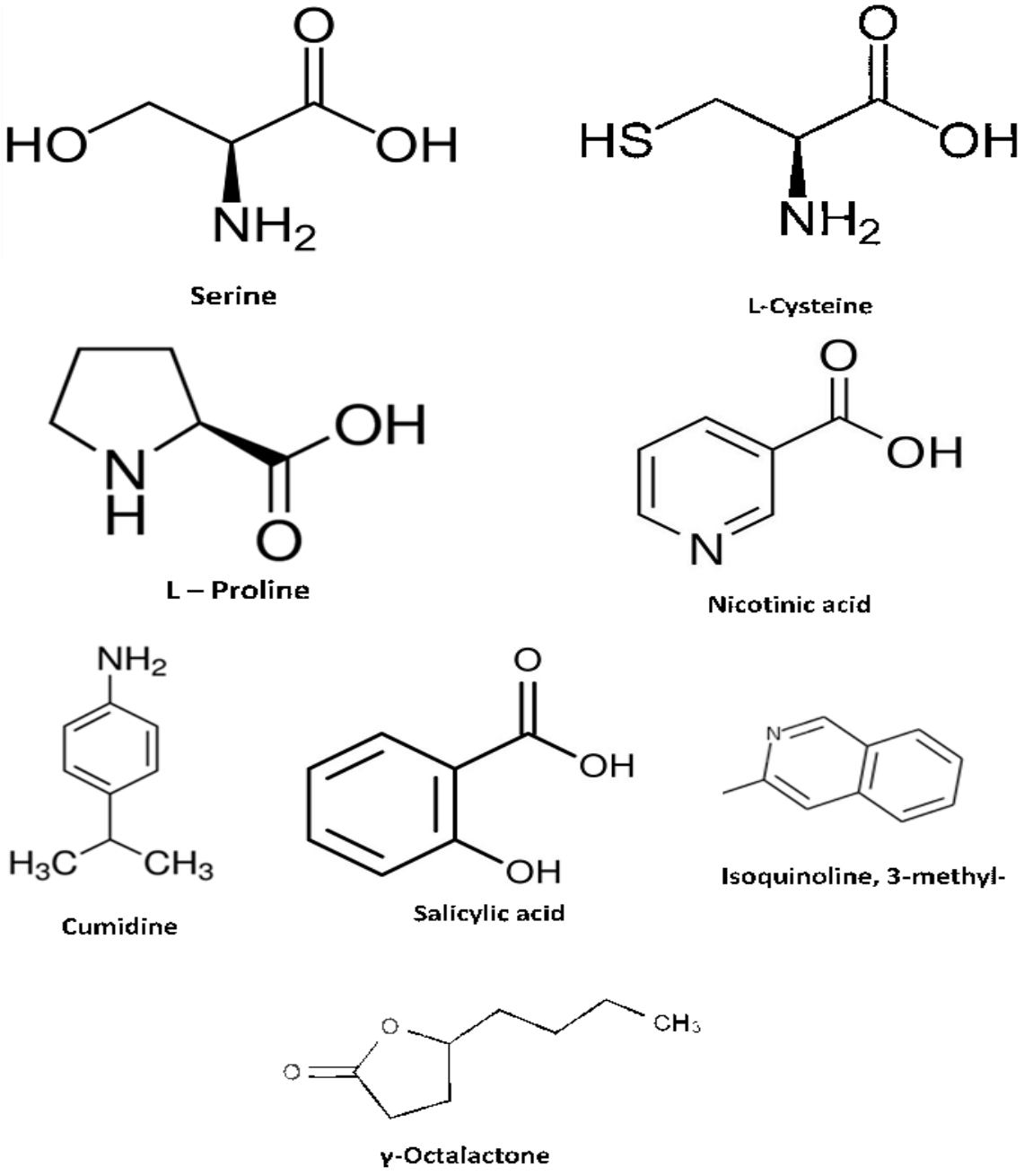


Figure 5.4.7: LC-MS identified compounds of *V. amygdalina* infusion

Analysis of the metabolites of the normal brain tissues revealed the presence of ganglioside, glucose, cardiolipin, triglyceride, inosine, molybdenum cofactor, monosaccharide, nucleotide, and iduronic acid metabolic intermediates as shown in **Table 5.4.3**. Incubation with d-glucose led to depletion of dTDP-D-glucose and ganglioside GM2 (d18:0/22:1(13Z)), with concomitant generation of 2-keto-glutaramic acid, cysteinyl-tyrosine, acetyl adenylate,

CL(18:1(11Z)/18:1(11Z)/18:1(11Z)/18:1(11Z)), Ganglioside GM1 (d18:1/25:0), 4-Methylnonacosane, Ganglioside GT3 (d18:1/16:0) and Ganglioside GT3 (d18:0/16:0). Treatment with the infusion led regeneration of dTDP-D-glucose, with concomitant depletion of P1,P4-Bis(5'-uridyl) tetraphosphate, CL(18:2(9Z,12Z)/18:2(9Z,12Z)/18:2(9Z,12Z)/16:1(9Z)), 2-keto-glutaramic acid and cysteinyl-tyrosine. It also led to the generation of S-formylglutathione and adenosine tetraphosphate. Metformin led to regeneration of the glucose-induced depleted metabolites, with concomitant depletion of cysteinyl-tyrosine, acetyl adenylate, CL(18:1(11Z)/18:1(11Z)/18:1(11Z)/18:1(11Z)), ganglioside GT3 (d18:1/16:0) and ganglioside GT3 (d18:0/16:0), and generation of superoxide, glycerol 3-phosphate and guanosine tetraphosphate adenosine.

Table 5.4.3: Identified metabolites in glucose treated brain tissues

Metabolites	**Normal Tissues**	**Glucose only Tissues**	***V. amygdalina* Treated Tissues**	**Metformin Treated Tissues**
Uridine 5'-diphosphate	X	X	X	X
Ganglioside GM2	X	X	X	X
1-Phosphatidyl-1D-myo-inositol 3-phosphate	X	X	X	X
Ganglioside GD3 (d18:0/26:0)	X	X	X	X
TG(24:1(15Z)/o-18:0/18:4(6Z,9Z,12Z,15Z))	X	X	X	X
3-Methylellagic acid 8-(4-acetylrhamnoside)	X	X	X	X
UDP-L-iduronate	X	X	X	X
UDP-4-keto-6-deoxy-D-glucose	X	X	X	X
Ganglioside GT3 (d18:1/26:0)	X	X	X	X
dTDP-D-glucose	X	–	X	X
Cyanidin 3-(6''-dioxalylglucoside)	X	X	X	X
Adenosine tetraphosphate	X	X	X	X
ADP-ribose 1''-2'' cyclic phosphate	X	X	X	X
CL(16:0/18:0/16:0/18:1(9Z))	X	X	X	X
Ganglioside GT1b (d18:0/14:0)	X	X	X	X
Ganglioside GM2 (d18:0/22:1(13Z))	X	–	–	X
CL(16:1(9Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z) /22:5(7Z,10Z,13Z,16Z,19Z)/16:1(9Z))	X	X	X	X
Sedoheptulose 1,7-bisphosphate	X	X	X	X
P1,P4-Bis(5'-uridyl) tetraphosphate	X	X	–	X
CL(18:2(9Z,12Z)/18:2(9Z,12Z)/18:2(9Z,12Z) /16:1(9Z))	X	X	–	X
Molybdopterin-AMP	X	X	X	X
Ganglioside GM1 (18:1/22:0)	X	X	X	X
2-Keto-glutaramic acid	–	X	–	X
Cysteinyl-Tyrosine	–	X	–	–
Acetyl adenylate	–	X	X	–
CL(18:1(11Z)/18:1(11Z)/18:1(11Z)/18:1(11Z))	–	X	X	–
Ganglioside GM1 (d18:1/25:0)	–	X	X	X
4-Methylnonacosane	–	X	X	–
Ganglioside GT3 (d18:1/16:0)	–	X	X	–
Ganglioside GT3 (d18:0/16:0)	–	X	X	X
S-Formylglutathione	–	–	X	–
Adenosine tetraphosphate	–	–	X	–
Superoxide	–	–	–	X
Glycerol 3-phosphate	–	–	–	X

Guanosine tetraphosphate adenosine	–	–	–	X

Key: – = absent, X = present

Pathway enrichment analysis of the identified metabolites revealed metabolic pathways for lactose synthesis, androstenedione, estrone, sucrose, amino sugar, androgen and estrogen, inositol, galactose, porphyrin, sphingolipid, and pyrimidine metabolisms in normal brain tissues (control) as shown in **Table 5.4.4**. These pathways were unaltered on incubation with d-glucose, with activation of pyruvate metabolism pathway. Although incubation with *V. amygdalina* infusion did not affect these pathways, the intensity fold of the pyruvate metabolism pathway was significantly lower than that of glucose-only treated tissues. Treatment with metformin deactivated the pyruvate metabolism pathway, with concomitant activation of de novo triacylglycerol biosynthesis, cardiolipin biosynthesis, degradation of superoxides, glycerol phosphate shuttle, mitochondrial electron transport chain, glycerolipid metabolism, and phospholipid biosynthesis pathways.

Table 5.4.4: Identified metabolic pathways in glucose treated brain tissues

Metabolic Pathways	**Normal Tissues**	**Glucose only Tissues**	***V. amygdalina* Treated Tissues**	**Metformin Treated Tissues**
Lactose Synthesis	X	X	X	X
Androstenedione Metabolism	X	X	X	X
Estrone Metabolism	X	X	X	X
Starch and Sucrose Metabolism	X	X	X	X
Amino Sugar Metabolism	X	X	X	X
Androgen and Estrogen Metabolism	X	X	X	X
Inositol Metabolism	X	X	X	X
Galactose Metabolism	X	X	X	X
Porphyrin Metabolism	X	X	X	X
Sphingolipid Metabolism	X	X	X	X
Pyrimidine Metabolism	X	X	X	X
Pyruvate Metabolism	–	X	X	–
De Novo Triacylglycerol Biosynthesis	–	–	–	X
Cardiolipin Biosynthesis	–	–	–	X
Degradation of Superoxides	–	–	–	X
Glycerol Phosphate Shuttle	–	–	–	X
Mitochondrial Electron Transport Chain	–	–	–	X
Glycerolipid Metabolism	–	–	–	X
Phospholipid Biosynthesis	–	–	–	X

Key: – = absent, X = present

Except the amino acids, the other identified compounds of *V. amygdalina* infusion were predicted to be able to cross the blood brain barrier (**Table 5.4.5**). All the identified compounds were predicted to be orally safe as they fell between classes 4 – 6, except for l-serine which fell on class 2.

Table 5.4.5: BBB Permeability and Predicted toxicity of compounds from *V. amygdalina* infusion

Compounds	BBB Permeability	Predicted LD50 (mg/kg)	Predicted Toxicity Class
L – Serine	No	2000	2
L – Cysteine	No	660	4
L - Proline	No	2078	5
Nicotinic Acid	Yes	3720	5
Cumidine	Yes	500	4
Salicylic acid	Yes	480	4
Isoquinoline, 3-methyl-	Yes	1230	4
γ-Octalactone	Yes	4390	5

5.4.5 Discussion

Diminished brain glucose uptake has been recognized as one of the complications of type 2 diabetes despite chronic hyperglycemia (Vagelatos and Eslick 2013; Whitlow et al. 2015). This has been attributed to down regulation of glucose transporters at the BBB, thus reducing facilitative transportation of glucose to the brain (Gejl et al. 2017; Hwang et al. 2017). *Vernonia amygdalina* amongst other medicinal plants have been reported for its antidiabetic and neuroprotective properties. To the best of our knowledge, this study reports for the first time the ability of *V. amygdalina* to stimulate brain glucose uptake and its effect on brain metabolic pathways and metabolites.

The increased brain glucose uptake by the infusion (**Figure 5.4.1**) indicates a glucose uptake facilitative potential of *V. amygdalina*. This can be attributed to the identified alkaloid, polyphenol and lactone constituents of the infusion (**Figure 5.4.7**), as they were predicted to be BBB permeable (**Table 5.4.5**). Their predicted permeability across BBB may facilitate the transportation of glucose to the brain by activation of glucose transporters notably GLUTs 1, 3 and 5 which are highly concentrated at the BBB (Simpson et al. 1994). Studies have reported the influence of phytochemicals particularly polyphenols on the activation of glucose transporters by medicinal plants (León et al. 2017; Williamson 2013). The reduced glucose uptake in brain tissues

incubated with metformin can be attributed to the fact that metformin exhibits its action by activation of GLUTs 2 and 4, which are less expressed at the BBB (Kellett and Brot-Laroche 2005; Rice et al. 2011). The increased glucose uptake by *V. amygdalina* can also be attributed to its ability to decrease ATPase activities in brain tissues (**Figure 5.4.4**), as decreased ATPase activity, particularly the Na^+/K^+ ATPase have been implicated in facilitating glucose transportation across the BBB (Falkowska et al. 2015; Magistretti and Allaman 2015). The high binding energy on docking d-glucose with ATPase (**Figure 5.4.5C** and **Table 5.4.1**) portrays a strong molecular interaction, which is evident by the increased ATPase activity (**Figure 5.4.4**) and decreased glucose uptake (**Figure 5.4.1**) in brain tissues incubated with glucose only. Similarly, the increased ATPase activity in the metformin treated brain tissues (**Figure 5.4.4**) corroborates its decreased glucose uptake (**Figure 5.4.1**).

Oxidative stress and inflammation have been reported for its influential role in the pathogenesis and progression of neuropathology (Das et al. 2009; Patel 2016), which have been attributed to the high consumption of O_2 and glucose dependence by the brain (Patel 2016). Although the brain's anatomy allows for a reductive environment which minimizes ROS generation, its low endogenous antioxidant system, redox-active metal load, polyunsaturated fatty acids, and excitotoxic and auto-oxidizable neurotransmitters dependence makes it prone to oxidative stress (Butterfield et al. 2001; Huang et al. 2004; Patel 2016). The depleted levels of GSH, NPT, and increased SOD and catalase activities, with concomitant increased MDA level in brain tissues incubated with glucose only (**Figures 5.4.2A – 2E**) depicts an occurrence of oxidative stress. This can be attributed to activation of the pyruvate metabolism pathway (**Table 5.4.4**). Activation of this pathway will lead to the production of lactate with concomitant generation of the essential cofactor, NAD^+. The continuous drive of this pathway lead to accumulation of lactate and increased generation of NAD^+ which sustains the glycolytic flux, which in turn increases the turnover of glycolysis production of the electron donor, NADH. This electron donor has been implicated in the production of ROS, as it inhibits the electron transport at complex II leading to the reduction of O_2 to $O_2^{\cdot-}$ (Brownlee 2001; Du et al. 2001). The activation of this pathway can also be attributed to the decreased glucose uptake, owing to the need for the brain to switch energy source from glucose to ketones. This is evident by the presence of the ketone metabolite, 2-keto-glutaramic acid in the glucose-only treated tissue (**Table 5.4.3**). 2-keto-glutaramic acid can also act as a substrate for the enzyme, alanine transaminase which catalyzes the reversible conversion of alanine to pyruvate. The

presence of sphingolipids and triglyceride derivatives (**Table 5.4.3**) in the glucose-only treated brain tissue, may be responsible to the increased MDA level (**Figure 5.4.2D**) as they can act as substrates for lipid peroxidation. The strong molecular interactions between d-glucose and catalase (**Figure 5.4.5B** and **Table 1**) suggests the potential of d-glucose to inhibit catalase activity.

The increased GSH and NTP levels, and SOD and catalase activities, with concomitant depletion of MDA level in the treated brain tissues indicate an antioxidative effect. The antioxidative effect of the infusion can be attributed to its identified phytochemical constituents particularly nicotinic acid, salicylic acid and γ-Octalactone, as these compounds have been reported for their antioxidant and neuroprotective activities (De La Cruz et al. 2004; Shoaib et al. 2017; Tupe et al. 2011). This can also be attributed to their predicted permeability across the BBB (**Table 5.4.5**), which corroborates other reports on the ability of polyphenols and alkaloids to cross the BBB (Youdim et al. 2003; Zhang et al. 2017). The standard antidiabetic drug, metformin caused an inhibition of the pyruvate metabolism, with concomitant activation of the glycerol phosphate shuttle and mitochondrial electron transport chain (**Table 5.4.5**). This indicates activation of a proper channel of transporting the electron donors generated by the glycolytic pathway, thus mopping up free radicals while generating ATPs for the brain use. This is corroborated by the superoxide and glycerol 3-phosphate metabolites (**Table 5.4.3**) and superoxide degradation pathway (**Table 5.4.4**), which is evident by its high SOD activity (**Figure 5.4.2B**). The activated lipid metabolic pathways in the metformin treated tissues, indicates a maintenance of tissue integrity which may also be responsible for the decreased MDA level (**Figure 5.4.2D**).

The increased NO level and myeloperoxidase activity (**Figures 5.4.3A** and **3B**) indicates an occurrence of proinflammation in the brain tissue incubated in glucose only. This is evident by the presence of nitrate functional group (**Figure 5.4.6** and **Table 5.4.2**). Similarly, the molecular interaction between d-glucose and myeloperoxidase (**Figure 5.4.5A** and **Table 5.4.1**) indicates the potential of the former to activate the latter. The reversed level and activity on incubation with *V. amygdalina* infusion and metformin indicates an anti-proinflammatory activity, which is evident by the absence of the nitrate functional groups (**Figure 5.4.5A** and **Table 5.4.1**). This corroborates previous reports on the anti-proinflammatory activity of *V. amygdalina* (Farombi and Owoeye 2011; Georgewill and Georgewill 2010), and can be attributed to the identified phytochemical constituents (**Figure 5.4.7**) as well as their predicted BBB permeability (**Table 5.4.5**). Thus, further indicating the neuroprotective effect of *V. amygdalina*.

The predicted toxicity of the identified phytochemical constituents (**Table 5.4.5**) may indicate a relative safety of the infusion when ingested orally.

5.4.6 Conclusion

These results suggest the ability of *V. amygdalina* to stimulate glucose uptake in brain tissue, with concomitant antioxidative and anti-proinflammatory activities. Thus, indicating its neuroprotective potential against diabetic brain. This can be attributed to the identified phytochemicals and their permeability across the BBB. Thus, further giving credence to the reports and folkloric use of this plant in the treatment of neurodegeneration diseases.

Acknowledgments

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CHAPTER 6

ANTI-OXIDATIVE, ANTI-DIABETIC, AND CYTOTOXIC ACTIVITIES OF *CLERODENDRUM VOLUBILE* L. *IN VITRO* AND *EX VIVO*

6.1 Clerodendrum volubile

Figure 6.1: *Clerodendrum volubile.* Common names*: obenetete* (Urhobo, Nigeria)*; eweta, dagba and marugbo* (Yoruba, Nigeria)*; and belentientien* (*Itsekiri, Nigeria*). Photo: Mr. Edega Erukainure at Warri, Nigeria on May 19, 2016.

6.1.2 Background

Clerodendrum volubile L. is amongst the underutilized leafy vegetable consumed and employed for medicinal purposes mostly by tribes of the Niger-Delta, Nigeria. It is a climbing shrub belonging to the Lamiaceae (Verbenaceae) family (Erukainure et al. 2011a). It climbs up to 3 m high, it is glabrous (except the inflorescences) with numerous flowers (Burkill 1985; Erukainure et al. 2011a). It is found in the deciduous forest and secondary jungle, across the Region, Senegal to Fernando Po (Burkill 1985; Shrivastava and Patel 2007).

6.1.3 Ethnopharmacology

The leaves of *C. volubile* are employed in the treatment of arthritis, rheumatism, dropsy, swellings, oedema, gout, general healing, pain-killers, pregnancy, antiaborifacients, sedatives, and diabetes (Burkill 1985; Erukainure et al. 2011a).

6.1.4 Biological activities

Clerodendrum volubile leaves have been reported for their antioxidative activities (Adefegha and Oboh 2011; Adefegha and Oboh 2016; Erukainure et al. 2016b; Molehin et al. 2017b); antihypertensive activity (Adefegha and Oboh 2016; Erukainure et al. 2018); antidiabetic activity (Adefegha and Oboh 2016; Erukainure et al. 2018; Erukainure et al. 2017a); immunomodulatory and anti-inflammatory activity (Erukainure et al. 2017a; Erukainure et al. 2016a; Erukainure et al. 2017d); and hypolipidemic activity (Erukainure et al. 2018; Erukainure et al. 2017a).

6.1.5 Phytochemistry

Identified phytochemicals in C. volubile leaves include hydroxycinnamic acids, flavonoids, trihydroxybenzoic acids, ellagitannins, and terpenes (Adefegha and Oboh 2016; Erukainure et al. 2014; Erukainure et al. 2018; Erukainure et al. 2017a; Erukainure et al. 2017b; Molehin et al. 2017a; Oboh et al. 2017a).

6.1.6 Aims and objective

The aims of this study are to investigate the *in vitro* and *ex vivo* antioxidative, antidiabetic, and cytotoxic activities of the extracts and fractions of *C. volubile*. A comprehensive review on its phenolic and medicinal properties was also carried out.

6.2 *Clerodendrum volubile*: Phenolics and Applications to Health

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Preface: This article reviewed the phenolic properties of *C. volubile* and its applications to health. It has been published as a chapter in "Polyphenols: Mechanisms of Action in Human Health and Disease". (Erukainure LO, Sanni O, Islam MS. (2018). Clerodendrum volubile: Phenolics and Applications to Health. In: Watson R, Preedy V, Zibadi S (Eds). Polyphenols: Mechanisms of Action in Human Health and Disease. 2nd Edn., Elsevier. DOI: 10.1016/B978-0-12-813006-3.00006-4)

6.2.1 Abstract

The medicinal and health benefits of most medicinal plants are attributed to their phenolic constituents. Phenolics are secondary metabolites consisting of an aromatic ring with one or more hydroxyl groups, and/or other functional groups. *Clerodendrum volubile* are amongst medicinal plants studied for their phenolic constituents and health benefits. It is folklorically employed in the treatment of various diseases and ailments such as type 2 diabetes, cancer, immuno-disorders, neurodegeneration and hypertension. In this review, the medicinal/health benefits of *C. volubile* were discussed in relationship to its phenolic constituents. Its possible mechanism of action was also proposed.

Keywords: Antioxidative Stress; Anticancer; Immunomodulation; Phenolics; and Type 2 Diabetes

6.2.2. Introduction

The health benefits of phenolics are well documented (Lin et al. 2016; Vermerris and Nicholson 2008). Phenolics are secondary metabolites in plants responsible for most of their health promoting properties (Tomás- Barberán and Espin 2001). They are utilized in plants as defense mechanism against pathogens and protection against ultraviolent rays (Beckman 2000). They also contribute to the color, acidity, taste, fragrance and oxidative permanence of plants (Pandey and Rizvi 2009).

Chemically, phenolics possess an aromatic ring with one or more hydroxyl groups, including functional derivatives such as methyl ethers, esters, and glycosides (Harborne 1989). They are classified into phenolic acids, hydroxycinnamic acids, flavonoids and lignans, which all arise from the amino acids, phenylalanine or tyrosine. The amino acids are deaminated to cinnamic acids, before entering the phenylpropanoid pathway (Pereira et al. 2009). One or more hydroxyl groups are then introduced into the phenyl rings to forming the common carbon skeleton, the C6-C3 phenylpropanoid unit (Pereira et al., 2009). This is well reflected in the C6 aromatic ring of hydroxybenzoic acids, C6–C3 structure of hydroxycinnamic acids, C6–C3–C6 structure of flavonoids, and C6–C4–C6 structure of lignans (Xiao et al. 2013).

6.2.3. Phenolics and Health

Several studies have reported the inverse correlation between phenolics intake and chronic human diseases such as cardiovascular diseases, diabetes, cancer, obesity and inflammation (Arts and Hollman 2005; Pandey and Rizvi 2009; Scalbert et al. 2005). This is dependent on their absorption and metabolism by the gastro-intestinal (GI) system into the circulatory system and transportation to the liver (Ozcan et al. 2014). Their structures play a major influential role in this regard by determining conjugation with other phenolics, degree of glycosilation/acylation, molecular size and solubility (Bravo 1998; Crozier et al. 2009; Ozcan et al. 2014).

These medicinal properties have been attributed to the potent antioxidative effect of phenolics, owing to their ability to (i) scavenge free radicals, (ii) chelate divalent cations and (iii) modulate endogenous antioxidant enzymes (Ozcan et al. 2014; Pandey and Rizvi 2009). These abilities are dependent on the hydroxylation of the aromatic rings. Phenolics have also been reported to bring about their medicinal effect by modulating intracellular signaling cascades involved in cellular metabolism (Crozier et al. 2009; Ozcan et al. 2014).

Clerodendrum volubile

Phenolics are widely distributed in plants and are responsible for most of their medicinal properties (Tomás- Barberán and Espin 2001). The phenolic contents of some of these plants have been analyzed and their protective activities reported. Amongst such plant is *Clerodendrum volubile* which belongs to the genus *Clerodendrum* L. under the Labiatae family.

The genus, *Clerodendrum* L. is very large and diverse with over 580 identified species comprising small trees, shrubs, and herbs which are widely distributed in the tropics and subtropics (Shrivastava and Patel 2007). Most of these species are employed as folk medicine in Asian and African continents (Shrivastava and Patel 2007).

Clerodendrum volubile is indigenously known as obenetete by the Urhobos and Itsekiris, as well as eweta, dagba or marugbo by the Yorubas all in Southern Nigeria (Erukainure et al. 2010; Ogunwa et al. 2016). It is often regarded as magic leaf owing to its use in folkloric medicine for the treatment and management of several diseases such as diabetes mellitus, cancers, arthritis, ulcers, and neurological disorders (Burkill 1985). Despite these folkloric claims, there are however limited scientific data on its biological activities and phenolic constituents.

This chapter discusses the studied biological activities and phenolic constituents of *C. volubile*.

6.2.4. Phytochemistry of *Clerodendrum volubile*

1. *Preliminary Phytochemical Screening*

Preliminary phytochemical screening of the leaves of *C. volubile* revealed the presence of the presence of flavonoids, tannins, and phenols as summarized in **Table 6.2.1.**

Table 6.2.1: Summary of Preliminary Studies on Phenolic Constituents of *Clerodendrum volubile*

Phytochemicals	Extracts	Plant Part	References
Flavonoids	Methanol; Aqueous; Ethanol; Diethyl ether	Leaf	(Akinpelu et al. 2016; Erukainure et al. 2011a; Jaiyesimi and Adekoya 2013; Ogunwa et al. 2015; Ogunwa et al. 2016; Senjobi et al. 2017)
Tannins	Methanol; Aqueous; Ethanol; Diethyl ether	Leaf	(Akinpelu et al. 2016; Erukainure et al. 2011a; Jaiyesimi and Adekoya 2013; Ogunwa et al. 2015; Ogunwa et al. 2016; Senjobi et al. 2017)
Phenol	Methanol; Aqueous; Ethanol; Diethyl ether	Leaf	(Akinpelu et al. 2016; Erukainure et al. 2011a; Ogunwa et al. 2015; Ogunwa et al. 2016; Senjobi et al. 2017)

I. Flavonoids

Flavonoids are hyroxylated phenolics with a benzo-γ-pyrone structure, and one of the most abundant groups of plant phenolics (Kumar and Pandey 2013). Their pharmacological roles have been well established and dependent on the structural class, hydroxylation degree, conjugations, substitution, and polymerization (Kumar and Pandey 2013). Thus, illustrating a structural dependent activity (Heim et al. 2002; Kumar and Pandey 2013). This is evident in their ability to donate electrons (Amić et al. 2003), chelate iron catalysis (Flora 2009), reduce Fe^{2+} (Catapano et al. 2017), attenuate oxidation (Aviram and Fuhrman 1998), and activate antioxidant enzymes (Masella et al. 2005).

Ogunwa (2016) revealed the presence of flavonoids in the aqueous, ethanol and methanol extracts of *C. volubile* leaves, with the ethanol extract having the highest concentration. Akinpelu et al. (2016), Ogunwa et al. (2016) and Senjobi et al. (2016) also reported similar observations in ethanol and methanol extracts. These studies correspond with a previous study by Erukainure et al. (2011) on the phytochemical contents of the leaves.

II. Tannin

Tannins are water-soluble polyphenolic biomolecules responsible for the astringency of most plants (Chung et al. 1998; Ukoha et al. 2011). They are mostly consist of a large number of hydroxyl and other functional groups, a characteristic that makes them cable of binding with macromolecules especially proteins (Chung et al. 1998). This binding ability have been reported to have a negative impact on animal nutrition as it decreases feed intake, feed efficiency, net metabolizable energy, and protein digestibility (Bele et al. 2010; Ukoha et al. 2011).

Tannins are classified as hydrolyzable and condensed tannins. Hydrolyzable tannins consist of a polyhydric alcohol and hydroxyl groups which are partially or wholly esterifies by gallic acid or hexahydroxydiphenic acid (Chung et al. 1998). While condensed tannins consist of a group of polyhydroxy-flavan-3-ol oligomers and polymers that are linked by carbon – carbon bonds between flavanol subunits (Schofield et al. 2001).

Erukainure et al. (Erukainure et al. 2011a) reported the presence of tannin in the aqueous extract of *C. volubile* leaves. This was further corroborated in a study by Ogunwa et al. (Ogunwa et al. 2016). Ogunwa et al. (2016) also compared the concentration in the aqueous and methanol extracts, and concluded the methanol extract had a higher concentration, which corresponded with previous reports (Jaiyesimi and Adekoya 2013). Senjobi et al. (Senjobi et al. 2017) however reported the highest concentration in diethyl ether, when they compared the tannin contents in aqueous, methanol and diethyl ether extracts. The presence of tannin in the ethanol extract reported by Akinpelu et al. (Akinpelu et al. 2016), however contradicts that of Ogunwa et al. (2016) who reported the absence of tannin in the extract.

Although tannins may be considered as anti-nutrients, their presence in *C. volubile* leaves also poses therapeutic benefits. Tannins have been reported for their antioxidant, anti-hemorrhagic, antiseptic, anti-inflammatory, antihelmintics, anticancer, antimicrobial and antiviral activities (Buzzini et al. 2008; Koleckar et al. 2008).

2. *High-performance liquid chromatography (HPLC) Fingerprinting*

Studies on the high-performance liquid chromatography (HPLC) analysis of various extracts of *C. volubile* leaves have revealed the presence of phenolic compounds as shown in **Figure 6.2.1** and **Table 6.2.2**.

Table 6.2.2: HPLC – identified phenolics in *C. volubile* leaves

Phenolics	**Compounds**	**Extracts**	**References**
Hydroxycinnamic acids	Caffeic acid	Phenolic-rich extract; ethyl acetate, ethanol and methanol extracts	(Adefegha and Oboh 2016; Molehin et al. 2017a; Oboh et al. 2017a)
	Chlorogenic acid	Phenolic-rich extract	(Adefegha and Oboh 2016; Oboh et al. 2017a)
	p-Coumaric acid	Phenolic-rich extract	(Oboh et al. 2017a)
Flavonoids	Rutin	Phenolic-rich extract; ethyl acetate, ethanol and methanol extracts	(Adefegha and Oboh 2016; Molehin et al. 2017a; Oboh et al. 2017a)
	Isoquercitrin	Phenolic-rich extract	(Adefegha and Oboh 2016)
	Quercitrin	Phenolic-rich extract; ethyl acetate, ethanol and methanol extracts	(Molehin et al. 2017a; Oboh et al. 2017)
	Quercetin	Phenolic-rich extract; ethyl acetate, ethanol and methanol extracts	(Adefegha and Oboh 2016; Molehin et al. 2017a; Oboh et al. 2017a)
	Kaempferol	Phenolic-rich extract; ethyl acetate, ethanol and methanol extracts	(Molehin et al. 2017a; Oboh et al. 2017)
	Apigenin	Phenolic-rich extract; ethyl acetate, ethanol and methanol extracts	(Molehin et al. 2017a; Oboh et al. 2017)

	Catechin	Phenolic-rich extract; ethyl acetate, ethanol and methanol extracts	(Molehin et al. 2017a; Oboh et al. 2017)
	Luteolin	Ethyl acetate, ethanol and methanol extracts	(Molehin et al. 2017a)
Trihydroxybenzoic acids	Gallic acid	Phenolic-rich extract; ethyl acetate, ethanol and methanol extracts	(Molehin et al. 2017a; Oboh et al. 2017)
Ellagitannins	Ellagic acid	Phenolic-rich extract	(Oboh et al. 2017a)

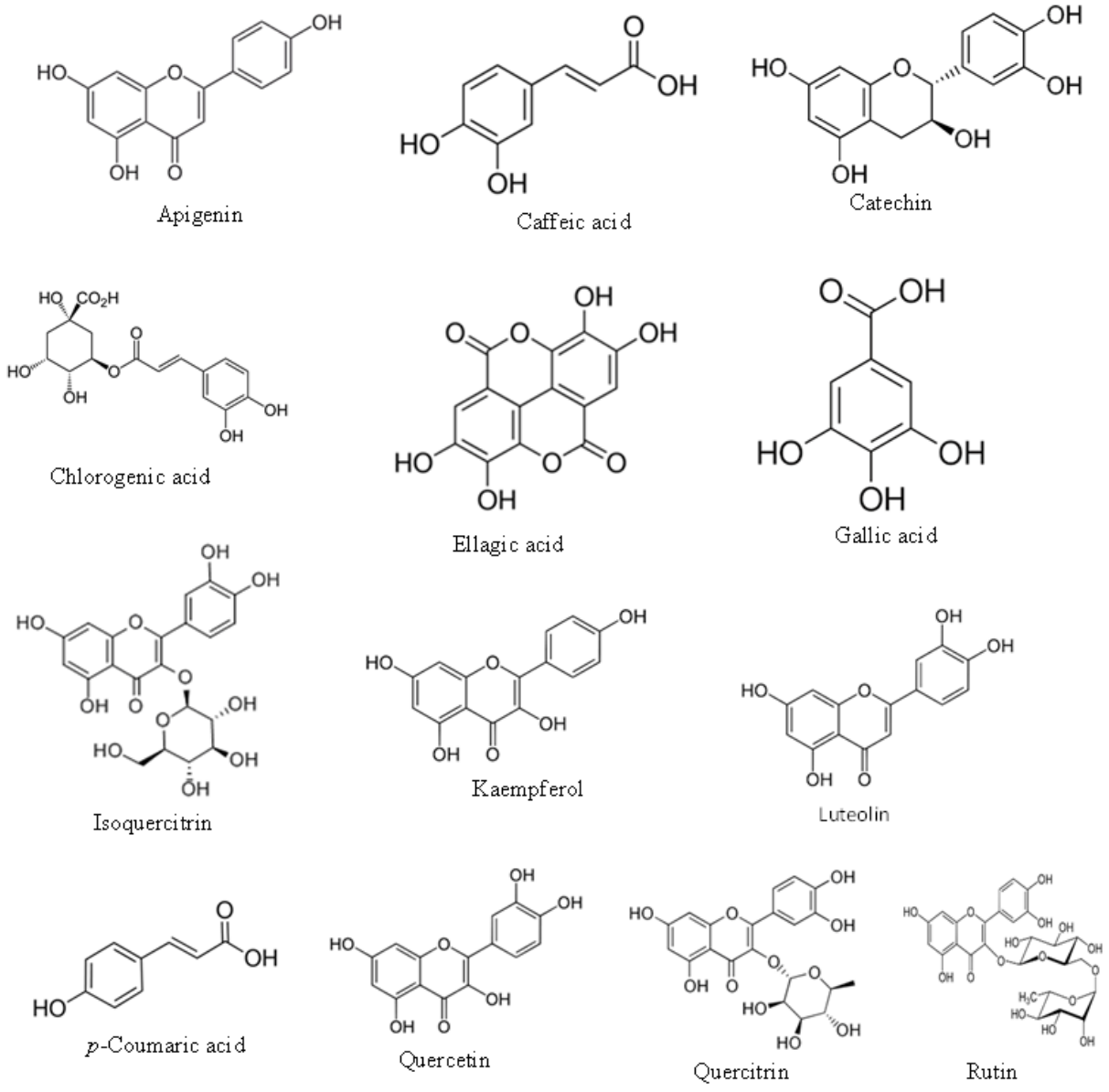


Figure 6.2.1: HPLC – identified phenolics in *C. volubile* leaves

I. Hydroxycinnamic acids

Hydroxycinnamic acids (HCAs) are natural phenylpropenoic acid compounds, which occurs as esters, glycosides, and/or conjugates of proteins (Chen and Ho 1997; Zhang et al. 2018). A few also exists as natural free acids (Chen and Ho 1997). They are major intermediates in the biosynthetic pathways of polyphenols (El-Seedi et al. 2012). They have been recognized as an

important source of antioxidants and play an influential role in the stability, flavor, color, nutritional bioavailability of foods rich in the compounds (Chen and Ho 1997; Rocha et al. 2012).

HCAs are derived from phenylalanine and tyrosine, comprising of a nine carbon (C6-C3) skeleton with a side chain double bond (with cis or a trans configuration), representing a phenylpropanoid structure (El-Seedi et al. 2012; Razzaghi-Asl et al. 2013). The presence of hydroxyl functional groups on the benzene ring, and the unsaturated bond of its ethylenic side chain are important sites for reactions with reactive oxygen species (ROS), portraying a structure - activity relationship (Barone et al. 2009; Razzaghi-Asl et al. 2013). Thus, their biological activity depends on the pattern of substitution of the aromatic moiety (Razzaghi-Asl et al. 2013).

The most common HCAs are cinnamic acid, caffeic acid, sinapic acid, *o*-coumaric acid, *m*-coumaric acid, *p*-coumaric acid and ferulic acid. Adefegha and Oboh (Adefegha and Oboh 2016) reported the presence of caffeic and chlorogenic acids in a phenolic rich extract of *C. volubile* leaves. This was confirmed by Oboh et al. (Oboh et al. 2017a), who also reported the presence of *p*-coumaric acid in the phenolic rich extract. Molehin et al. (Molehin et al. 2017a) also reported the presence of caffeic acid in the ethyl acetate, ethanol and methanol extracts of the leaves.

II. Flavonoids

The presence of flavonoids reported the preliminary screening of *C. volubile* leaves was further confirmed by HPLC analysis of the leaf extracts.

Adefegha and Oboh (41), and Oboh et al. (42) reported the presence of quercetin and its glycone rutin in the phenolic rich extract of the leaves. This was further confirmed by Molehin et al. (43) in the ethyl acetate, ethanol and methanol extracts. Quercetin is one of the most studied dietary flavonoid with diverse pharmacological applications particularly antioxidant, anticancer and neuroprotection (Maalik et al. 2014). It consists of 5 hydroxyl groups and 2 benzene rings that is linked by a heterocyclic pyrane ring, forming the backbone for other flavonoids such as naringenin, hesperidins and tangeritin (Kumar and Pandey 2013; Lakhanpal and Rai 2007; Materska 2008). Although quercetin is an aglycon, it also exists in most plants as glycone (Lakhanpal and Rai 2007). Aside rutin, Adefegha and Oboh (41), and Oboh et al. (42) reported the presence of isoquercitrin and quercitrin. These qlycones brings about their biological activities by undergoing hydrolysis to release quercetin (Comalada et al. 2005).

Other well studied flavonoids identified in *C. volubile* leaves are kaempferol, apigenin, catechin, and luteolin (Adefegha and Oboh 2016; Molehin et al. 2017a; Oboh et al. 2017a). Apigenin and luteolin belong to the flavonoid class, flavones which is distinguished from other classes by the absence of a 3-hydroxyl group (Kumar and Pandey 2013). Kaempferol and catechin are flavonols, whose benzene ring is condensed with a α-pyrone or a dihydro-derivative (Kumar and Pandey 2013). It is distinguished from flavones by the presence of a 3-hydroxyl group and C2–C3 double bond (Kumar and Pandey 2013; Narayana et al. 2001).

III. Trihydroxybenzoic acids

Gallic acid is the most popular of trihydroxybenzoic acids. Also known as 3,4,5-trihydroxybenzoic acid, gallic acid has been reported in most plants. Adefegha and Oboh (41) and Oboh et al. (42) reported its presence in the phenolic rich extract of *C. volubile* leaves. Molehin et al. (43) also compared its concentration in the ethyl acetate, ethanol and methanol extracts of C. *volubile* leaves, with the ethyl acetate extract having the least concentration.

Gallic acid is biosynthesized from 3-dehydroshikimate in a reaction catalyzed by shikimate dehydrogenase to give 3,5-didehydroshikimate, which then undergoes tautomerization to form gallate (Dewick and Haslam 1969; Muir et al. 2011).

It consists of 3 hydroxyl groups and a carboxylic acid group attached to a benzene ring. The bonding of the hydroxyl groups in an ortho position results to a coplanar and bent configuration, which is favorable for antioxidative activities (Badhani et al. 2015; Sroka and Cisowski 2003)

IV. Ellagitannins

Ellagitannins are esters of hexahydroxydiphenoic acid and monosaccharide, and are classified under hydrolysable tannins (Lipińska et al. 2014). They are recognized as the largest group of tannins, with over 500 compounds reported (Khanbabaee and van Ree 2001; Landete 2011). Their easy labilization in solution, makes them prone to hydrolysis and polymerization reactions. On hydrolysis with acid and/or base, their hexahydroxydiphenoyl (HHDP) group undergoes a spontaneous rearrangement to the gallic acid dimeric derivative, ellagic acid (Landete 2011).

The presence of ellagic acid has been reported in many plants. Oboh et al. (2017a) reported its presence in the phenolic rich extract of C. *volubile* leaves, thus confirming the previous reports on

the presence of tannins in the leaves (**Table 6.2.1**) (Akinpelu et al. 2016; Erukainure et al. 2011a; Jaiyesimi and Adekoya 2013; Ogunwa et al. 2015; Senjobi et al. 2017).

Ellagic acid consists of a hydrophilic domain made up of 4 phenolic groups and 2 lactones, and a lipophilic domain made up of four rings (García-Nino and Zazueta 2015). These domains play an influential role in its biological activities, particularly the hydrophilic which can hydrogen bond and accept electrons (García-Nino and Zazueta 2015; Sepúlveda et al. 2011). Thus, denoting a structure – activity relationship.

3. Bioavailability

Several studies have investigated the absorption and metabolism of these phenolics, thereby providing useful insights to their health benefits. Their bioavailability is summarized in Table 6.2.3.

Table 6.2.3: Bioavailability of HPLC-identified phenolics in *C. volubile* leaves

Phenolics	Bioavailability	Compounds	References
Hydroxycinnamic acids	Chlorogenic acid is hydrolyzed to caffeic and quinic acids in the stomach and intestine. Highest dose ingestion is associated with reduced bioavailability. *p*-Coumaric acid has a higher bioavailability than chlorogenic and caffeic acids. In rats, it is absorbed in all sections of the gastrointestinal tract, with the jejunum having the highest absorption rate. In the liver, it is conjugated with glucuronide, sulfate and sulfoglucuronide.	Caffeic acid Chlorogenic acid *p*-Coumaric acid	(Pei et al. 2016; Zhao and Moghadasian 2010)
Flavonoids	It is absorbed from both the small intestine and colon, with absorption from the latter being the most efficient. They are conjugated with glucoronic acid or sulphate, after absorption from the small intestine. They may also undergo O-methylation. Those that to the colon are metabolized by microflora enzymes.	Rutin Isoquercitrin Quercitrin Quercetin Kaempferol Apigenin	(Dangles and Dufour 2006; Viskupičová et al. 2008)

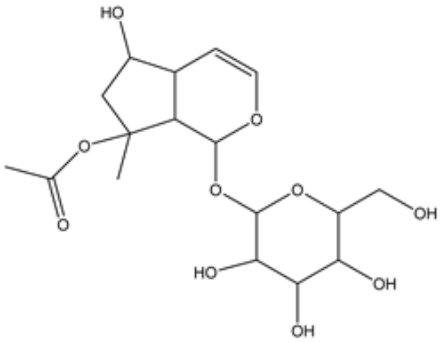
		Catechin Luteolin	
Trihydroxybenzoic acids	It is readily absorbed from the gastrointestinal tract resulting to peak plasma concentration 1 – 2 hours after ingestion. It is mostly excreted unchanged in urine. Once absorbed, they undergo methylation, decarboxylation and dehydroxylation	Gallic acid	(Shahrzad and Bitsch 1998)
Ellagitannins	It is absorbed from the stomach and/or the proximal small intestine. At the distal part of the small intestine and the colon, they are metabolized by microflora enzymes. They are then acted on by Phase II UGTs and/or methyltransferases before excretion in urine	Ellagic acid	(Rodriguez-Mateos et al. 2014; Seeram et al. 2006)

4. *Isolation and Structural Elucidation*

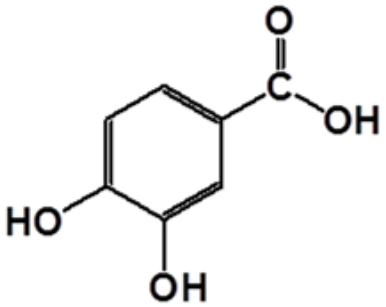
In addition to HPLC fingerprinting, some phenolic compounds depicted in Figure **6.2.2** and **Table 6.2.4** have been isolated and structurally elucidated via different chromatography and spectroscopy techniques. The isolation of these compounds affirms the reported presence of phenol and flavonoids in the preliminary phytochemical investigation (**Table 6.2.1**) (Erukainure et al. 2011a; Ogunwa et al. 2015; Senjobi et al. 2017).

Table 6.2.4: Isolated compounds from *C. volubile* leaves

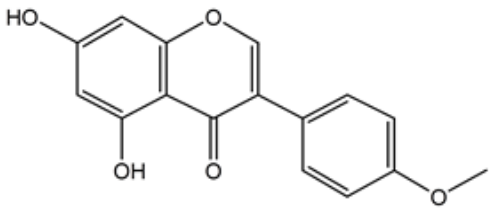
Isolated Compounds	**Common Name**	**Fraction**	**Reference**
5-hydroxy-7-methyl-1-(3,4,5-trihydroxy-6-(hydroxymethyl) tetrahydro-2H-pyran-2-yloxy)-1,4a,5,6,7,7a-hexahydrocyclopenta[c]pyran-7-yl	Ajugoside	Butanol	(Erukainure et al. 2014)
3, 4–dihydroxybenzoic acid	Protocatechuic acid	Ethyl acetate	(Erukainure et al. 2017a)
5,7-Dihydroxy-6,4'-dimethoxyflavone	Pectolinarigenin	Dichloromethane	(Erukainure et al. 2017b)
4'-Methoxy-5,7-dihydroxy isoflavone	Biochanin		(Erukainure et al. 2018)
5,7,4'-Trimethoxykaempferol			(Erukainure et al. 2018)



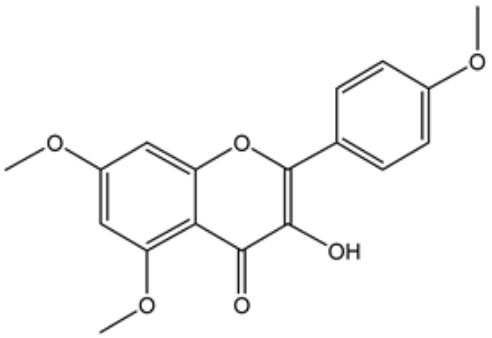
5-hydroxy-7-methyl-1-(3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yloxy)-1,4a,5,6,7,7a-hexahydrocyclopenta[c]pyran-7-yl (Ajugoside)



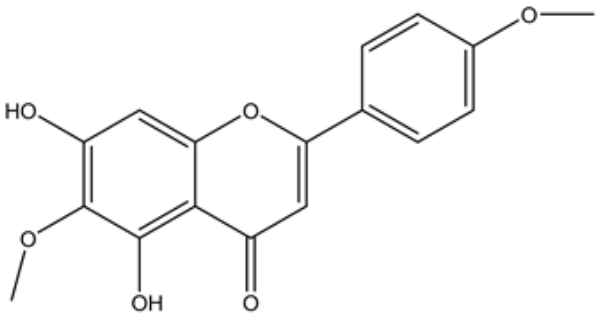
3, 4–dihydroxybenzoic acid (Protocatechuic acid)



4'-Methoxy-5,7-dihydroxy isoflavone (Biochanin)



5,7,4'-Trimethoxykaempferol



5,7-Dihydroxy-6,4' -dimethoxyflavone (Pectolinarigenin)

Figure 6.2.2: Isolated phenolics from leaves of *C. volubile* leaves

6.2.5. Biological Activities of *Clerodendrum volubile*

Although *C. volubile* have been employed in folkloric medicine for the treatment of several diseases and ailments, there are however fewer studies to back its medicinal properties. To the best of our knowledge, the studies done so far on the biological activities of *C. volubile* are summarized in **Table 6.2.5**.

Table 6.2.5: Studied biological activities of *C. volubile* leaves

Biological Activities	Experimental Type	Target Organ/cells	Mechanism	Plant Part	Phenolic Compounds	Non-Phenolic Compounds	References
Free radical scavenging/Reducing power/Fe^{2+} chelating	*In vitro*		Scavenges DPPH, NO, OH-, SO and ABTS; chelates and reduces Fe^{2+}	Leaves Flower	Chlorogenic acid Caffeic acid Rutin Isoquercitrin Quercitrin Quercetin Kaempferol Gallic acid Catechin Luteolin Apigenin Ellagic acid *p*-Coumaric acid	Triacetin 3,6-dimethyl-3-octanol 2R-Acetoxymethyl-1,3,3-trimethtyl–4t–(3-methyl-2-buten-1-yl)-1c-cyclohexanol Stigmastan-3,5-diene	(Adefegha and Oboh 2011; Adefegha and Oboh 2016; Erukainure et al. 2010; Erukainure et al. 2016a; Molehin et al. 2017a; Oboh et al. 2017a; Ogunwa et al. 2016)
Antioxidative stress	*Ex vivo*	Brain; Liver	Increases SOD and catalase activities;	Leaves	Ajugoside Chlorogenic acid		(Adefegha and Oboh 2016; Erukainure et

			decreases lipid peroxidation		Caffeic acid Rutin Isoquercitrin Quercitrin Quercetin Kaempferol Gallic acid Catechin Apigenin Ellagic acid *p*-Coumaric acid		al. 2014; Oboh et al. 2017a)
	In vivo	Liver; Pancreas	Elevates GSH level; increases GPx, SOD, catalase activities; decreases lipid peroxidation	Leaves	Protocatechuic acid Biochanin 5,7,4'-Trimethoxykaempferol		(Erukainure et al. 2018; Erukainure et al. 2017a; Molehin et al. 2017b)
Anti-inflammatory	*In silico*		Molecularly interacts with COX-2 and	Flowers; Leaves	Protocatechuic acid	Triacetin	(Erukainure et al. 2017a; Erukainure et al. 2016a)

			TNF-α with negative predicted free binding energy			3,6-dimethyl-3-octanol 2R-Acetoxymethyl-1,3,3-trimethtyl–4t–(3-methyl-2-buten-1-yl)-1c-cyclohexanol	
	In vivo	Paws	Time dependently reduces linear circumference of paws				(Jaiyesimi and Adekoya 2013)
Immunomodulation	*Ex vivo*	Blood	Suppresses phagocytic oxidative burst in neutrophils and macrophages	Flowers; Leaves; Stem	Protocatechuic acid Pectolinarigenin	Triacetin 3,6-dimethyl-3-octanol 2R-Acetoxymethyl-1,3,3-trimethtyl–4t–(3-methyl-2-buten-1-yl)-1c-cyclohexanol Stigmastan-3,5-diene Fatty acids	(Erukainure et al. 2017a; Erukainure et al. 2016a; Erukainure et al. 2017d)
Hypolipidemic	*In vivo*	Serum	Reduces cholesterol, LDL-C, triglyceride	Leaves	Protocatechuic acid Biochanin		(Akinpelu et al. 2016; Erukainure et al. 2018; Erukainure et

			and vLDL-C levels; elevates HDL-C level		5,7,4'-Trimethoxykaempferol	al. 2017b; Molehin et al. 2017b)
Analgesic	*In vivo*	Abdominal cavity	Time dependently reduces writhing count; and inhibits abdominal constriction	Leaves		(Senjobi et al. 2017)
Anti-hypertensive	*In vitro*		Inhibits ACE	Leaves	Biochanin 5,7,4'-Trimethoxykaempferol Chlorogenic acid Caffeic acid Rutin Isoquercitrin Quercitrin Quercetin	(Adefegha and Oboh 2016; Erukainure et al. 2018)

	In Silico		Molecularly docks with ACE with a negative free binding energy		Biochanin 5,7,4'-Trimethoxykaempferol		Erukainure et al., 2018
Neuroprotection	*Ex vivo*	Brain	Inhibits AChE, BChE, and MAO	Leaves	Apigenin Caffeic acid Catechin Chlorogenic acid Ellagic acid Gallic acid Kaempferol *p*-Coumaric acid Quercetin Quercitrin Rutin		(Oboh et al. 2017a)
Anticancer	*In vitro*	Breast cancer cells (MCF-7)	Inhibits cell proliferation, arrests cell cycle progression,	Leaves		Oleic acid Octadecanoic acid n-Hexadecanoic	(Erukainure et al. 2016b)

			downregulates MMP-9 expression, and attenuates oxidative stress			acid 6-methyl-2-heptanone	
Antidiabetes	*In vitro*		Inhibits α-glucosidase, α-amylase	Leaves	Chlorogenic acid Caffeic acid Rutin Isoquercitrin Quercitrin Quercetin Kaempferol		(Adefegha and Oboh 2016; Erukainure et al. 2018)
	In silico		Molecularly docks with α-glucosidase with negative binding energy		Biochanin 5,7,4'-Trimethoxykaempferol		(Erukainure et al. 2018; Erukainure et al. 2017a)
	In vivo	Pancreas; Serum	Reduces blood glucose level; improves β-cell distribution and function, glucose		Protocatechuic acid		

			tolerance; Increases serum insulin and pancreatic Ca^{2+} levels				
Toxicity	*In vitro*	CC-1 cells	Increases cell proliferation	Flower; Leaves	Pectolinarigenin	Triacetin 3,6-dimethyl-3-octanol 2R-Acetoxymethyl-1,3,3-trimethtyl–4t–(3-methyl-2-buten-1-yl)-1c-cyclohexanol Stigmastan-3,5-diene	(Erukainure et al. 2016a; Erukainure et al. 2017b)
	In silico		Predicted toxicity class ranging from classes III – VI; Predicted inhibitors of cytochrome P (CYPs) 1A2, 2D6 and 3A4	Leaves; Stem	Biochanin 5,7,4'-Trimethoxykaempferol Protocatechuic acid Pectolinarigenin	3-Eicosyne Succinic acid, 2,4-dimethylpent-3-yl ethyl ester (2,4,6-Trimethylcyclohexyl) methanol 4-Penten-1-ol, propanoate	(Erukainure et al. 2018; Erukainure et al. 2017a; Erukainure et al. 2017b; Erukainure et al. 2017d)

					3-Methyl-2-butenoic acid, 2-ethylcyclohexyl ester	
	In vivo	Serum	Decreases AST, ALT, ALP, and TB levels; Improves hepatic architecture in carbon tetrachloride-induced toxicity	Leaves Biochanin 5,7,4'-Trimethoxykaempferol Protocatechuic acid		(Erukainure et al. 2018; Erukainure et al. 2017a; Molehin et al. 2017b)

1. *Antioxidative Activities*

Oxidative stress has been implicated in the pathogenesis and progression of most diseases and their complications. It arises from an imbalance between free radicals generation and the endogenous antioxidants, with the former being the depleted. These free radicals are constituted of the reactive oxygen species (ROS) and reactive nitrogen species (RNS), which are continuously produced by oxidative metabolism, immune-activities, and cellular bioenergetics (Tan et al. 2018). Lipid peroxides, nitric oxide (NO), superoxide anion ($O_2^{\bullet -}$), hydroxy radicals, ($OH^{\bullet}$), hydrogen peroxide (H_2O_2), hypochlorite (ClO^-), and singlet oxygen ($^1O_2$) constitute the most studied free radicals. Due to their unstable nature, free radicals react very fast with the cells' lipids and proteins leading to physiological and biochemical changes characterized by increased cell differentiation, proliferation, growth, and death (apoptosis and/or necrosis). The body has developed an endogenous antioxidant system to defend its self against excessive free radicals, thereby mitigating oxidative stress (Rahal et al. 2014). This system consists of enzymatic and non-enzymatic antioxidants. Superoxide dismutase (SOD), catalase, xanthene oxidase, glutathione peroxidase, and glutathione reductase are amongst the most studied antioxidant enzymes, while glutathione, ascorbic acid, and tocopherol for non-enzymatic. They act by scavenging free radicals, thus preventing oxidative damage. In addition to the endogenous antioxidant system, the body defends itself against free radicals by utilizing phytochemicals obtained from food and other natural products.

Plant based antioxidant activities are well documented, with phenolics playing a major role. The ability of *C. volubile* leaves to scavenge free radicals have been reported. Erukainure et al. (2010) first reported the DPPH (1,1-diphenyl-2 picrylhydrazyl) radical scavenging activity of *C. volubile*, which he attributed to its total phenolic and flavonoids contents. This was also confirmed by Adefegha and Oboh (Adefegha and Oboh 2011; Adefegha and Oboh 2016), Ogunwa et al. (16), and Molehin et al. (43). DPPH is a readily available commercial transient free radical used in the study of scavenging potentials of natural products (Mishra et al. 2012). Ogunwa et al. (16) and Molehin et al. (Molehin et al. 2017a) also reported the ability of the leaf extract to scavenge another commercial free radical, ABTS (2, 2- azinobis (3-ethylbenzo-thiazoline- 6-sulfonate)). The leaves have also been reported to scavenge $O_2^{\bullet -}$, $OH^{\bullet}$, and H_2O_2 (Adefegha and Oboh 2011; Adefegha and Oboh 2016; Molehin et al. 2017a; Ogunwa et al. 2016). Molehin et al. (43), and Adefegha and

Oboh (41) attributed these activities to the identified phenolics (Table 2). Phenolics have been recognized as strong antioxidants (Pereira et al. 2009). This has been attributed to their hydroxyl groups, which donates an electron to the free radical. This leads to the formation of a more chemical stable product, as well as interaction of the hydroxyl groups with ∏-electrons of the benzene ring leading to delocalization of the radical (Pereira et al. 2009). These reactions quench the effect of the free radical, thus attenuating the oxidative damage in the cells.

The antioxidant properties of phenolics have also been attributed to their ability to chelate metal ions involved in redox metabolism (Yang et al. 2001). Metals such as iron (Fe), copper (Cu), chromium (Cr), and cobalt (Co) have been shown to exacerbate the formation of $O_2^{\bullet -}$ and $OH^{\bullet}$ via the Fenton reaction, leading to peroxidation of the membrane lipids and fragmentation of DNAs (Jomova and Valko 2011). Adefegha and Oboh (41, 61) reported the antiperoxidative effect of the phenolic rich extract of *C. volubile* leaves against Fe^{2+}-induced lipid peroxidation in rat liver and pancreas. Oboh et al. (42) further reported similar effect in rat brain. The reducing and chelating properties of *C. volubile* leaves on iron have also been reported (Adefegha and Oboh 2011; Adefegha and Oboh 2016; Erukainure et al. 2010; Molehin et al. 2017a), thus demonstrating the protective potential of the leaves against iron toxicity. The pro-oxidant properties of iron can be attributed to its ability to exist in both ferrous (Fe^{2+}) and ferric (Fe^{3+}) ionic states (Rajpathak et al. 2009).

The ability of *C. volubile* leaves to mitigate oxidative stress in disease states and toxicity has also been reported. Erukainure et al. (Erukainure et al. 2016b) reported an increased GSH level, SOD and catalase activities and decreased malondialdehyde (MDA) in breast cancer cells treated with the leaf extract. The antioxidative protective properties of the leaf extract were also reported in diabetic oxidative pancreas and serum (Erukainure et al. 2018), which was attributed to the isolated flavones, biochanin and 5,7,4'-trimethoxykaempferol. Molehin et al. (Molehin et al. 2017b) reported an aggravative GSH level, SOD, catalase, and GPx activities with a concomitant reduced MDA level in carbon tetrachloride-induced hepatotoxicity. These activities can be attributed to the ability of the hydrophobic benzenoid rings and hydroxyl groups of phenolics to interact with proteins (Pereira et al. 2009)

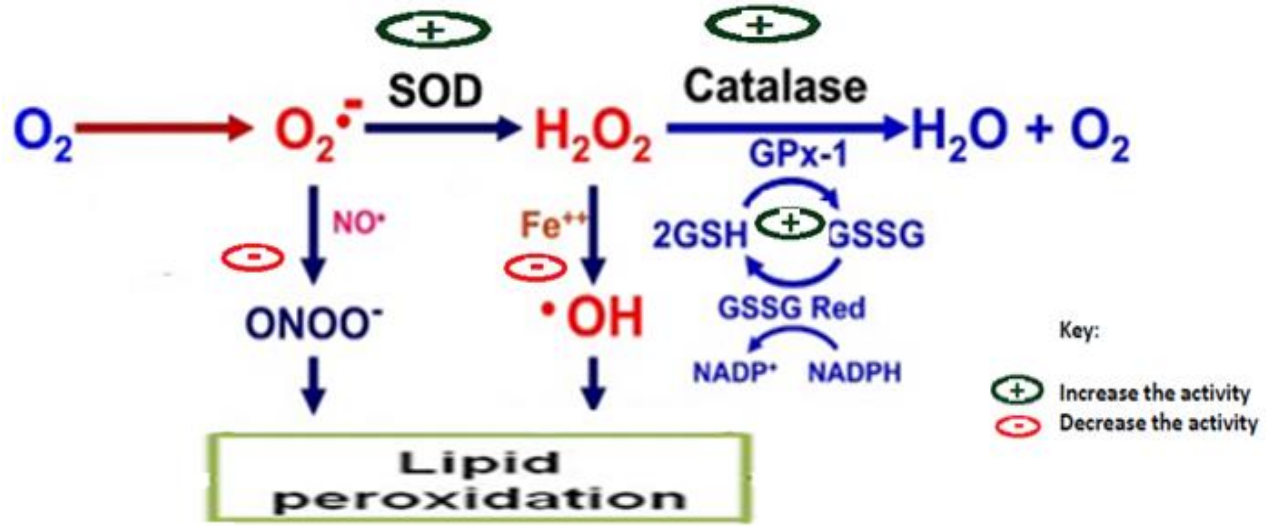


Figure 6.2.3: Antioxidative mechanism of *C. volubile* leaves

2. *Antihypertensive Activity*

Hypertension is the most common cardiovascular disease and has been recognized as a major contributor to global morbidity and motility, thus a global health epidemic (Adefegha and Oboh 2016; Li et al. 2015; Miranda et al. 2016). It is characterized by high blood pressure which could lead to other cardiovascular complications, kidney failure, neurodegeneration and other complications (Li et al. 2015; Maione et al. 2013).

Activation of angiotensin-I converting enzyme (ACE) has been implicated in the progression of hypertension owing to its regulation (Oboh et al. 2017c). It is activated after stimulation of the angiotensin 1 receptor, leading to an increased level of angiotensin II a potent vasoconstrictor (Adefegha and Oboh 2011; Oboh et al. 2017c)

Adefegha and Oboh (41, 61) reported the ability of *C. volubile* leaves to inhibit ACE, which they attributed to the total phenol content and identified phenolics of the extracts (table 2). Erukainure et al. (Erukainure et al. 2018) further confirmed this ability and attributed it to the isolated flavones, biochanin and 5,7,4'-trimethoxykaempferol. The molecular interactions of these compounds with ACE were further investigated *in silico*. 5,7,4'-trimethoxykaempferol interacted with ACE by forming hydrogen bond with the residues HIS 387, TYR 523, LYS 511 (67). While biochanin interacted via HIS 383, GLU 411, HIS 387, TYR 523 (67). These results demonstrate the anti-hypertensive properties of *C. volubile* leaves, which can be attributed to the phenolic contents.

An inverse relationship has been correlated with increased phenolics intake and decreased hypertension (Godos et al. 2017; Miranda et al. 2016). Phenolics have been shown to inhibit ACE by chelating its hydroxyl groups with the Zn^{2+} moiety of ACE (Li et al. 2015). Structure-inhibition studies on their relationship, revealed an increased inhibitory activity with increasing hydroxyl groups and less structural steric hindrance (Li et al. 2015).

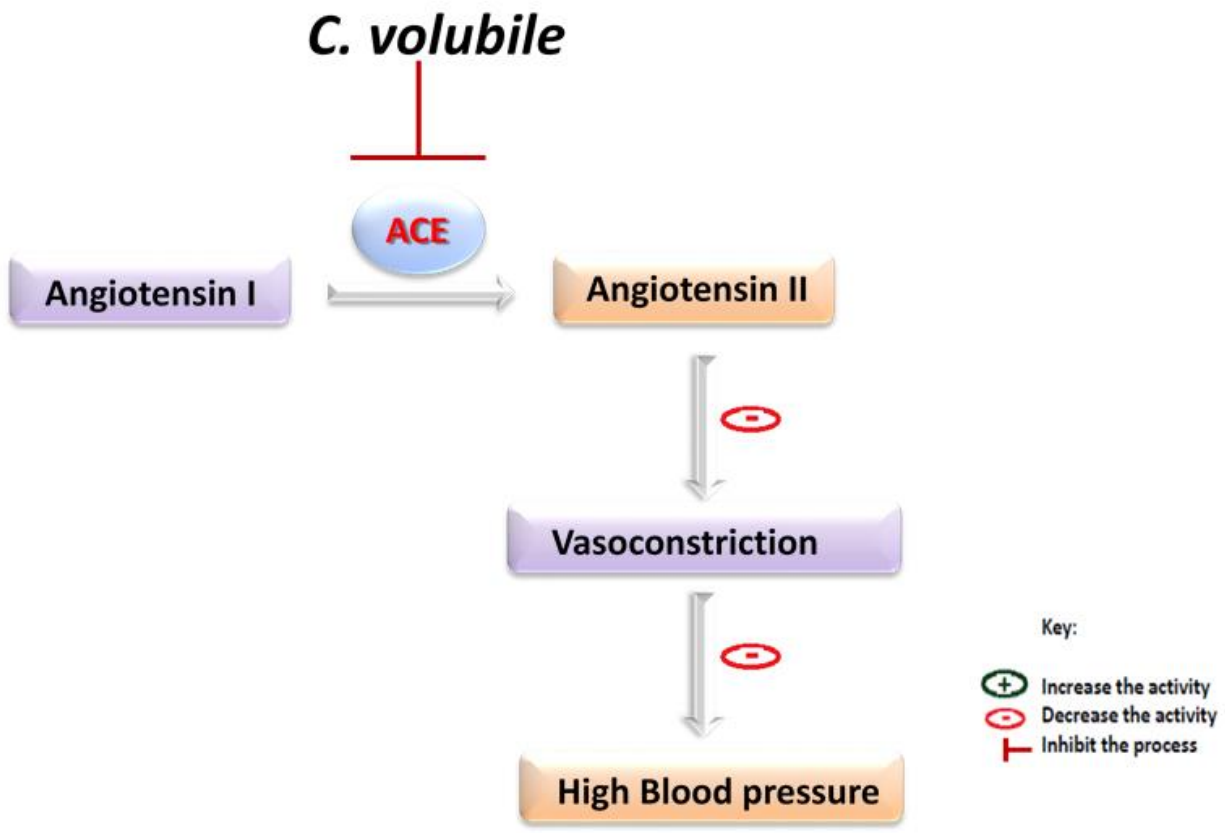


Figure 6.2. 4: Antihypertensive activity of *C. volubile* leaves

6.5.3 *Antidiabetic Activity*

Diabetes mellitus (DM) is a global epidemic with 425 million people reported with the disease in 2017, and sub-Saharan Africa (SSA) accounting for 3.76% (IDF 2018). It is a metabolic disease that affects carbohydrate, fatty acid and lipids metabolism owing to inability of the pancreatic β cells to secret insulin (type 1 diabetes) or the body not being able to effectively utilize the insulin secreted (type 2 diabetes [T2D]) (Erukainure et al. 2013). Both types of diabetes are characterized by increased blood glucose, with T2D being the most prevalent (IDF 2018). Insulin resistance and

β-cell dysfunction has been recognized as the hallmarks of T2D, leading to chronic hyperglycemia (Prentki and Nolan 2006).

Owing to cost of treatment, there have been increased interest in the use of medicinal plants for the treatment and management of T2D. The leaves of *C. volubile* have been investigated for their antidiabetic properties. Adefegha and Oboh (41) investigated the ability of its phenolic rich extract to inhibit α-glucosidase and amylase, key enzymes linked to T2D. They concluded the extract exhibited a dose-dependent inhibitory effect of the enzymes, depicting an anti-type 2 diabetic potential. The observed activity was attributed to the HPLC-identified phenolics (table 2). Similarly, Erukainure et al. (Erukainure et al. 2018) reported the inhibitory activities of the methanol extract and its fractions on α-glucosidase, which they attributed to biochanin and 5,7,4'-trimethoxykaempferol. Erukainure et al. (Erukainure et al. 2018; Erukainure et al. 2017a) also investigated the molecular interactions of protocatechuic acid, biochanin and 5,7,4'-trimethoxykaempferol with α-glucosidase *in silico*. Protocatechuic acid interacted with interacted with the active site residues: ASP 146 (4.3 Å), GLY 149 (3.6 Å) and LYS 164 (5.3 Å) via hydrogen bonding with the two meta and para oxygen atoms of its two free hydroxyl groups and oxygen of –OH of the –COOH (Erukainure et al. 2017a). 5,7,4’-trimethoxykaempferol interacted with residues ASP 203, ARG 526, and PHE 450, with the –OH and –COOH groups donating hydrogen bonds to ASP 203 and ARG 526 respectively (Erukainure et al. 2018). While biochanin interacted with residues ARG 526, TRP 406, ASP 443, and HIS 600, with the –OH and –COOH groups donating hydrogen bonds to ASP 443 and ARG 526 respectively (Erukainure et al. 2018). This reflects a structure-activity relationship. Unsaturation at 2,3-position and the presence of 4-carbonyl group has been reported to increase the inhibitory property of phenolics on *α*-amylase (Adisakwattana et al. 2009). Similarly, hydroxylation and galloylation has been shown to improve their inhibitory effect (Xiao et al. 2013). Inhibition of α-glucosidase and α-amylase has been employed by most antidiabetic drugs such as acarbose in the reduction of blood glucose level (Erukainure et al. 2017c). These enzymes breakdown dietary carbohydrate to glucose, thereby increasing the postprandial blood glucose level. Their inhibition would therefore reduce the breakdown of dietary carbohydrate, thus reducing blood glucose level.

Erukainure et al. (Erukainure et al. 2018; Erukainure et al. 2017a) reported the ability of the ethyl acetate and dichloromethane (DCM) fractions to reduce blood glucose level, and they attributed it

to the isolated phenolics, protocatechuic acid, biochanin and 5,7,4'-trimethoxykaempferol. The fractions improved β – cell function and increased serum insulin level. Further investigation with the DCM fraction revealed an improved distribution of pancreatic β-cells as well and glucose tolerance (Erukainure et al. 2018). The Ca^{2+} level of the pancreatic tissues was also increased.

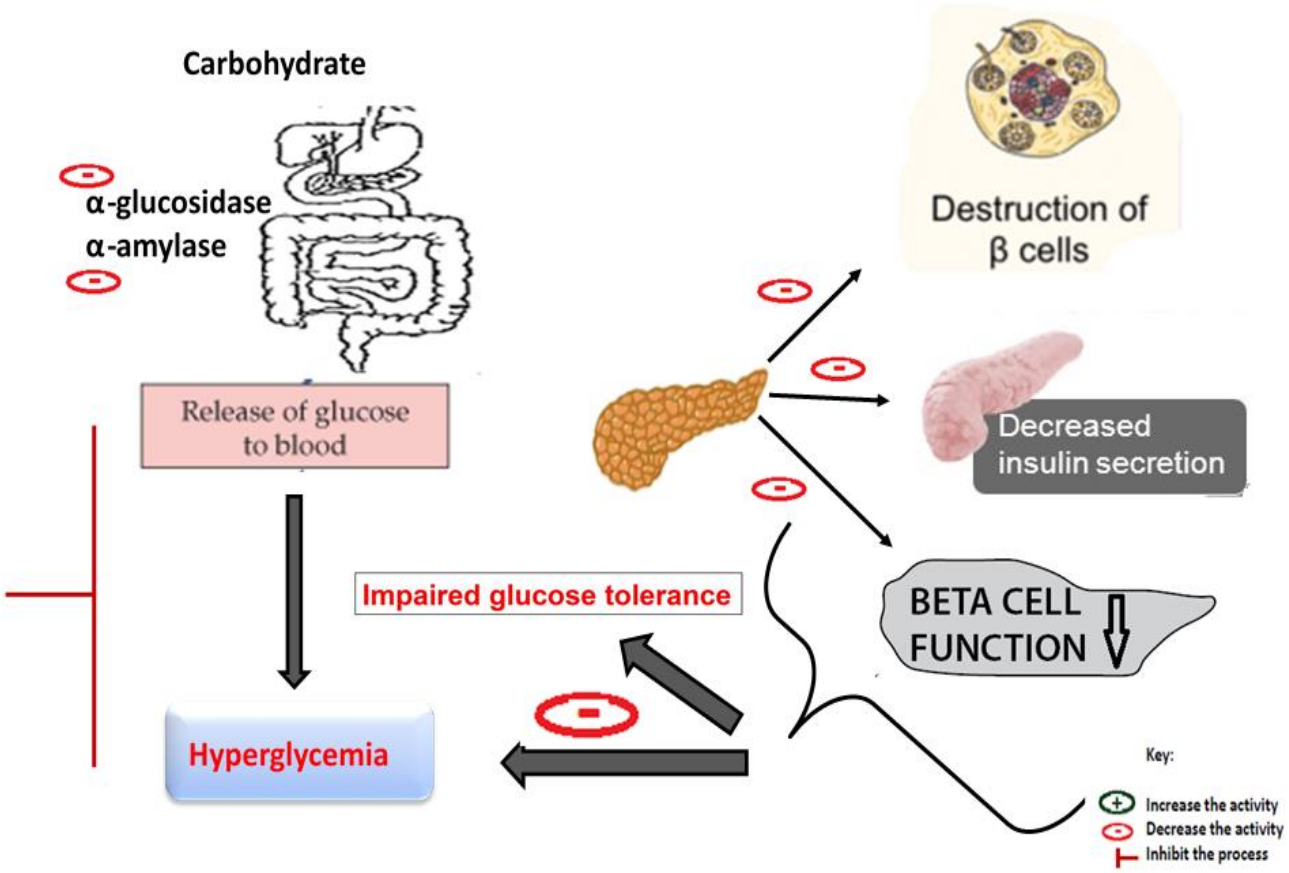


Figure 6.2.5: Antidiabetic activity of *C. volubile* leaves

4. *Immunomodulatory and Anti-inflammatory Activity*

Polymorphonuclear neutrophils (PMNs) are among the known phagocytes and the body's first line of defense against pathogens invasions (Harbort et al. 2015). Their ability to modulate both pro- and anti-inflammatory activities indicates a major influence on the immune system (Ciz et al. 2012). They (PMNs) release large volumes of ROS via NADPH oxidase activity upon stimulation, a process known as respiratory oxidative burst (Erukainure et al. 2017a; Erukainure et al. 2016a; Erukainure et al. 2017b). This process has been recognized as an efficient microbicidal

mechanism. Excessive oxidative burst has however been implicated in the recruitment of inflammatory cytokines, which leads to pathogenic inflammatory processes and related disorders with physiological consequences (Ciz et al. 2012).

Over the years, medicinal plants have been employed as immunomodulators due to their antioxidant and anti-inflammatory properties (Erukainure et al. 2017b). Erukainure et al. (Erukainure et al. 2017a; Erukainure et al. 2016a; Erukainure et al. 2017d) reported the ability of *C. volubile* leaves, stem and flowers to quench polymorphonuclear neutrophils (PMNs) respiratory oxidative burst, thereby portraying an immunomodulatory activity. They attributed the activity to the isolated phenolics, protocatechuic acid and pectolinarigenin which also displayed potent anti-oxidative burst activity. The authors demonstrated the ability of pectolinarigenin to suppress T-cell proliferation, portraying an anti-inflammatory potential (Erukainure et al. 2017b). The leaves have been reported to reduce the linear circumference of rat paws, which also portrays an anti-inflammatory activity (Jaiyesimi and Adekoya 2013).

Computational simulation of protocatechuic acid with T-cell showed that the para hydroxyl group hydrogen bonded to GLU 406, while the hydroxyl group (–OH) of the carboxylic acid (–COOH) bonded to PRO 437. Dual hydrogen bond interactions were observed on the carbonyl group with HIS 405 and HIS 415. There was a metal bond between the ligand and ZN 1. Electrostatic, covalent and van der Waals interactions were also observed between the ligand and with residues GLY 349, LEU 350, HIS 409 and TYR 436 (Erukainure et al. 2017a). This corresponds with previous reports that the scavenging activities of phenolics are greatly influenced by the number of hydroxyl substituents (Ciz et al. 2008). Ciz et al. (Ciz et al. 2008) further proposed that their C-2,3 double bond might play an influential role in the inhibition of phagocytic ROS production.

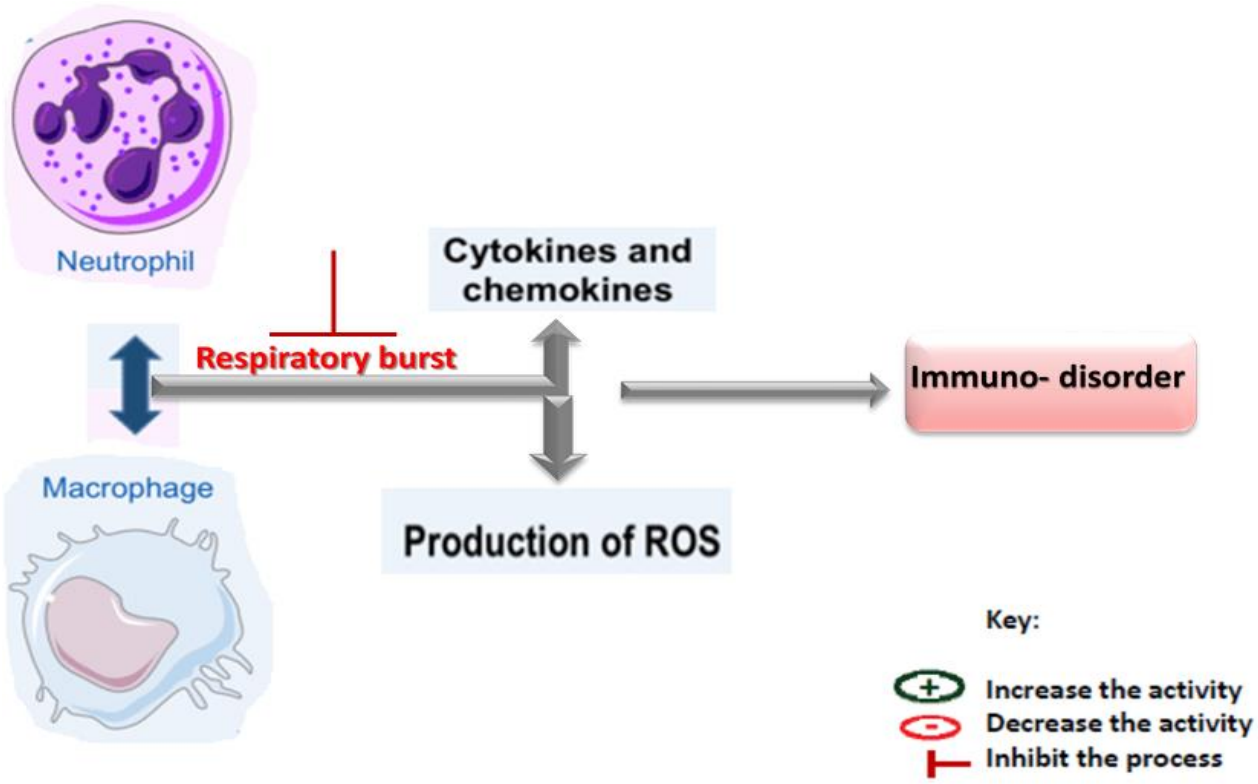


Figure 6.2.6: Immunomodulatory activity of *C. volubile* leaves

5. *Hypolipidemic Activity*

Hyperlipidemia is a medical condition characterized by elevated levels of serum cholesterol and/or low-density lipoprotein (LDL) cholesterol as well as alteration of other lipid parameters such as triglyceride, very low-density lipoprotein (vLDL) cholesterol and high-density lipoprotein (HDL) cholesterol (Erukainure et al. 2011b; Onwe et al. 2015). It has been recognized as a major cardiovascular risk factor which could give rise to cardiovascular diseases (CVD) and other atherosclerosis related conditions (Shattat 2015).

It is divided into two subclasses, primary and secondary hyperlipidemia. The former is genetic related, while the latter arises from complications from other disease condition (Shattat 2015). Medicinal plants and diets have been used in the treatment and control of secondary hyperlipidemia, this has been attributed to their ability to modulate blood lipid profile, particularly reducing blood cholesterol and increasing HDL levels (Erukainure et al. 2011b).

The hypolipidemic properties of the leaves of *C. volubile* has been reported. Akinpelu (Akinpelu et al. 2016) reported the ability of the leaf ethanol extract to lower plasma cholesterol, LDL, vLDL and triglyceride levels, as well as increased HDL level in Poloxamer-407 induced hyperlipidemic rats. Molehin et al. (Molehin et al. 2017b) reported similar effects in carbon tetrachloride-induced hepatotoxicity. Erukainure et al. (Erukainure et al. 2018; Erukainure et al. 2017a) reported the ability of the leaf fractions to mitigate increased serum cholesterol, LDL, vLDL and triglyceride levels, while increasing HDL level concomitantly in type 2 diabetic rats. This was attributed to the isolated phenolics, protocatechuic acid, biochanin, and 5,7,4'-trimethoxykaempferol (Erukainure et al. 2018; Erukainure et al. 2017a)

6. Toxicity

The safety of medicinal plants has been a major concern, owing to issues of standardization, characterization and preparation (Ezuruike and Prieto 2014; Shan et al. 2007). Plants consist of a cocktail of phytochemicals which are extractable based on the type of extracting solvent. Different plant extracts exert different biological function, which can be toxic (Ezuruike and Prieto 2014).

The toxicity of different extracts of *C. volubile* leaves have been reported. Erukainure et al. (Erukainure et al. 2017a; Erukainure et al. 2016a; Erukainure et al. 2017b) reported the cytotoxic effect of the fractions and isolated compounds of the leaves and flowers. They showed that DCM fractions were cytotoxic against CC-1 cells but the isolated compounds, biochanin, 5,7,4'-trimethoxykaempferol, protocatechuic acid and pectolinarigenin had no cytotoxic effect.

The toxicity of these isolated compounds was also predicted *in silico*. The toxicity classes of biochanin, 5,7,4'-trimethoxykaempferol, and pectolinarigenin were predicted to be V, while protocatechuic acid was IV. These classes further showed the relative safety of the compounds. Biochanin and 5,7,4'-trimethoxykaempferol were predicted as potent inhibitors of CYPs 1A2, 2C9, 2D6, and 3A4, thus suggesting caution in co-usage with other prescribed drugs metabolized by the enzymes (Erukainure et al. 2018).

In vivo studies showed the ability of the leaf extract to lower serum hepatic (AST and ALT) and renal (urea) biomarkers level in type 2 diabetic rats (Erukainure et al. 2018). Molehin et al. (Molehin et al. 2017b) reported similar effects in carbon tetrachloride-induced hepatotoxic rats.

They further reported the ability of the methanol extract to improve hepatic architecture in carbon tetrachloride-induced hepatoxicity (Molehin et al. 2017b).

6.2.6. Proposed Mechanism of Action of *C. volubile*

Based on the biological activities studied so far, *C. volubile* may bring about its health beneficiary effect by [1] modulation of the redox homeostasis, leading to decreased oxidative stress which in turn decreases the pathogenesis and complications of diabetes, hepatotoxicity, cancer and neurodegeneration; [2] down regulation of MMP-9 expression and concomitant arrest of the cell cycle, leading to decreased proliferation of cancer cells; [3] inhibition of ACE activity leading to decreased production of angiotensin II, thereby inhibiting vasoconstriction of the blood vessels; [4] inhibition of oxidative burst and inflammatory cytokines, thereby inhibiting immune-disorders and inflammations implicated in the pathogenesis of pancreatic β – cell dysfunction and insulin resistance. This in turn leads to increased insulin production and glucose tolerance; [5] inhibition of key carbohydrate catabolic enzymes leading to decreased postprandial blood glucose level; and [6] inhibition of cholinergic and monoaminergic enzymes implicated in neurodegeneration.

This is summarized in **Figure 6.2.7.**

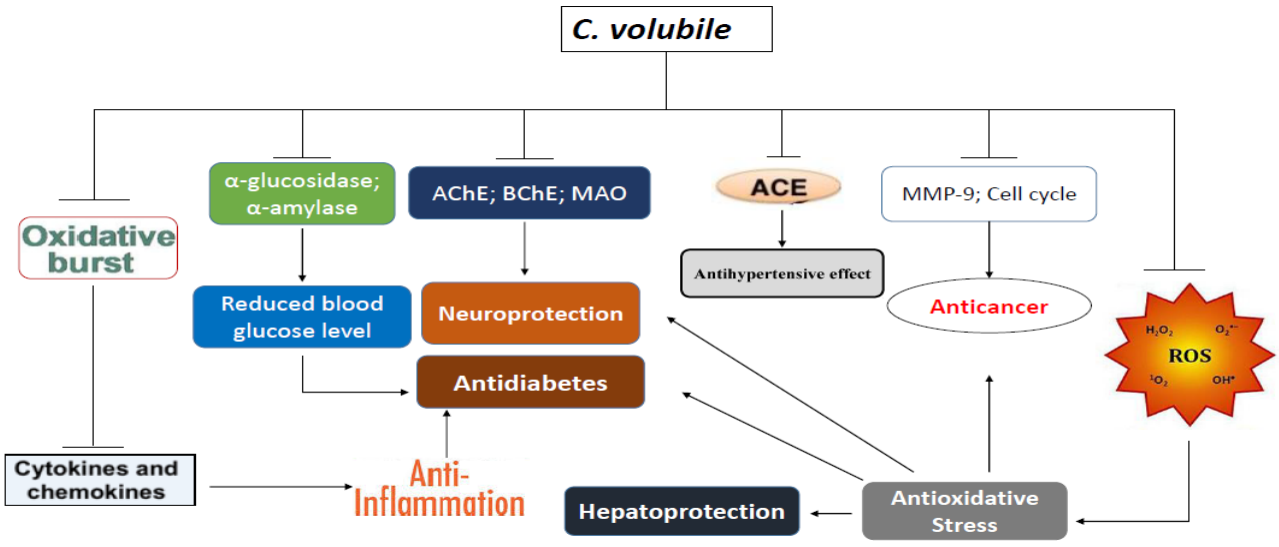


Figure 6.2.7: Proposed mechanism of action of C. volubile and its medicinal activities

6.2.8 Conclusion

Studies so far on *C. volubile* authenticate its folkloric use in the management and treatment of various ailments. These health benefits can be attributed to the phenolics identified so far in the plant. However, cautions should be considered when using the plant with other drugs metabolized by CYPs 1A2, 2C9, 2D6, and 3A4. More studies should be conducted on the molecular mechanisms behind the pharmacological properties of the plant.

6.3 Flowers of Clerodendrum volubile Modulates Redox Homeostasis and Suppresses DNA Fragmentation in Fe2+ - induced Oxidative Injury; and Inhibits Carbohydrates and Lipids Catabolic Enzymes linked to Type 2 Diabetes

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Preface: This article investigated the antioxidant, antidiabetic, chemical and predicted toxicity properties of the ethyl acetate, ethanol and aqueous extracts of *C. volubile* flowers *in vitro*, *ex vivo*, and *in silico*.

6.3.1 Abstract

The antioxidative and antidiabetic properties of *Clerodendrum volubile* flowers were investigated *in vitro*. The flowers were sequentially extracted with solvents of increasing polarity (n-hexane, ethyl acetate, ethanol and aqueous). The concentrated extracts were subjected to *in vitro* antioxidant assays using the 2,2'-diphenyl-1-picrylhydrazyl (DPPH) scavenging and Ferric reducing antioxidant power (FRAP) protocols. Their inhibitory activities were investigated on α-glucosidase, pancreatic lipases, pancreatic ATPase and glucose-6-phospatase activities. Their antioxidative and anti-apoptotic effects on Fe^{2+}-induced oxidative injuries were also investigated in pancreatic and hepatic tissues *ex vivo*. The extracts showed potent free radical scavenging activity and significantly ($p<0.05$) inhibited all studied enzymes. The GSH level was significantly ($p<0.05$) elevated in both tissues with concomitant increase in Superoxide Dismutase (SOD) and Catalase activities as well as reduced levels of malondialdehyde (MDA). The extracts significantly ($p<0.05$) suppressed DNA fragmentation in hepatic tissue. These activities were dose – dependent.

The ethanol extract showed the best activity and can be attributed to the synergetic effect of its chemical constituents identified via GC-MS. These results suggest the antioxidative, antidiabetic and anti-obesogenic potentials of *C. volubile* flowers.

Keywords: Anti-hyperglycemia; *C. volubile*; Oxidative Stress; and Type 2 Diabetes

6.3.2 Introduction

Over the years there have been increasing interesting in the use of medicinal plants in the treatment and management of diabetes mellitus. This can be attributed to the high cost of treating diabetes, coupled to its increasing prevalence and toxic side effects of synthesized drugs. In 2015, 415 million people were estimated to be diabetic which accounted for 12% of the global health expenditure and 5 million deaths (I.D.F. 2016).

Type 2 diabetes (T2D) occurs owing to inability of the body to effectively utilize the insulin produced, leading to chronic hyperglycemia. It accounts for more than 90% of all diabetes, and has been recognized as a major contributor to global mortality and morbidity (I.D.F. 2018). Oxidative stress has been implicated in the pathogenesis and progression of type 2 diabetes. It arises due to hyperglycemia – induced elevated reactive oxygen species (ROS) production, causing an imbalance in endogenous antioxidant defense system (Tiwari et al. 2013).

To the best of our knowledge, there remains a scientific dearth in the antioxidative and antidiabetic potential of *C. volubile* flowers. This paper thus aims to report the anti-oxidative potentials of the ethyl acetate, ethanol and aqueous extracts of *C. volubile* flowers on Fe^{2+} - induced oxidative pancreatic and hepatic injuries. As well as their antidiabetic potentials by investigating their inhibitory activity on key carbohydrate and lipid digestive enzymes linked to T2D.

6.3.3 Materials and Methods

Kindly refer to Chapter 2, subsections: 2.1.1, 2.1.3, 2.1.5, 2.1.6, 2.2 – 2.4, 2.6, 2.10.1, 2.11.1 and 2.13 for details.

6.3.4 Results

Total phenol analysis revealed the ethyl acetate extract to have the highest phenolic content as depicted in **Figure 6.3.1**. This was followed by ethanol, with the aqueous extract having the lowest.

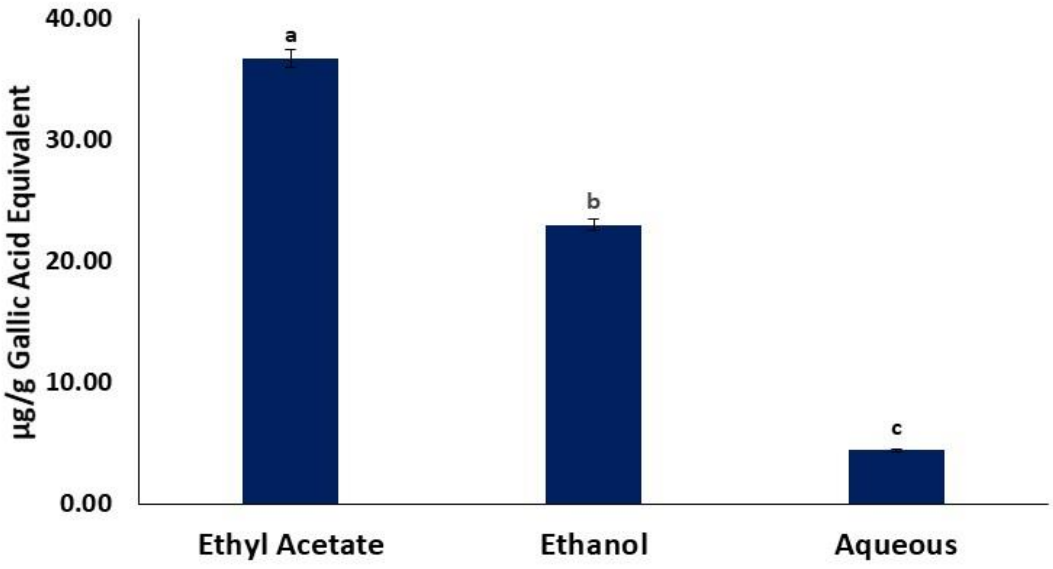
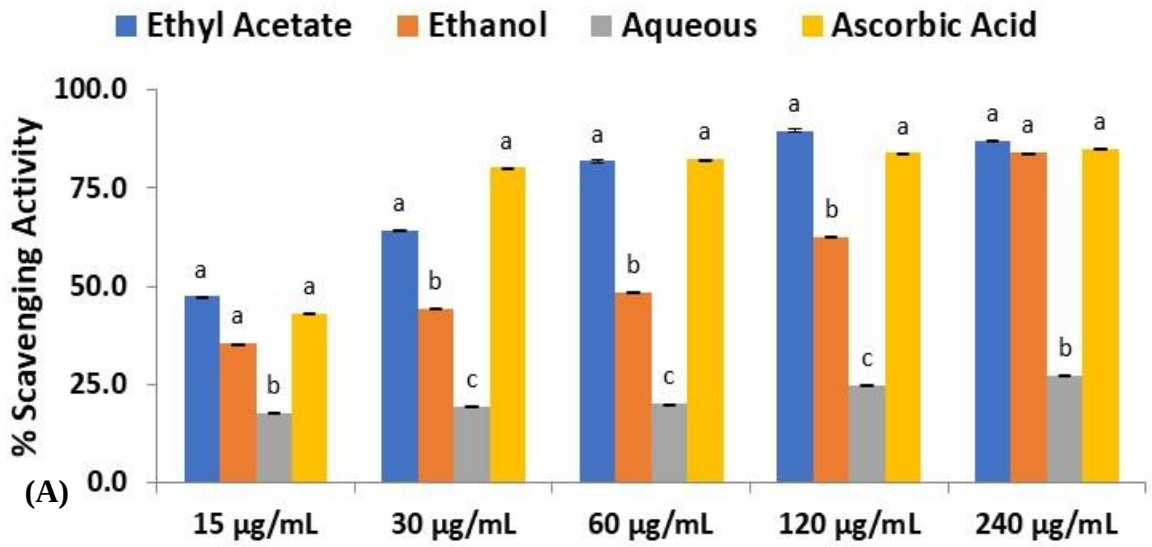


Figure 6.3.1: Total phenolic contents of the *C. volubile* flower extracts. Data are presented as mean ± SD. [abc]Values with different letter above the bars for a given extract are significantly different from each other ($p < 0.05$, Tukey's HSD-multiple range post-hoc test, IBM SPSS for Windows).

Significant ($p<0.05$) dose dependent DPPH scavenging and FRAP activities were observed for all extracts as shown in **Figures 6.3.2A** and **6.3.2B** respectively. The low IC_{50} value of the ethanol extract portrays it as the best activity.

IC_{50} (µg/mL): Ethyl Acetate: 12.23; Ethanol: 8.99; Aqueous: >1000; Ascorbic Acid: 8.32



IC_{50} (µg/mL): Ethyl Acetate: 2.32; Ethanol: 1.43; Aqueous: 2.17; Ascorbic Acid: 1.47

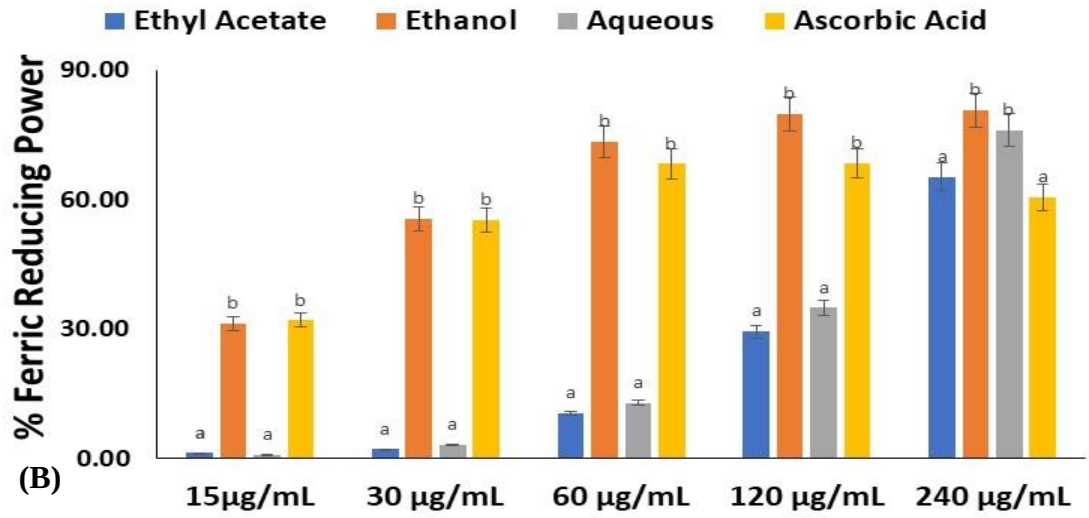
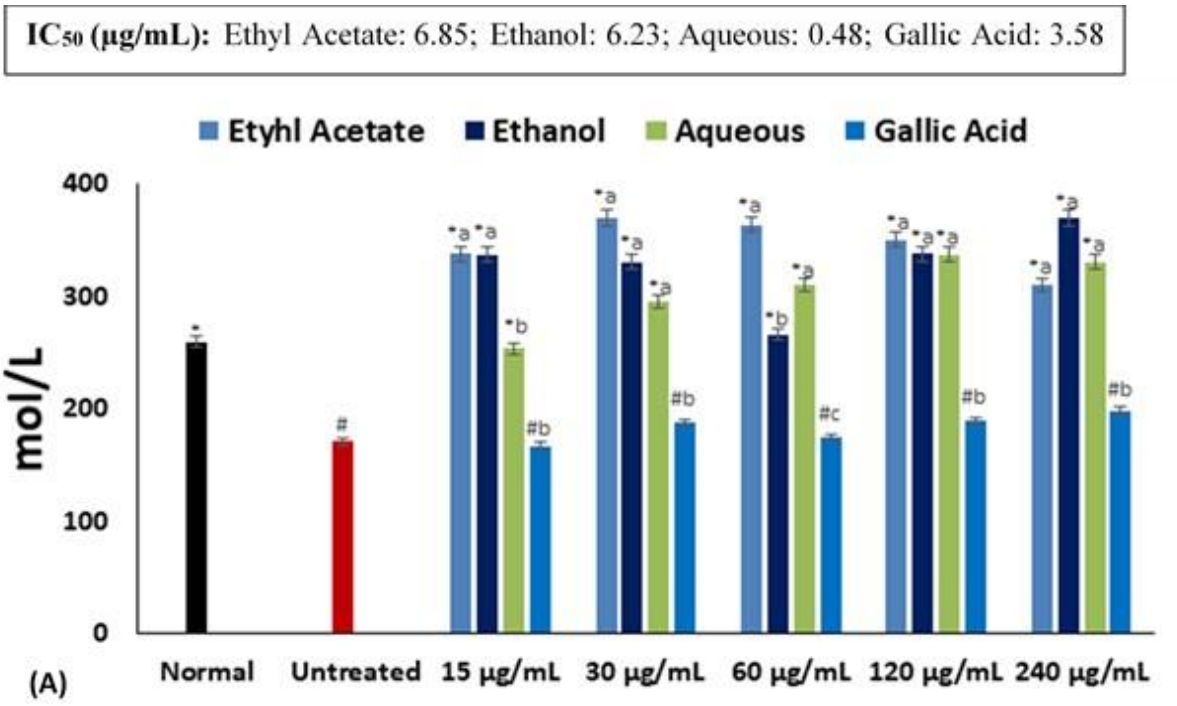


Figure 6.3.2: (A) DPPH and (B) Ferric reducing antioxidant power (FRAP) activity of *C. volubile* flower extracts. Data are presented as mean ± SEM. [abc]Values with different letter above the bars for a given extract are significantly different from each other ($p < 0.05$, Tukey's HSD-multiple range post-hoc test, IBM SPSS for Windows).

Incubation with $FeSO_4$ led to induction of oxidative injury as portrayed by the significant ($p<0.05$) decreased GSH level in pancreatic tissue as depicted in **Figure 6.3.3A**. The effect was more pronounced in hepatic tissue (**Figure 6.3.3B**). A significant ($p<0.05$) reversible effect was observed on treatment with the extracts in both tissues depicting their therapeutic potentials. The aqueous extract showed the best activity in the pancreatic tissues, while the ethanol extract was the best for hepatic tissues.



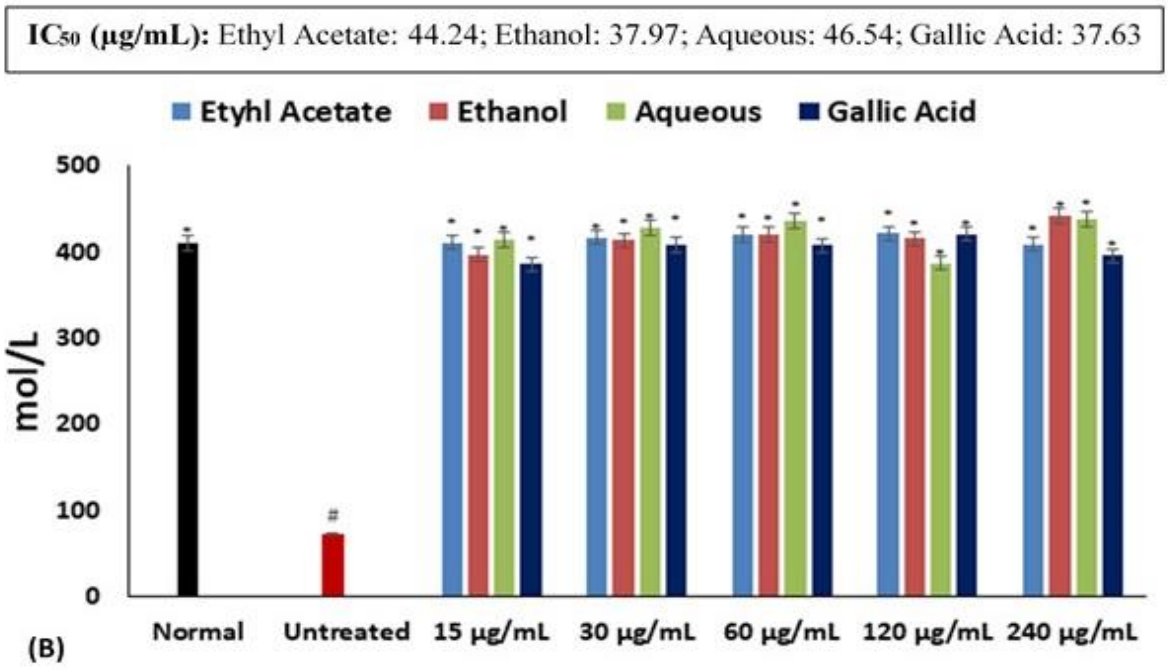
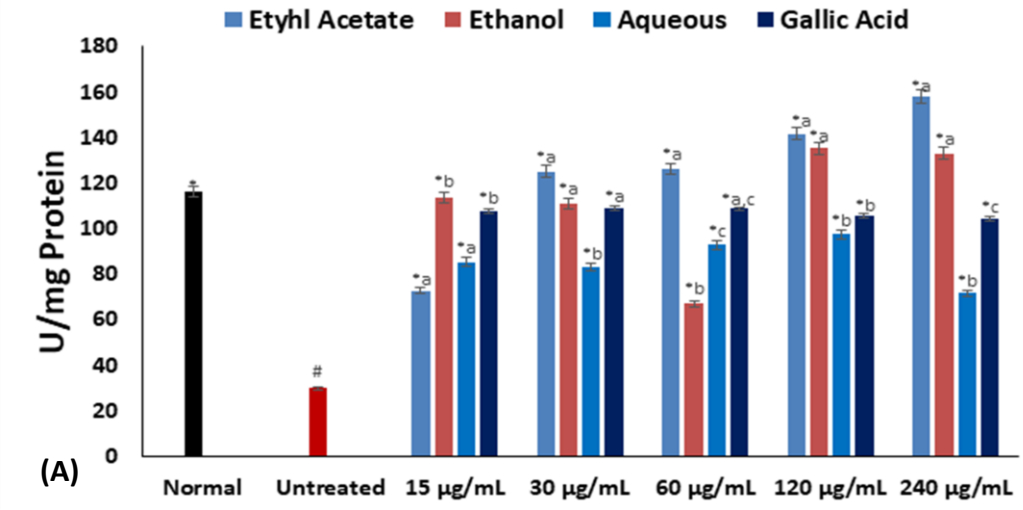


Figure 6.3.3: Effect of *C. volubile* flower extracts on GSH level in oxidative (A) pancreatic and (B) hepatic injuries. Data are presented as mean ± SEM. *Significantly different from untreated sample and #Significantly different from normal sample; abcValues with different letter above the bars for a given extract are significantly different from each other ($p < 0.05$, Tukey's HSD-multiple range post-hoc test, IBM SPSS for Windows).

Induction of oxidative injury led to significant ($p<0.05$) reduced SOD activities in both tissues as shown in **Figures 6.3.4A** and **6.3.4B**. A dose–dependent increased activities was observed on treatment with the extracts in both tissues, with the ethanol extract showing the best activity for both tissues as depicted by its low IC_{50} values.

IC_{50} (µg/mL): Ethyl Acetate: 1.05; Ethanol: 0.25; Aqueous: 3.22; Gallic Acid: 10.28



IC_{50} (µg/mL): Ethyl Acetate: 1.17; Ethanol: 0.38; Aqueous: 0.60; Gallic Acid: 0.87

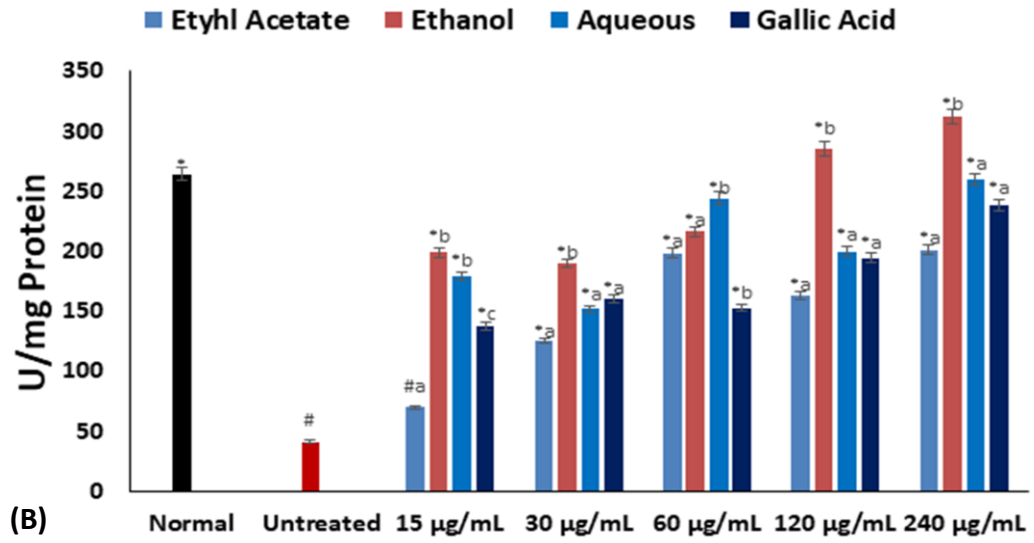
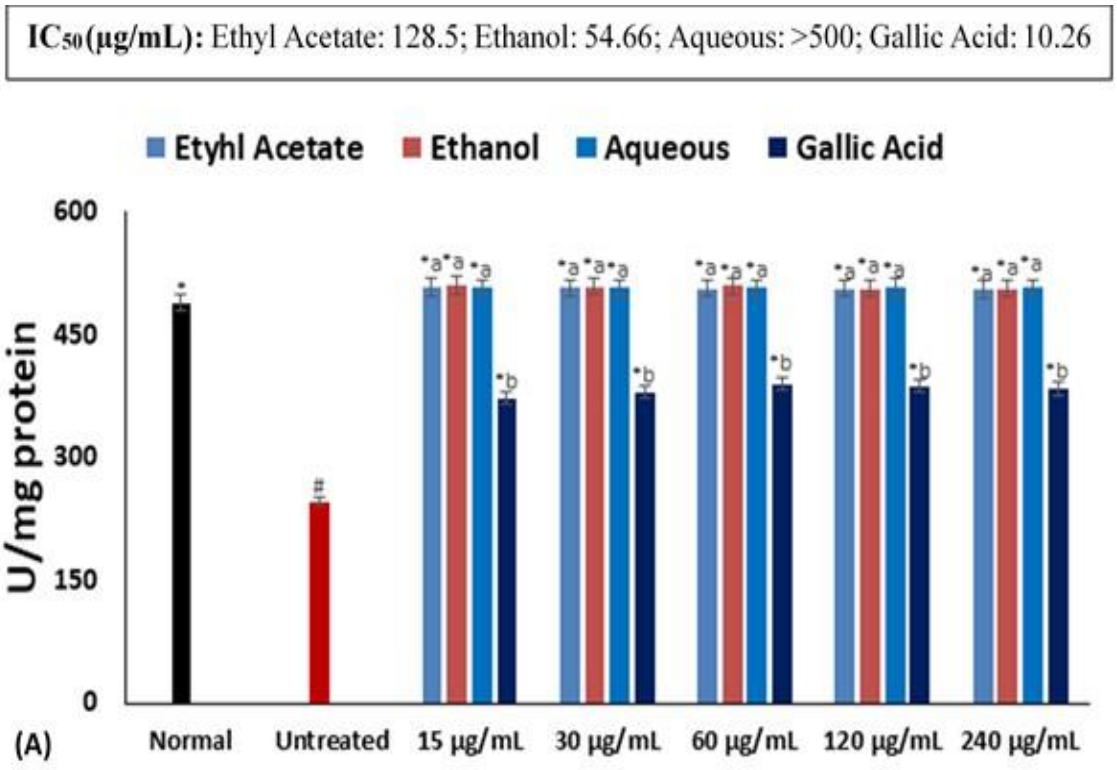


Figure 6.3.4: Effect of *C. volubile* flower extracts on SOD activity in oxidative (A) pancreatic and (B) hepatic injuries. Data are presented as mean ± SEM. $^{\#}$Significantly different from normal sample and *Significantly different from untreated sample; ^{abc}Values with different letter above the bas for a given extract are significantly different from each other ($p < 0.05$, Tukey's HSD-multiple range post-hoc test, IBM SPSS for Windows).

Significant ($p<0.05$) reduced catalase activities were observed in both tissues on induction of oxidative injury with $FeSO_4$ as depicted in **Figures 6.3.5A** and **6.3.5B**. An increased activity was observed on treatment with the extracts in a dose – dependent pattern, with the ethanol showing the best activity for pancreatic tissues (**Figure 6.3.5A**) and aqueous extract for hepatic tissues (**Figure 6.3.5B**) as portrayed by their low IC_{50} values.



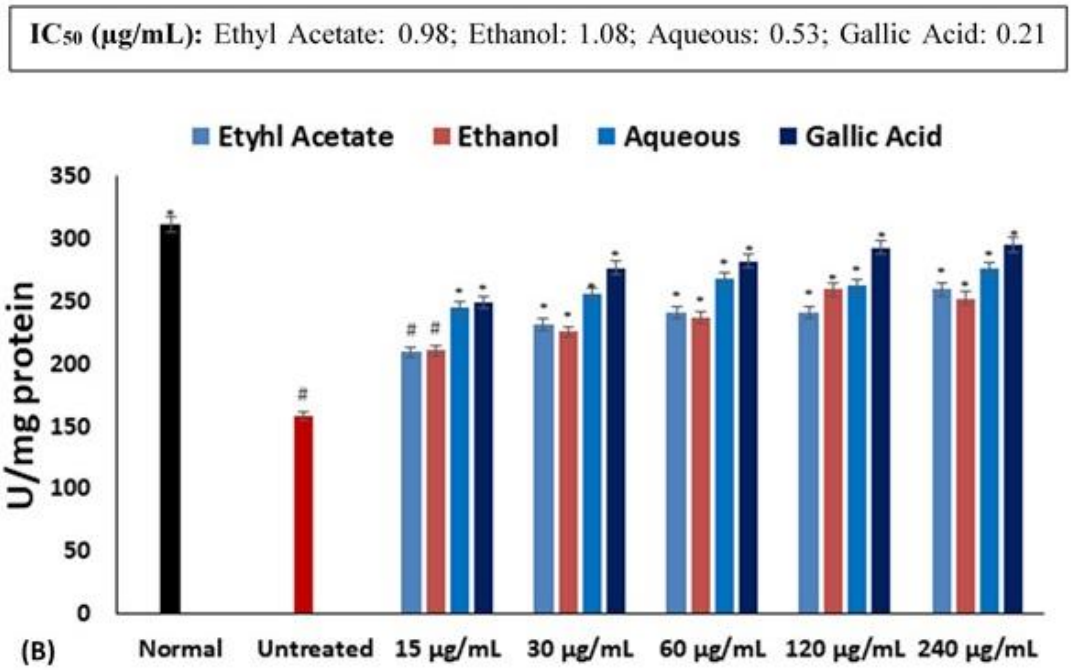
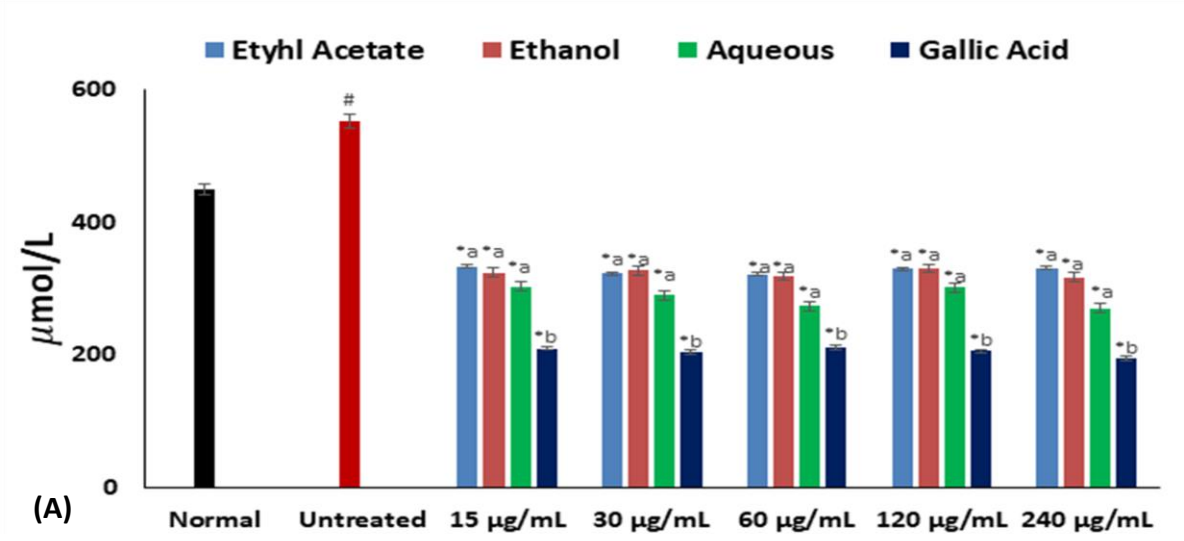


Figure 6.3.5: Effect of *C. volubile* flower extracts on catalase activity in oxidative (A) pancreatic and (B) hepatic injuries. Data are presented as mean ± SEM. *Significantly different from untreated sample and #Significantly different from normal sample ($p < 0.05$, Tukey's HSD-multiple range post-hoc test, IBM SPSS for Windows).

There were significant ($p<0.05$) increase in MDA levels on induction of oxidative injury with $FeSO_4$ in both tissues as shown in **Figure 6.3.6A** and **6.3.6B**. This was significantly ($p<0.05$) reduced on treatment with the extracts in a dose–dependent pattern in pancreatic tissue, with the aqueous showing the best activity for pancreatic tissues (**Figure 6.3.6A**) and ethanol for hepatic tissues (**Figure 6.3.6B**) as revealed by their low IC_{50} values.

IC_{50} (µg/mL): Ethyl Acetate: 16.92; Ethanol: 64.56; Aqueous: 12.30; Gallic Acid: 127.56



IC_{50} (µg/mL): Ethyl Acetate: 1.98; Ethanol: 1.50; Aqueous: 1.97; Gallic Acid: 1.24

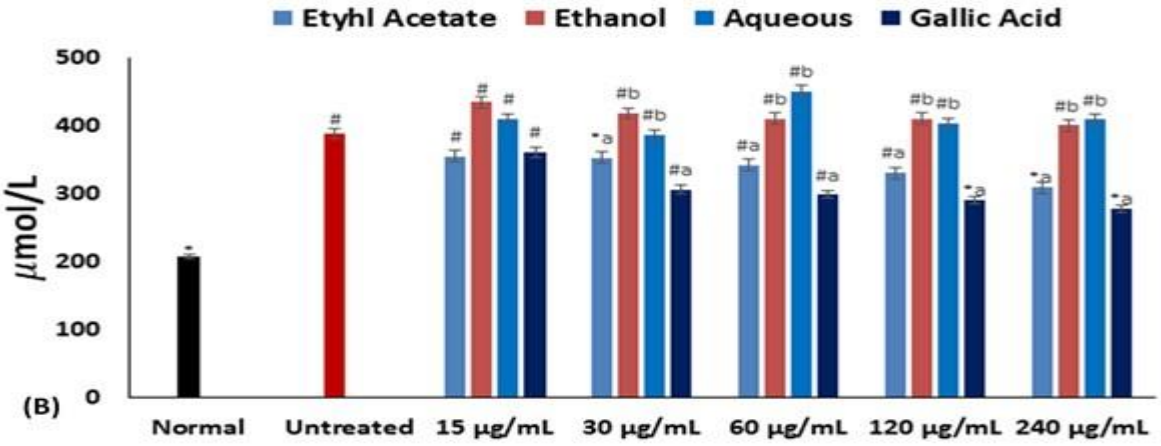


Figure 6.3.6: Effect of C. volubile flower extracts on lipid peroxidation in oxidative (A) pancreatic and (B) hepatic injuries. Data are presented as mean ± SEM. *Significantly different from untreated sample and #Significantly different from normal sample; abValues with different letter above the bas for a given extract are significantly different from each other ($p < 0.05$, Tukey's HSD-multiple range post-hoc test, IBM SPSS for Windows).

All extracts showed significant ($p<0.05$) inhibitory activity on α – glucosidase and pancreatic lipase as depicted in **Figures 6.3.7A** and **6.3.7B**, with the aqueous extract showing the best

inhibitory activity which compared favorably to acarbose and orlistat respectively as portrayed by its low IC_{50} values.

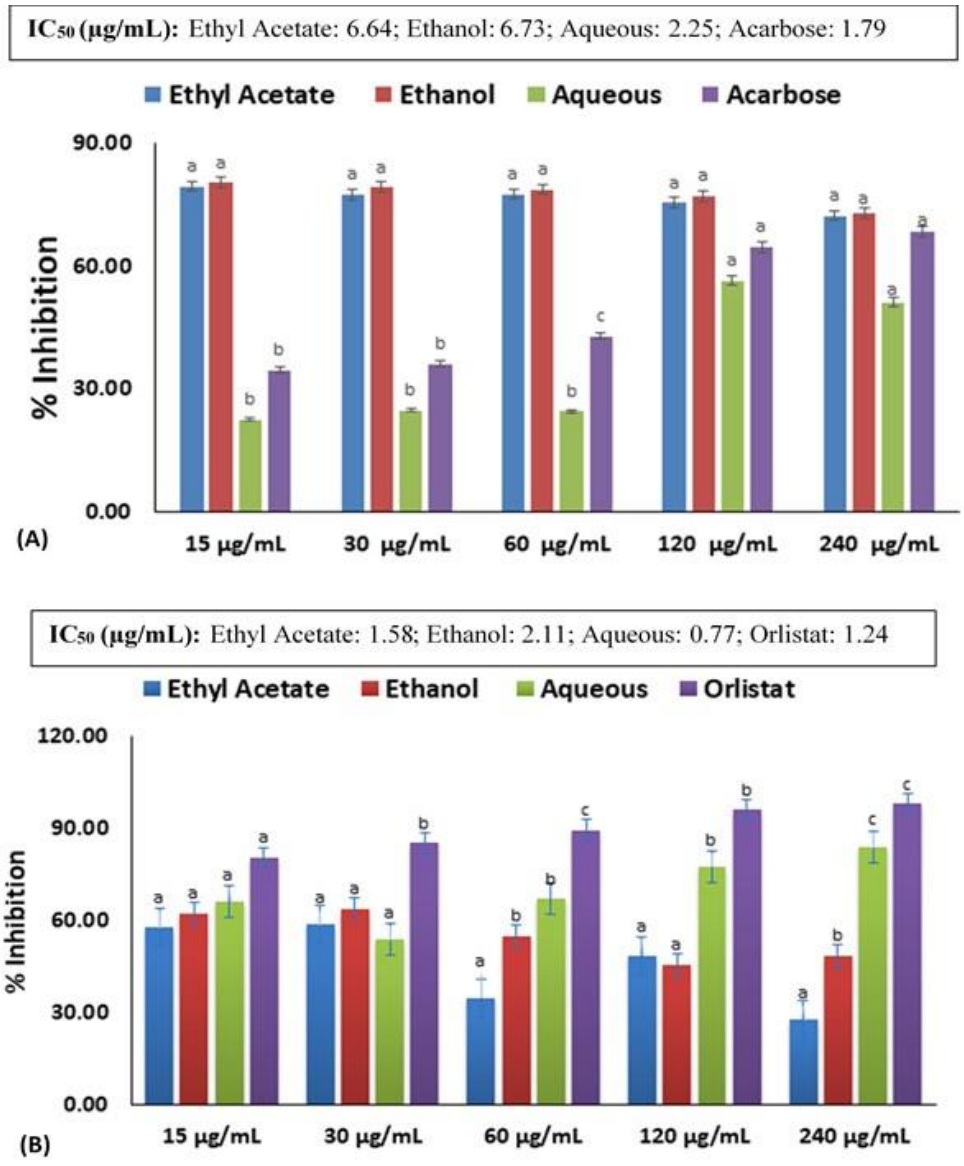


Figure 6.3.7: (A) α-glucosidase and (B) pancreatic lipase inhibitory activities of *C. volubile* flower extracts. Data are presented as mean ± SEM. [abc]Values with different letter above the bas for a given extract are significantly different from each other ($p < 0.05$, Tukey's HSD-multiple range post-hoc test, IBM SPSS for Windows).

There was a significant ($p<0.05$) increase in ATPase activity on induction of oxidative injury in pancreatic tissues as depicted in **Figure 6.3.8**. This was significantly ($p<0.05$) reduced on

treatment with the extracts, with the ethyl acetate showing the best activity as revealed by its low IC_{50} value.

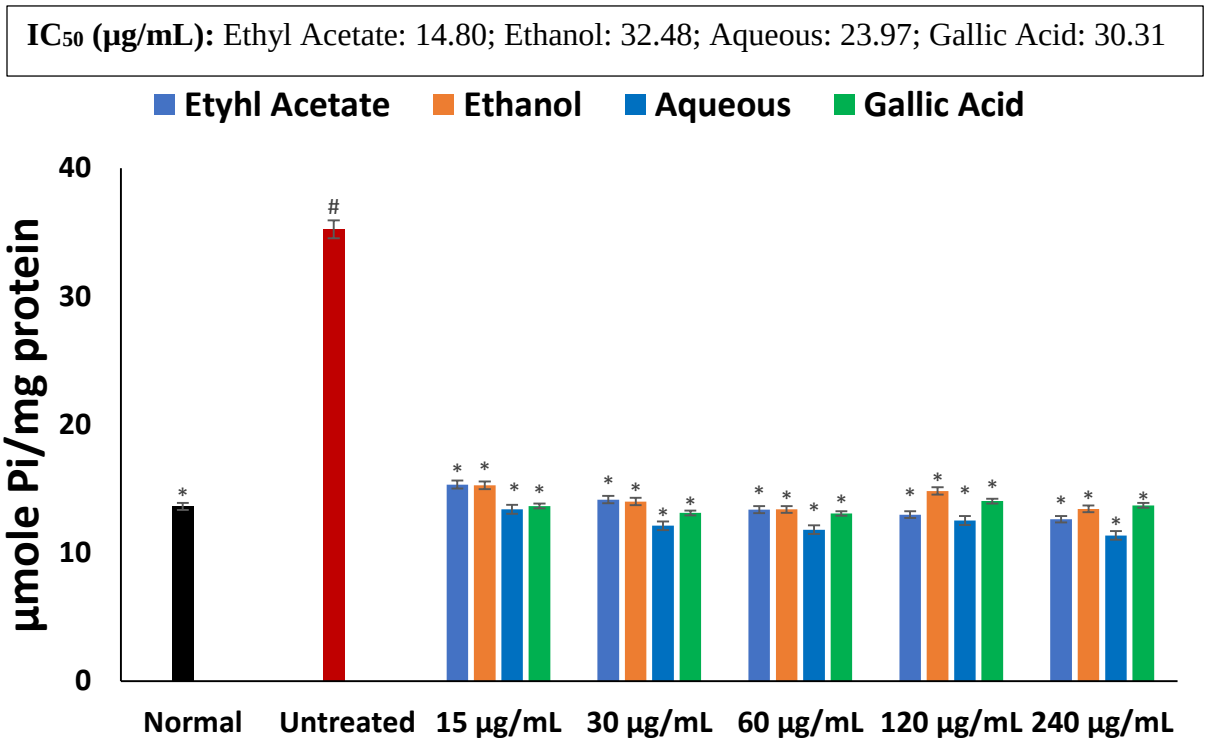


Figure 6.3.8: Effect of *C. volubile* flower extracts on ATPase activity in oxidative pancreatic injury. Data are presented as mean ± SEM. *Significantly different from untreated sample and #Significantly different from normal sample ($p < 0.05$, Tukey's HSD-multiple range post-hoc test, IBM SPSS for Windows).

A significant ($p<0.05$) increase in glucose-6-phosphate activity was observed on induction of oxidative injury in hepatic tissues as shown in **Figure 6.3.9**. All extracts showed significant ($p<0.05$) dose – dependent inhibition of the activity to near normal, with the ethyl acetate showing the best activity as revealed by its low IC_{50} value.

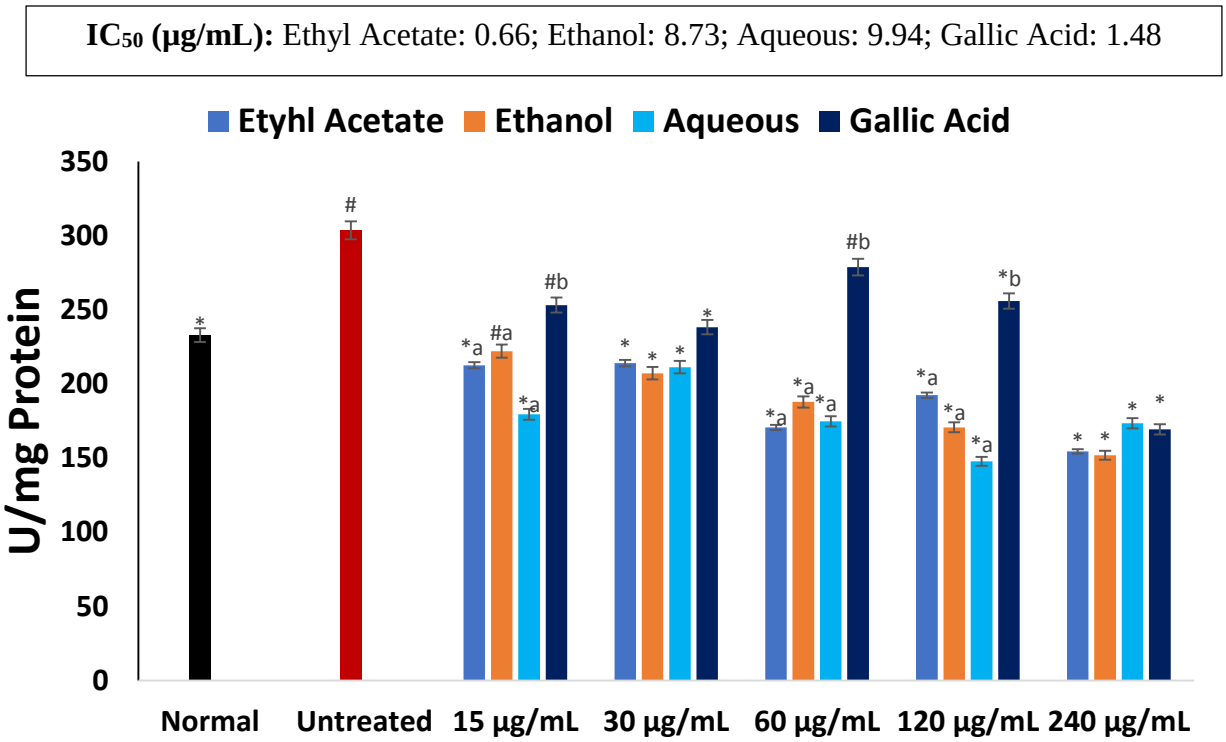


Figure 6.3.9: Effect of C. volubile flower extracts on Glucose-6-Phosphatase activity in oxidative hepatic injury. Data are presented as mean ± SEM. *Significantly different from untreated sample and #Significantly different from normal sample; abValues with different letter above the bas for a given extract are significantly different from each other ($p < 0.05$, Tukey's HSD-multiple range post-hoc test, IBM SPSS for Windows).

Induction of oxidative injury led to significant ($p<0.05$) increased DNA fragmentation in both tissues as depicted in **Figures 6.3.10A** and **6.3.10B**. The extracts showed better suppressive activities in hepatic tissues (**Figure 6.3.10B**) when compared to pancreatic tissues (**Figure 6.3.10A**), with the ethyl acetate showing the best activity as revealed by its low IC_{50} value.

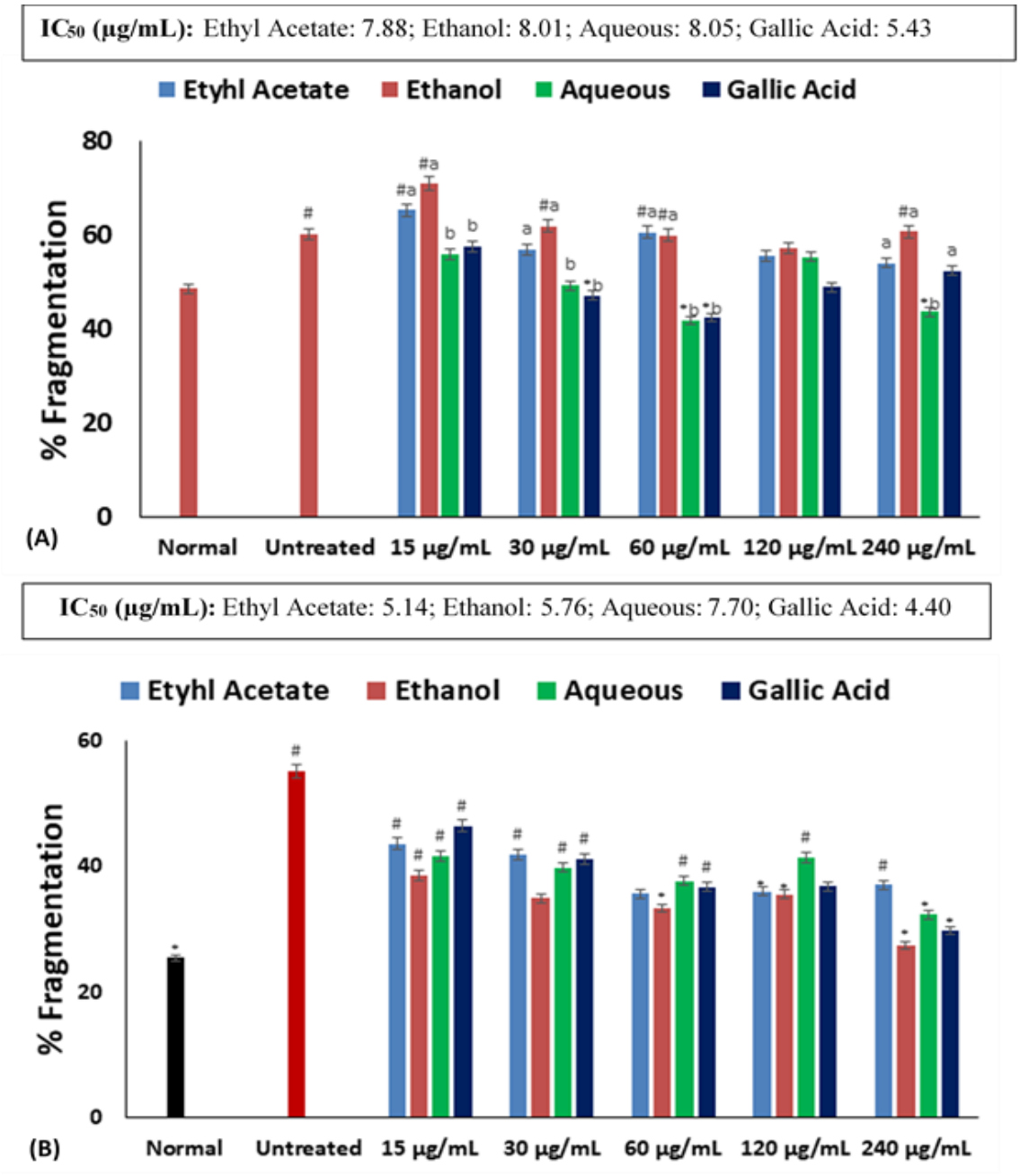


Figure 6.3.10: Effect of *C. volubile* flower extracts on DNA fragmentation in oxidative (A) pancreatic and (B) hepatic injuries. Data are presented as mean ± SD. *Significantly different from untreated sample and #Significantly different from normal sample ($p < 0.05$, Tukey's HSD-multiple range post-hoc test, IBM SPSS for Windows).

GC – MS analysis of the ethanol extract revealed the presence of 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one, 2-methylbenzofuran, D-allose, Methyl-*β*-D-glucopyranoside, Phytol

acetate, Trans-phytol, Octadecanoic acid, 9-(4-methoxyphenyl)xanthene, Stigmasterol and Sitosterol as shown in **Figure 6.3.11** and **Table 6.3.1**.

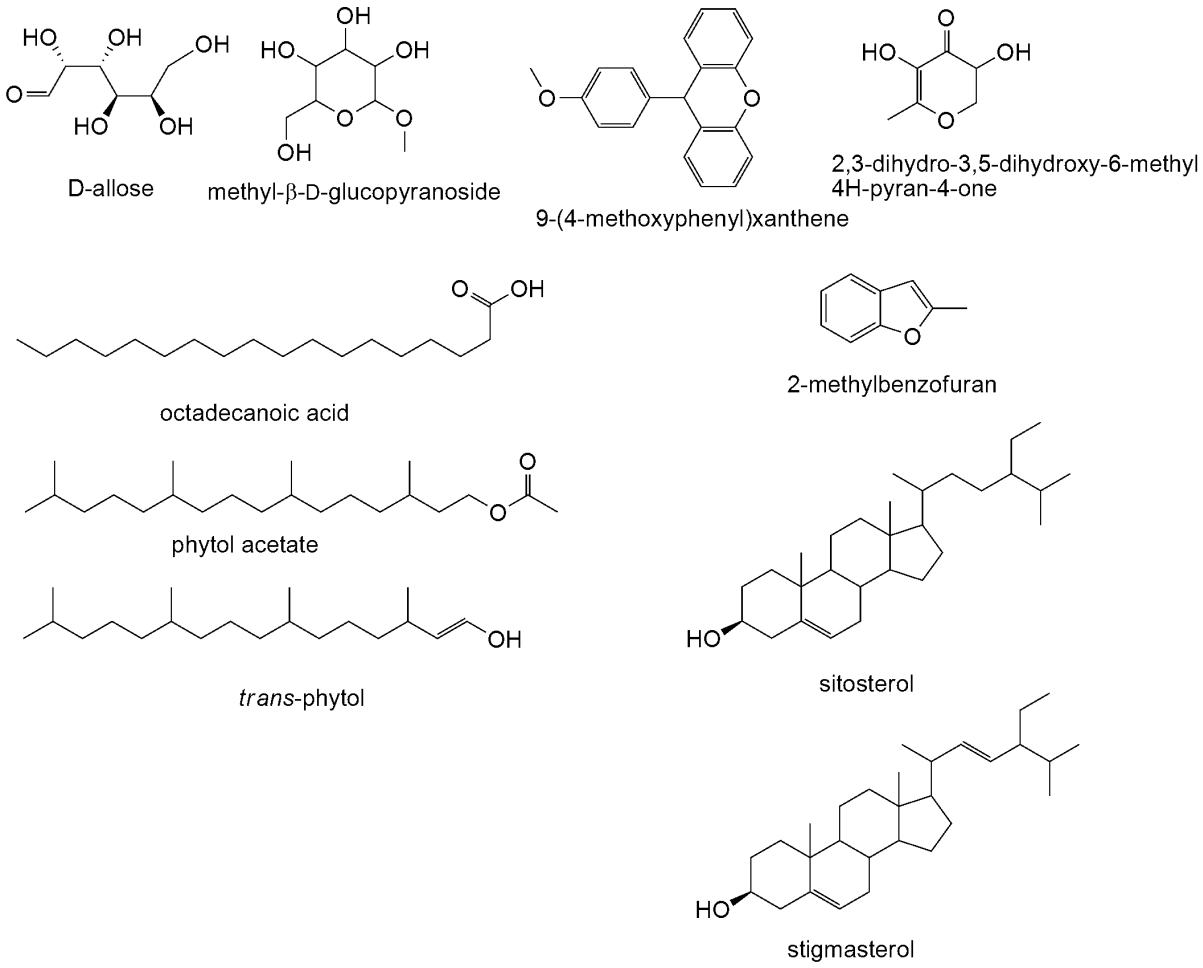


Figure 6.3.11: Structures of the compounds identified in the ethanol extract of *C. volubile* flower.

Table 6.3.1: Identified compounds of the ethanol extract of *C. volubile* flower by GC-MS

Compounds	RT (min)	Molecular mass	Relative abundance (%)
2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one	7.79	144	1.23
2-methylbenzofuran	8.33	132	0.75
D-allose	12.47	180	1.65
Methyl-β-D-glucopyranoside	14.05	194	2.16
Phytol acetate	16.42	338	2.12
Trans-phytol	19.10	296	1.47
Octadecanoic acid	19.56	284	2.95
9-(4-methoxyphenyl)xanthene	22.39	288	2.28
stigmasterol	28.97	412	0.71
sitosterol	29.77	414	0.69

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

Prediction of oral toxicity of the identified compounds on ProTox (tox.charite.de) web-based server revealed D-allose and Octadecanoic acid fall into class 3 as shown in **Table 6.3.2**. 2-methylbenzofuran and Trans-phytol fell into class 5. Methyl-β-D-glucopyranoside and Phytol acetate were on class 6, while the others were class 4.

Table 6.3.2: Oral Toxicity Prediction of Identified compounds of the ethanol extract of *C. volubile* flower by GC-MS

Ligands	Predicted LD50	Toxicity Class	Prediction Accuracy
2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one	595mg/kg	4	68.07%
2-methylbenzofuran	2960mg/kg	5	67.38%
D-allose	51mg/kg	3	70.97%
Methyl-β-D-glucopyranoside	23000mg/kg	6	72.90%
Phytol acetate	8000mg/kg	6	72.90%
Trans-phytol	5000mg/kg	5	100%
Octadecanoic acid	130mg/kg	3	100.00%
9-(4-methoxyphenyl)xanthene	370mg/kg	4	68.07%
Stigmasterol	890mg/kg	4	72.90%
Sitosterol	890mg/kg	4	72.90

6.3.5 Discussion

The high cost of treating diabetes coupled to the economic burden of most developing countries have led to an increased growth in the utilization of antidiabetic medicinal plants. Several studies have attributed the potency of these plants to the presence of phytochemicals particularly the phenols and flavonoids (Oboh and Ademosun, 2011). In this study, the antidiabetic potentials of *C. volubile* flowers were investigated using *in vitro* and *ex vivo* models. Its chemical constituents were also reported.

Several studies have correlated total phenolic content with increased free radical scavenging activities (Saeed et al., 2012; Anahita et al., 2015; Hossain and Shah, 2015). In this study, the ethyl acetate and ethanol extracts showed the highest total phenolic contents (**Figure 6.3.1**) and the highest scavenging activities (**Figure 6.3**.2a). However, the ethyl acetate which showed the highest total phenolic content did not display the best scavenging activity. The observed DPPH scavenging activity corresponds with earlier reports by Erukainure et al. (2016) on the free radical scavenging activities of fractions of the methanolic extract of the flower.

GSH has been recognized as a major maker of oxidative stress as it is the first line of the endogenous antioxidant system and its reduced level has been reported in the pathogenesis of T2D complications (Tiwari et al. 2013). The reduced levels in the untreated tissues therefore indicates an occurrence of oxidative stress (**Figure 6.3.3A** and **6.3.3B**), which can be attributed to the oxidation of Fe^{2+} to Fe^{3+}. The increased levels in the treated tissues indicates an antioxidative protective potential by the extracts, which also correlates with their observed *in vitro* antioxidant activities (**Figures 6.3.2A** and **6.3.2B**).

The SOD and catalase levels in pancreatic tissue has been reported to be much lower than that of the hepatic tissues, thus making it more susceptible to oxidative injury (Davari et al. 2013). Iron induced oxidative injury has been associated with increased production of superoxide ($O_2^{\cdot -}$) and hydroxyl ($^{\cdot}OH$) radicals via Fenton and Haber Weiss reactions (Aslan et al. 2000). SOD protects against oxidative cellular injury by catalyzing the dismutation of $O_2^{\cdot -}$ to H_2O_2. Catalase then converts it to oxygen and water. The reduced activities of SOD and catalase on in the untreated tissues further indicates an incidence of oxidative injury (**Figures 6.3.4A – 6.3.5B**). Their

significant ($p<0.05$) increase on treating with the extracts, further corroborates their antioxidative properties.

Accumulation of H_2O_2 owing to decreased catalase activity has been implicated in lipid peroxidation. In Fe^{2+} oxidative toxicity, the unstable cations react continuously with the accumulating H_2O_2 via Fenton reaction to yield hydroxyl radicals ($^{\bullet}OH$) (Aslan et al. 2000). The generated $^{\bullet}OH$ attacks the membrane lipids, there by initiating a peroxidative chain reaction (Aslan et al. 2000; Kanias and Acker 2010). The increased MDA level in the untreated tissues indicates an occurrence of lipid peroxidation (**Figures 6.3.6A** and **6.3.6B**). Lipid peroxidation is also considered as a marker of oxidative stress and even argued as an early marker for diabetes (Arora et al., 2013). The decreased MDA level after treatment with the extracts particularly in the pancreatic tissue (**Figure 6.3.6A**), therefore indicates an anti-peroxidative effect which corroborates with the increased GSH levels (**Figure 6.3.3A** and **6.3.3B**) as well as SOD and catalase activities (**Figure 6.3.4A** – **6.3.5B**).

Most antidiabetic drugs bring about their functions by inhibiting key metabolic enzymes linked to type 2 diabetes. The dose – dependent inhibition of intestinal α – glucosidase by the extracts (**Figure 6.3.7A**) indicates an anti-hyperglycemic potential as a result of delay in carbohydrate digestion. Studies have demonstrated that inhibition of α – glucosidase causes delayed and prolonged carbohydrate digestion, thus reducing the rate of glucose absorption (Bischoff ; Bischoff 1995). The extracts also portrayed an anti-obesogenic activity as depicted by their dose – dependent inhibition of pancreatic lipase (**Figure 6.3.7B**). Pancreatic lipase is a major inhibitory target in the treatment and management of obesity, as it hydrolyzes triglycerides into monoglycerides and free fatty acids (Lunagariya et al. 2014).

Inhibition of pancreatic ATPase activity particularly Na^+, K^+-ATPase has been suggested as a possible mechanism for glucose – initiated insulin secretion via membrane depolarization (Owada et al. 1999). The increased ATPase activity in the untreated pancreatic tissue may depict an impaired membrane depolarization thus signifying a decrease in insulin secretion (**Figure 6.3.8**). Consequently, the significant ($p<0.05$) reduced activity to near normal on treatment with the extracts may portray increased membrane polarization, implying an increased insulin secretion

An increased glucose-6-phosphatase activity has been implicated in type 2 diabetes as it plays a major role in the homeostasis of blood glucose levels (Ghosh et al. 2004; Van Schaftingen and Gerin 2002). Its increased activity on induction of oxidative stress in the hepatic tissues indicate an occurrence of increased gluconeogenesis and glycogenolysis (**Figure 6.3.9**). The observed dose – dependent inhibition by the extracts also portrays an anti-hyperglycemia activity.

Studies have implicated hyperglycaemia and hyperlipidemia in the apoptosis of pancreatic β – cells and hepatocytes in T2D, with oxidative stress being the driving key (Donath et al. 1999). It is characterized by DNA fragmentation and cellular shrinkage. The increased fragmentation on incubation with Fe^{2+} indicates an occurrence of apoptosis (**Figures 6.3.10A** and 6**.3.10B**). This can be attributed to the oxidation of Fe^{2}+ to Fe^{3+} as evident by the reduced GSH level (**Figures 6.3.3A** and **6.3.3B**) and antioxidant enzymes (**Figures 6.3.4A** – **6.3.5B**). This also corresponds to reports that oxidative stress significantly influences apoptosis (Allameh et al. 2010). The extracts significantly ($p<0.05$) reduced the process particularly in the hepatic tissue (**Figures 6.3.10B**), indicating an apoptotic activity.

The identified chemical constituents of the ethanol extract (**Figures 6.3.11**) has been reported for their antioxidative activities, particularly the phytol derivatives (Santos et al. 2013) and plant steroids (El-Kashef et al. 2015; Rao et al. 2013). The antidiabetic properties of sitosterol has also been documented (Karan et al. 2012). Thus, the antioxidative and antidiabetic activities of the extract can be attributed to the synergistic effect of the identified chemical compounds.

The increasing interest in the use of medicinal plants over synthesized drugs have led to concerns about its toxicity and safety (Ezuruike and Prieto 2014; Yüce et al. 2006). Extracts from different plant parts have been shown to elicit different effects in the body, which could be toxic (Ezuruike and Prieto 2014). Due to ethical questions and animal rights in most developed countries, new computational methods for determining the toxicity of chemical compounds have been emerging. Amongst such is the ProTox server, which predicts the oral toxicity of small molecule compounds based on chemical similarities between compounds with known toxic effects and the presence of toxic fragments (Drwal et al. 2014). The toxicity classes (**Table 6.3.2**) of the identified compounds may indicate the ethanol extract is safe for oral consumption.

6.3.6 Conclusion

The inhibitory activities and exacerbation of antioxidant activities suggest an anti-hyperglycemic and anti – oxidative potential of *C. volubile* flowers, with the ethanol extract showing the best activity. These can be attributed to the synergistic effect of the identified compounds. These findings further ascertain its folkloric use in the treatment and management of T2D and its complications. However, further studies are required on the underlying molecular mechanisms.

6.4 *Clerodendrum volubile* inhibits Key Enzymes linked to Type 2 Diabetes but Induces Cytotoxicity in Human Embryonic Kidney (HEK-293) Cells via Exacerbated Oxidative Stress and Proinflammation

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Preface: This article investigated the antioxidant, antidiabetic, and cytotoxic properties of the dichloromethane (DCM) fraction of C. voluble leaves in vitro, as well as the mechanism by which it elicits its cytotoxicity. It has been published in Biomedicine and Pharmacotherapy. (**Erukainure OL**, Narainpersad N, Singh M, Olakunle S, Islam MS. (2018). Clerodendrum volubile inhibits key enzymes linked to type 2 diabetes but induces cytotoxicity in human embryonic kidney (HEK293) cells via exacerbated oxidative stress and proinflammation. Biomed Pharmacother., 106: 1144–1152).

6.4.1 Abstract

The toxicity and safety associated with the use of medicinal plants remains a major concern. In this study, the antidiabetic properties of the dichloromethane (DCM) fraction of *C. volubile* leaves were investigated *in vitro*. Its cytotoxic effect and mechanism of toxicity were also investigated in Human Embryonic Kidney (HEK-293) cells. The fraction was subjected to *in vitro* antioxidant assays using the 2,2'-diphenyl-1-picrylhydrazyl (DPPH) scavenging and Ferric reducing antioxidant power (FRAP) protocols. Its enzyme-inhibitory properties were investigated on α-glucosidase and α-amylase activities. Gas Chromatography Mass Spectroscopy (GC-MS) and Fourier Transform Infrared (FTIR) spectroscopic analysis were used to identify its phytoconstituents. Cytotoxicity was determined via MTT assay. The treated cells were assayed for reduced glutathione (GSH), non-protein thiol, nitric oxide and malondialdehyde (MDA) levels, as well as Superoxide Dismutase (SOD), catalase, myeloperoxidase and ATPase activities. Cell

apoptosis and/or morphological changes were determined using the acridine orange and ethidium bromide (AO/EB) dual staining method. The fraction showed significant ($p<0.05$) antioxidant and enzyme-inhibitory activity. It showed significant ($p<0.05$) cytotoxic effect against HEK-293 cells with concomitant depletion of antioxidative and elevation of proinflammatory biomarkers. Morphological changes were examined in the cells with an apoptotic index of 0.84. 1,1-Dodecanediol, diacetate was identified as the most predominant compound, while aromatics and amines as the most functional groups present in the fraction. These results suggest the antidiabetic and cytotoxic effects of *C. volubile* leaves. The toxicity can be attributed to induced oxidative stress and proinflammation with concomitant depletion of ATP leading to apoptosis of the cells.

Keywords: Apoptosis; Cytotoxicity; Oxidative Stress; Proinflammation; and Type 2 Diabetes

6.4.2 Introduction

Type 2 diabetes accounts for more than 90% of all diabetes, making it the most prevalent and a major contributor to diabetes-related mortality and morbidity (Deshpande et al. 2008; IDF 2015). Unlike type 1 diabetes, it arises owing to inability of the body to utilize the insulin produced and/or insufficient production of insulin (Deshpande et al., 2008). It is characterized by pancreatic β-cell dysfunction and insulin resistance, leading to hyperglycemia (Cerf 2013; Kahn 2003). Chronic hyperglycemia gives rise to increased production of free radicals, which when surpasses the endogenous antioxidant system induces oxidative stress (Tiwari et al. 2013). Oxidative stress has been implicated in the pathogenic micro- and macro-vascular complications associated with type 2 diabetes (Lipinski 2001; Pi et al. 2010; Saeed et al. 2012). Mitigation of oxidative stress and inhibition of carbohydrate and lipid metabolizing enzymes particularly the alpha – glucosidase, alpha – amylase and pancreatic lipase have been demonstrated to be therapeutic in the management of type 2 diabetes and its complications (Ademiluyi and Oboh 2013; Oboh et al. 2017b).

There are concerns about the toxicity and safety of medicinal plants as they consist of a myriad of phytochemicals which could elicit herbal – drug, herbal – food or herbal – herbal interactions (Ezuruike and Prieto 2014; Yüce et al. 2006). Issues of standardization, characterization, and preparation remain a question to most health practitioners (Shan et al. 2007). Methods of extraction have also been reported to elicit different biological actions in the body, some of which may be toxic owing to the type of phytochemicals extracted (Ezuruike and Prieto 2014; Liu 2004). Thus,

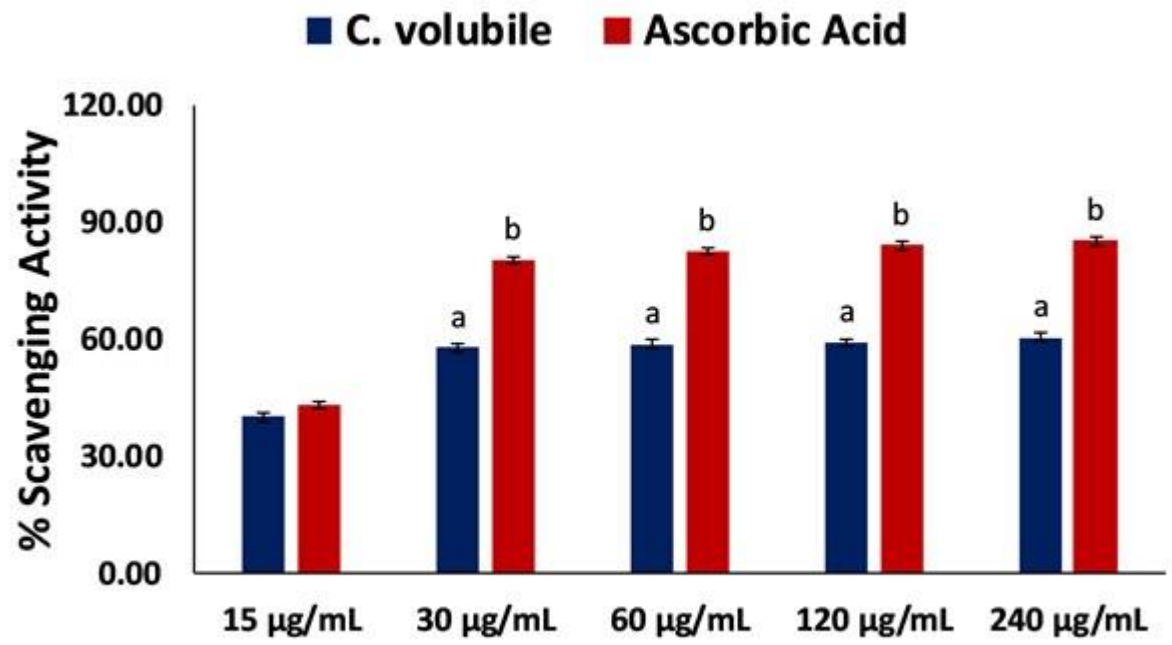
this study aims to report the ability of *C. volubile* to inhibit key enzymes linked to type 2 diabetes vis-à-vis alpha-glucosidase, alpha-amylase and pancreatic lipase as well as its cytotoxic effect and mechanism of toxicity on Human Embryonic Kidney (HEK-293) cells.

6.4.3 Materials and Methods

Kindly refer to Chapter 2, subsections: 2.1.1, 2.1.3, 2.1.5, 2.1.6, 2.2 – 2.4, 2.6, 2.9.3 – 2.9.4, 2.10.1, 2.10.4, 2.11.1 and 2.13

6.4.4 Results

The DCM fraction of *C. volubile* leaves showed significant ($p<0.05$) scavenging activity against DPPH radical with an IC_{50} value of 25.51 µg/mL as depicted in **Figure 6.4.1A** and **Table 6.4.1**. This is further portrayed by its significant ($p<0.05$) FRAP activity with an IC_{50} value of 144.67 µg/mL (**Figure 6.4.1B** and **Table 6.4.1**). These activities were dose – dependent and concomitant with increasing concentration.



(A)

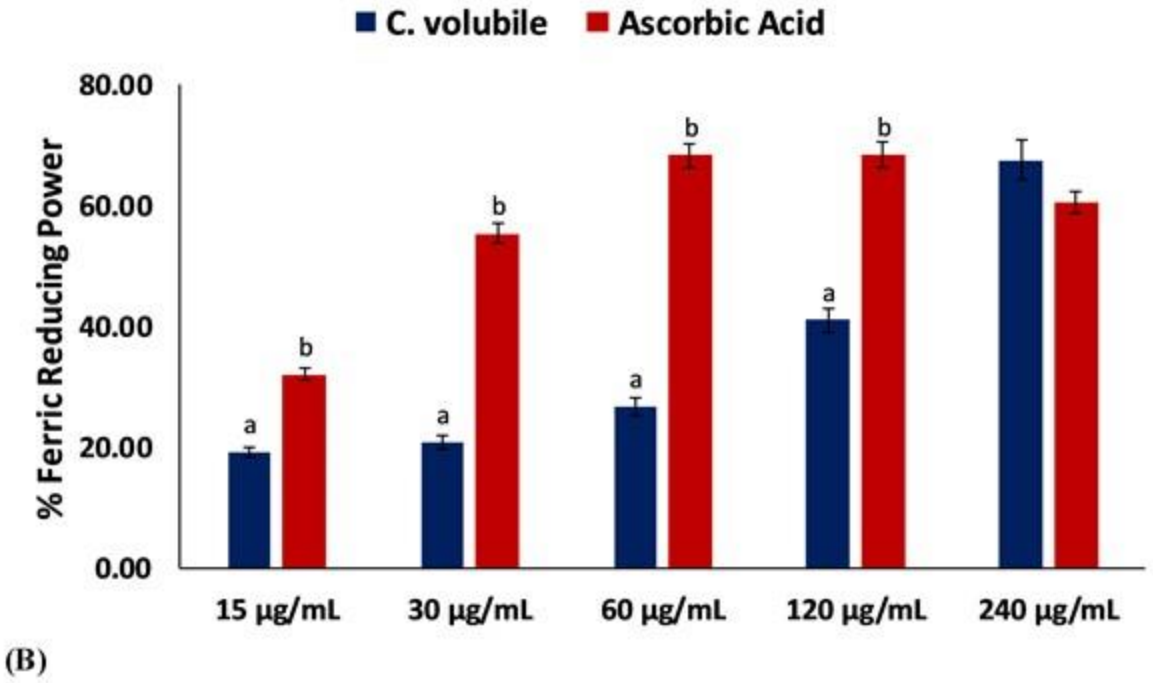


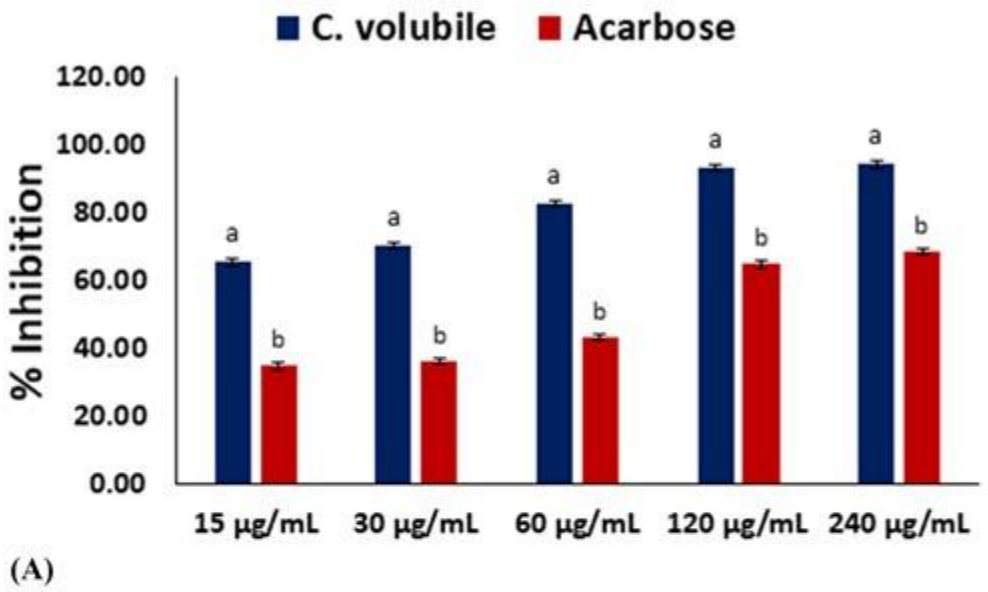
Figure 6.4.1: (A) DPPH scavenging and (B) FRAP activities of *C. voluble* DCM fraction. Data = mean ± SD; n = 3. [ab]Values with different letter above the bars for a given concentration are significantly different from each other

Table 6.4. 1: IC_{50} values of biological activities of *C. volubile*

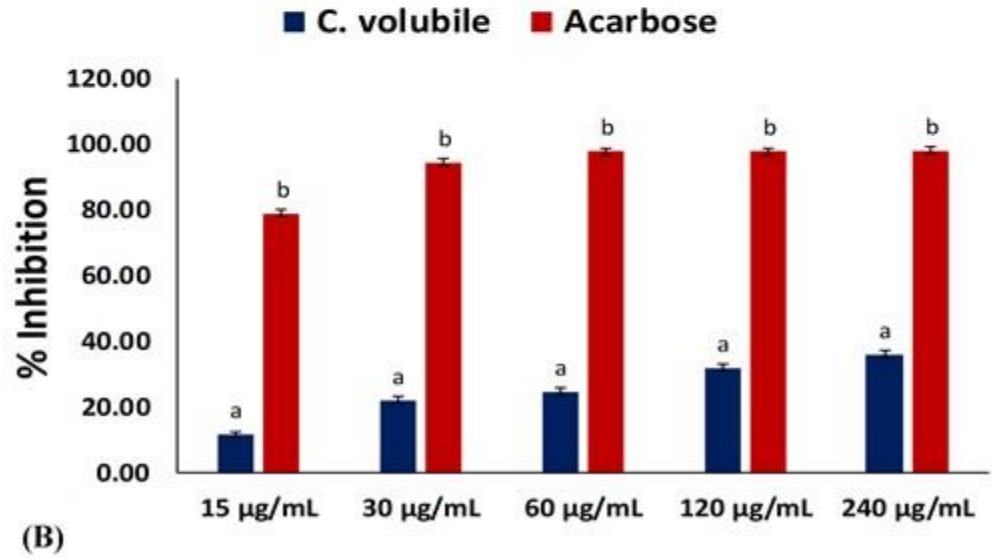
Biological Activities	*C. volubile* (µg/mL)	Ascorbic acid (µg/mL)	Acarbose (µg/mL)	Orlistat (µg/mL)
DPPH	25.51	8.31	–	–
FRAP	144.67	29.98	–	–
Alpha glucosidase	4.14	–	62.42	–
Alpha amylase	1,114.46	–	0.04	–
Lipase	0.02	–	–	0.15
MTT	229.52	–	–	–

Value = mean; n = 3

The fraction showed significant ($p<0.05$) higher inhibitory effect on α-glucosidase activity with an IC_{50} value of 4.14 µg/mL compared to that of the standard drug (62.42 µg/mL) as shown in **Figure 6.4.2A** and **Table 6.4.1**. It also showed a favorable effect on pancreatic lipase activity with an IC_{50} value of 0.02 µg/mL (**Figure 6.4.2C** and **Table 6.4.1**). However, the fraction had little or no inhibitory effect on α-amylase activity as depicted in **Figure 6.4.2B**.



(A)



(B)

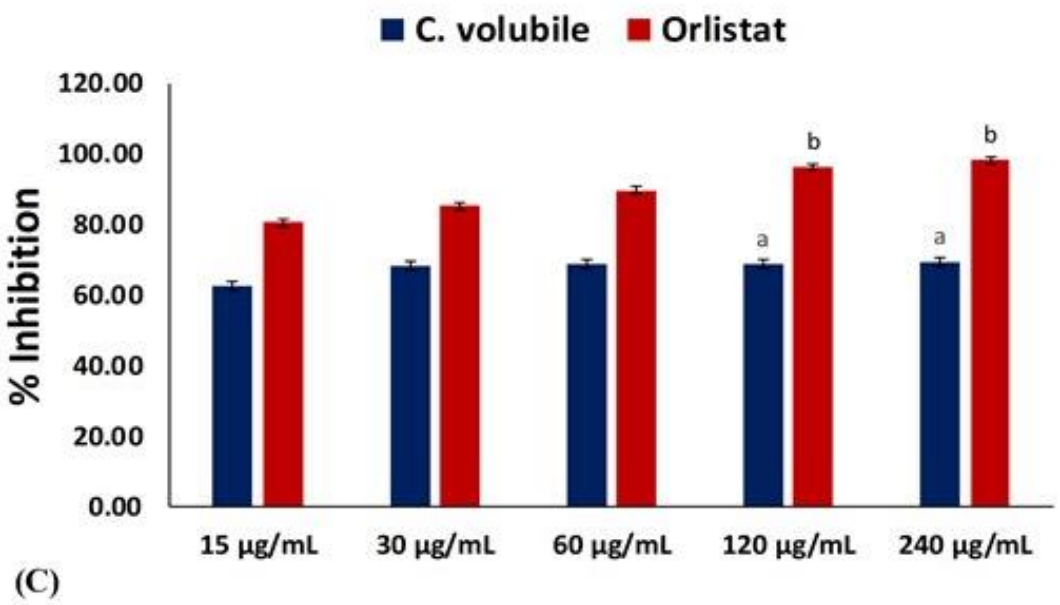


Figure 6.4.2: (A) Alpha glucosidase, (B) alpha amylase and (C) pancreatic lipase inhibitory activities of *C. volubile*. Values = mean ± SD; n = 3. [ab]Values with different letter above the bas for a given extract are significantly ($p < 0.05$) different from each other

As depicted in **Figure 6.4.3**, treatment of HEK-293 cells with the fraction caused a dose dependent inhibition of cell viability with an IC_{50} value of 229.52 µg/mL (**Table 6.4.1**), indicating a cytotoxic effect.

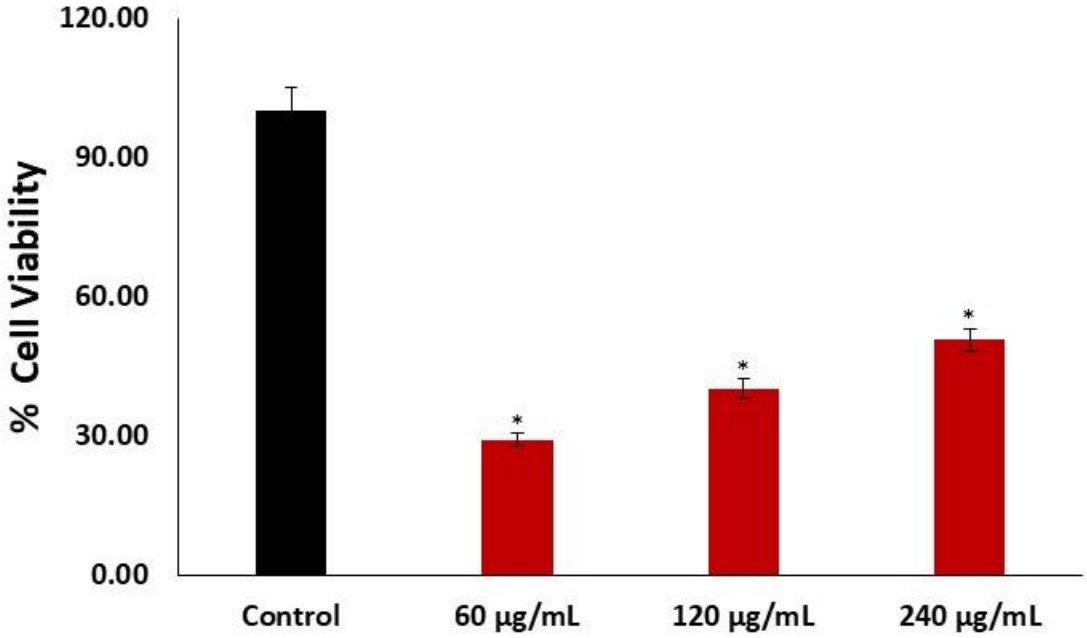
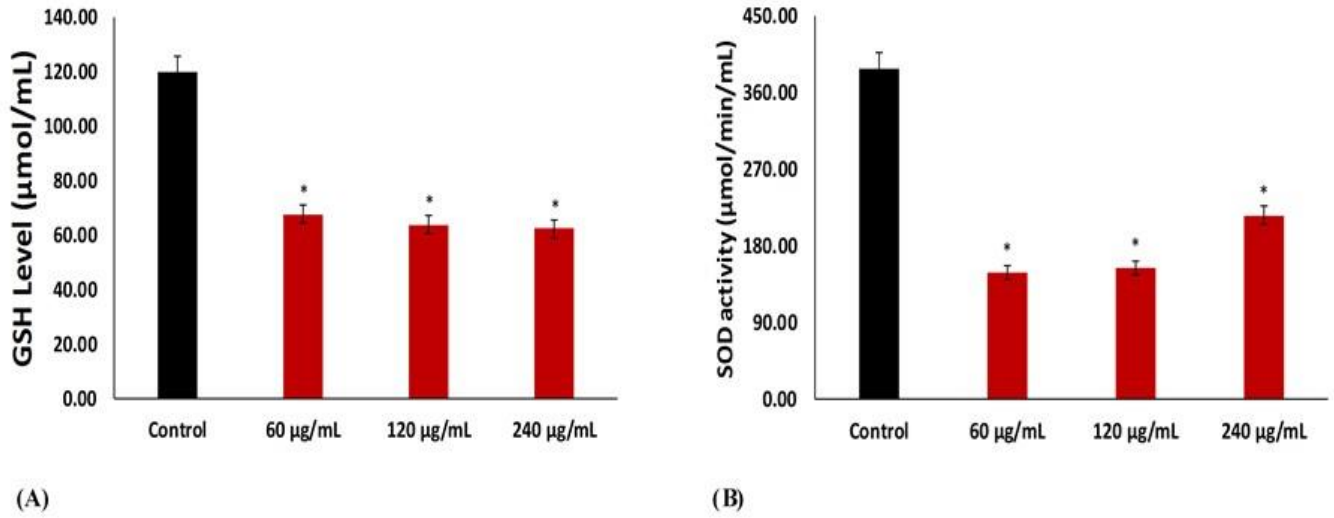


Figure 6.4.3: Effect of C. volubile on cell viability of HEK-293 cell lines. Values = mean ± SD; n = 3. *Significantly ($p < 0.05$) different from control.

Treatment with the fraction led to significant ($p<0.05$) depletion of GSH and MDA levels in the cells, with concomitant reduction in SOD and catalase activities as shown in **Figures 6.4.4A – 6.4.4D**.



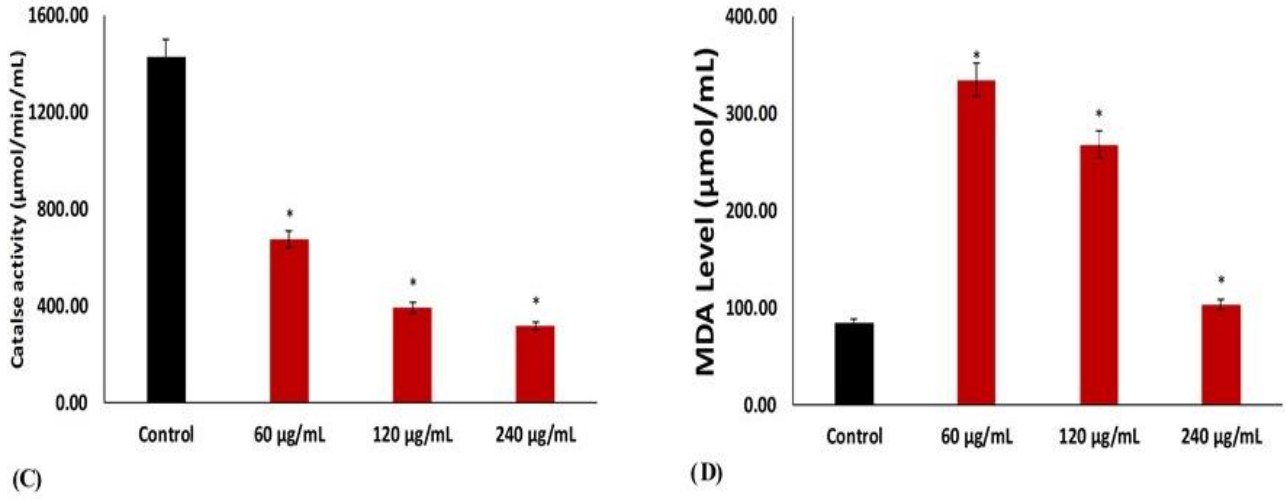
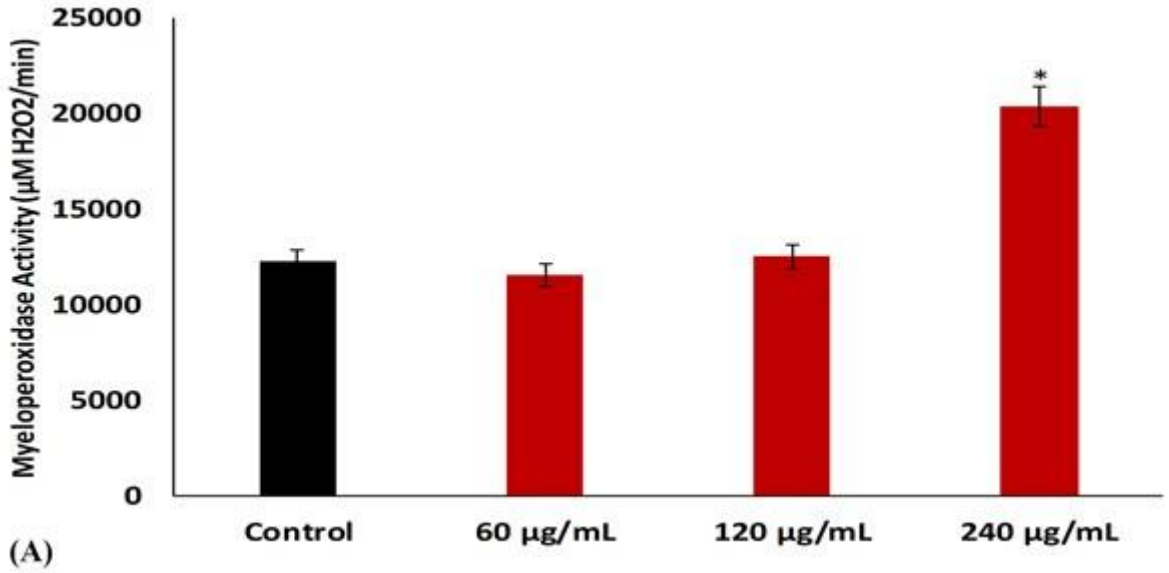
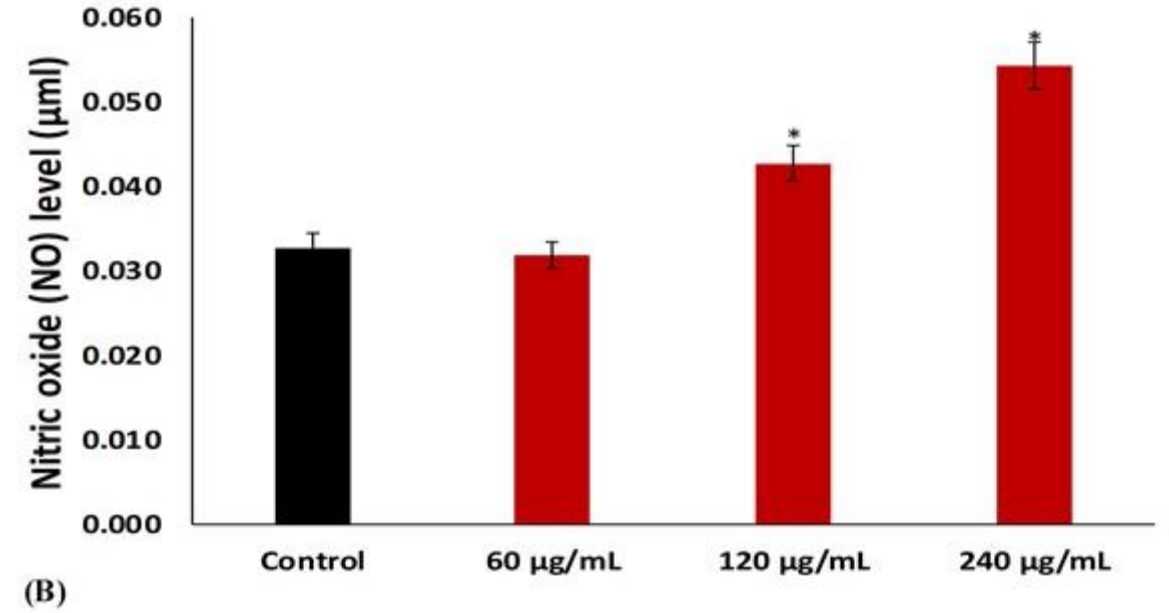


Figure 6.4.4: Effect of *C. volubile* on (A) GSH level, (B) SOD activity, (C) catalase activity and (D) MDA level in HEK-293 cell lines. Values = mean ± SD; n = 3. *Significantly ($p < 0.05$) different from control.

Myeloperoxidase activity and NO level were significantly ($p<0.05$) higher at the highest treatment concentration (240 µg/mL) as depicted in **Figures 6.4.5A and 6.4.5B**. The NPT levels were however significantly ($p<0.05$) depleted in all treatments as shown in **Figure 6.4.5C**.





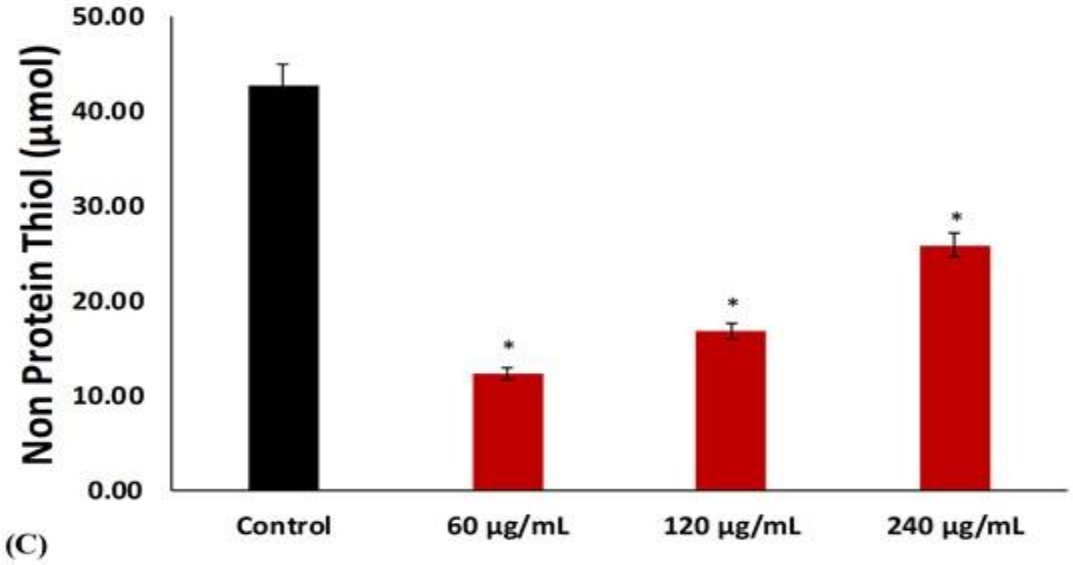


Figure 6.4.5: Effect of *C. volubile* on (A) myeloperoxidase activity, (B) nitric oxide and (C) non protein thiol levels in HEK-293 cell lines. Values = mean ± SD; n = 3. *Significantly ($p < 0.05$) different from control.

Treatment with the fraction led to significant ($p<0.05$) depletion of ATPase activity in the cells as depicted in **Figure 6.4.6**.

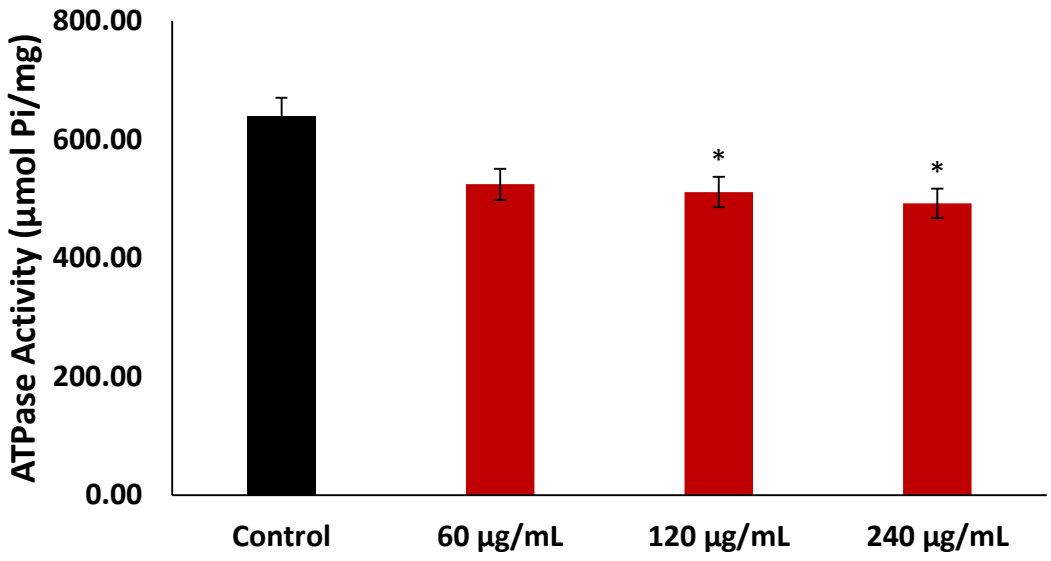
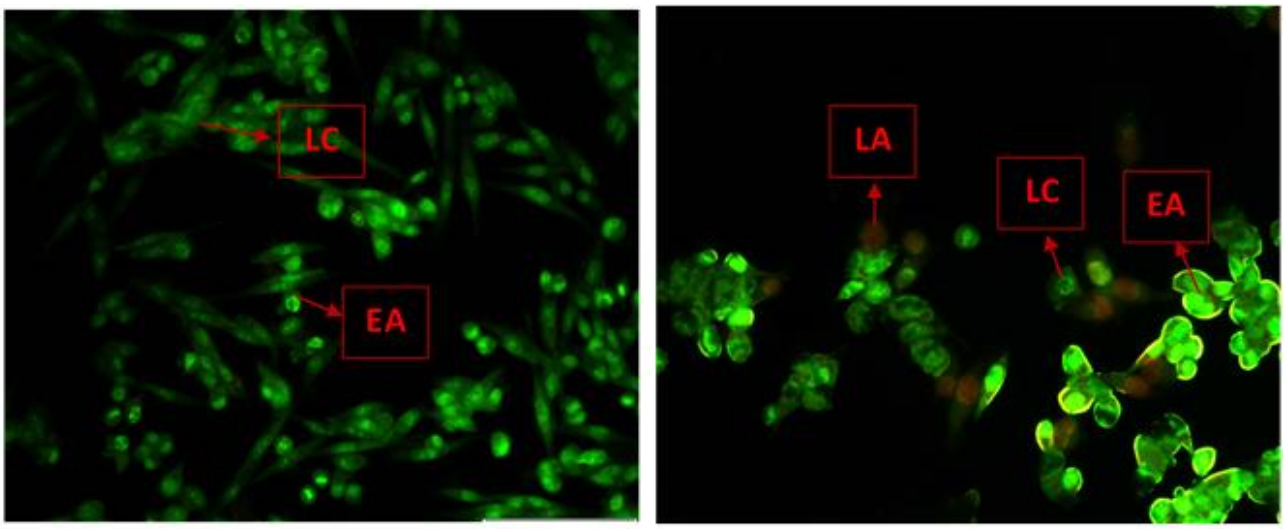


Figure 6.4.6: Effect of *C. volubile* on ATPase activity in HEK-293 cell lines. Values = mean ± SD; n = 3. *Significantly ($p < 0.05$) different from control.

There were morphological changes in the fraction – treated cells with an apoptotic index of 0.84 as depicted in **Figure 6.4.7A** and **6.4.7B**. Live, early apoptotic, and late apoptotic cells were portrayed by green, bright green/yellow, and red/orange colours respectively. These corresponded to the observed condensed chromatin, fragmented nucleus and shrink cells compared to the round homogenous nuclei in the control (untreated cells). There were little or no morphological changes in the untreated tissue.



(A) Control **(B) *C. volubile***

Figure 6.4.7A: Fluorescent micrographs of dual acridine orange/ethidium bromide stained cells showing *C. volubile* induced morphological changes in HEK-293 cell lines. Magnification = 200 µM. **Keys:** LC = Life Cells; EA = Early Apoptosis; LA = Late Apoptosis

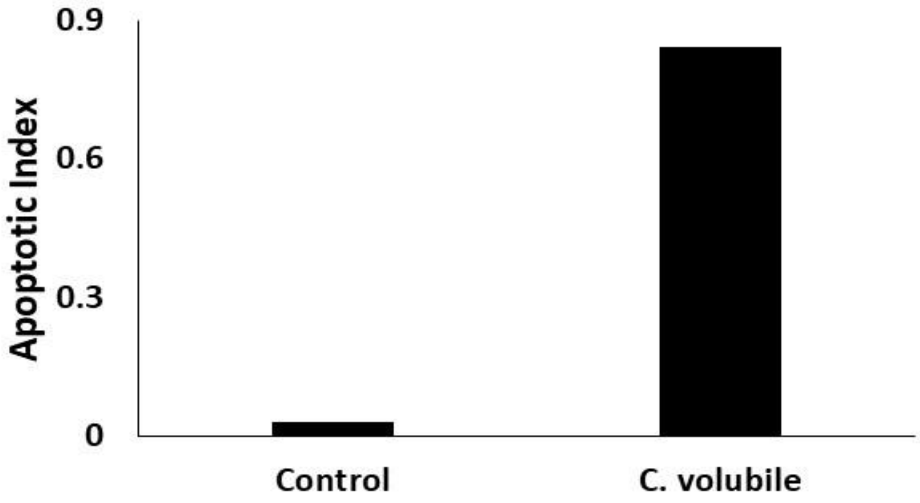


Figure 6.4.7B: Apoptotic indices of dual acridine orange/ethidium bromide stained HEK-293 cell lines

GC-MS analysis of the fraction revealed 1,1-Dodecanediol, diacetate as the most predominant compound as depicted in **Figure 6.4.8** and **Table 6.4.2**. Other compounds identified are

hexadecanoic acid; oxalic acid, allyl pentadecyl ester; nitric acid, decyl ester; 4,6-O-Furylidene-d-glucopyranose; and 2,3,4-trimethyl-1-pentanol.

Table 6.4.2: GC-MS identified compounds in *C. volubile*

Retention Time (Min)	**Compounds**	**Relative Abundance**
30.5	Hexadecanoic acid	13.42%
33.6	Oxalic acid, allyl pentadecyl ester	13.32%
34.8	1,1-Dodecanediol, diacetate	33.15%
36.5	Nitric acid, decyl ester	23.37%
40.0	4,6-O-Furylidene-d-glucopyranose	3.48%
54.5	2,3,4-trimethyl-1-pentanol	13.26%

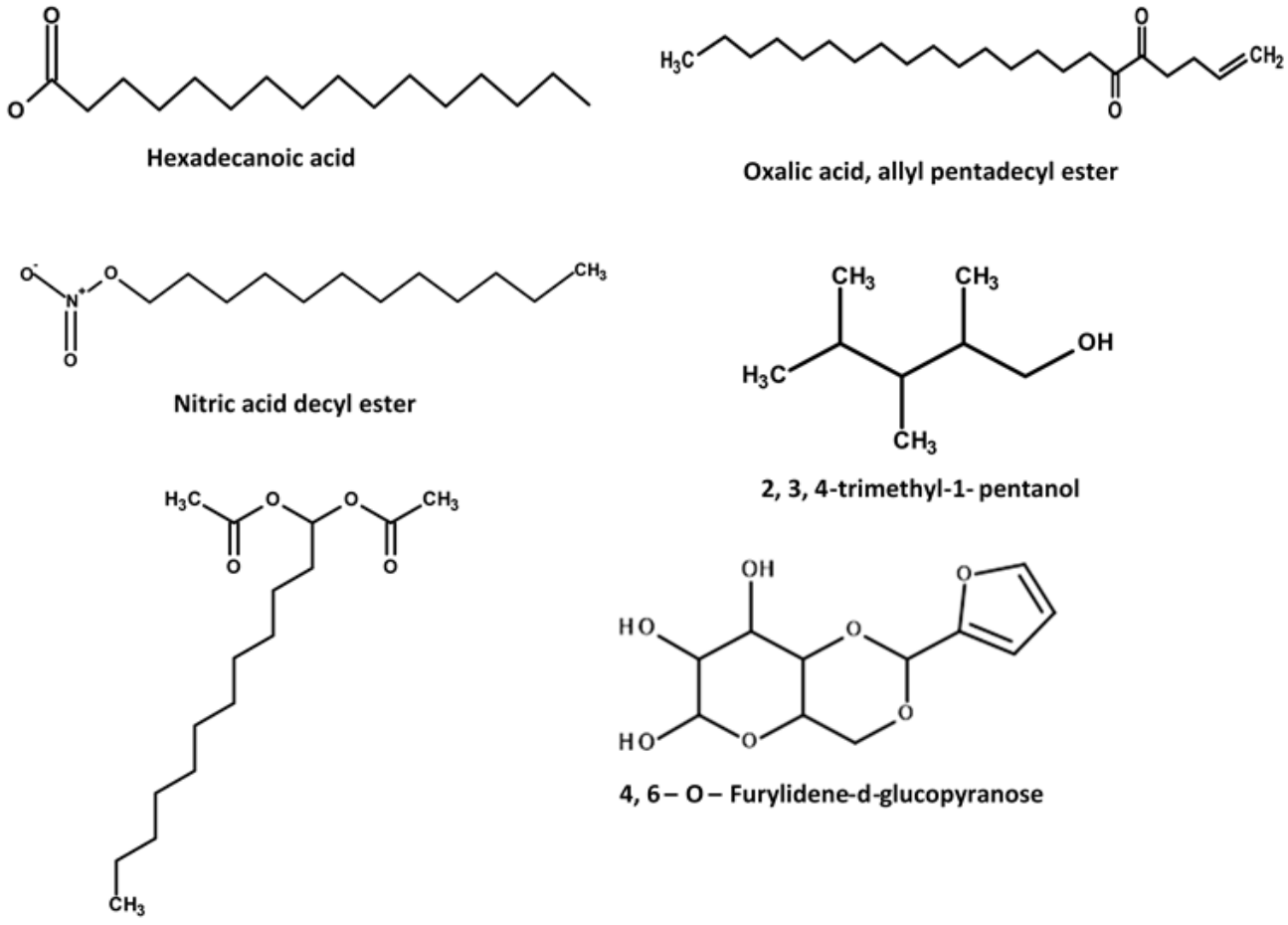


Figure 6.4.8: GC-MS identified compounds in *C. volubile*

FTIR analysis revealed aromatics and amines as the most functional groups present in the fraction as shown in **Figure 6.4.9** and **Table 6.4.3**. Other functional groups identified are alkyl halides, alkenes, aldehydes, ketones, alkanes, and amides.

Table 6.4.3: Quantitative analysis of FTIR spectroscopy of *C. volubile*

Frequency (cm^{-1})	**Functional Group**	**Chemical Bonds**
788.43	Alkyl halides	C–Cl stretch
834.73	Aromatics	C–H "oop"
895.37	1°, 2° amines	N–H wag
912.58	Carboxylic acids	O–H bend
968.19	Alkenes	=C–H bend
1035.15	Aliphatic amines	C–N stretch
1080.78	Alcohols, carboxylic acids, esters, ethers	C–O stretch
1121.92	Aliphatic amines	C–N stretch
1161.62	Alkyl halides	C–H wag ($–CH_2X$)
1223.19	Aromatic amines	C–N stretch
1300.09	Nitro compounds	N–O symmetric stretch
1400.31	Aromatics	C–C stretch (in–ring)
1455.17	Alkanes	C–H bend
1498.37	Nitro compounds	N–O asymmetric stretch
1619.61	1° amines	N–H bend
1699.04	α,β–unsaturated aldehydes, ketones	C=O stretch
1735.09	Aldehydes, saturated aliphatic	C=O stretch
2850.79	Alkanes	C–H stretch
2919.58	Alkanes	C–H stretch
3394.29	1°, 2° amines, amides	N–H stretch

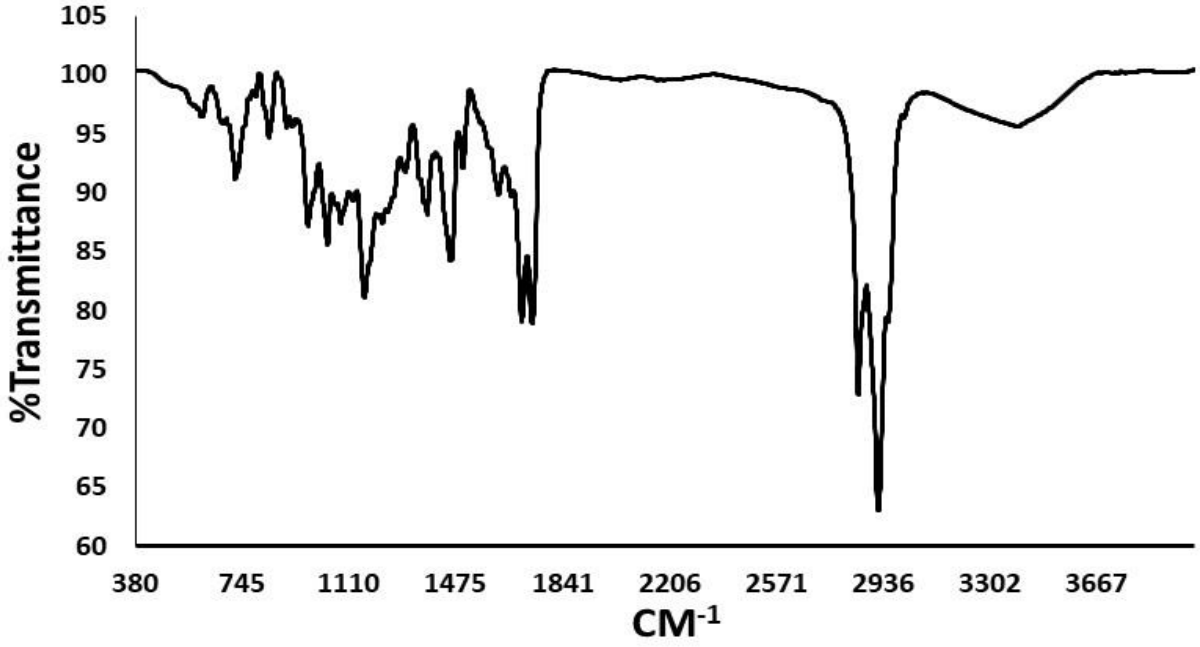


Figure 6.4.9: FTIR spectra of *C. volubile*

Hexadecanoic acid; oxalic acid, allyl pentadecyl ester; 1,1-Dodecanediol, diacetate and nitric acid, decyl ester were predicted to as CYP3A4 substrates, while Hexadecanoic acid and oxalic acid, allyl pentadecyl ester were predicted as inhibitors of CYP2A1 and CYP2C9 as depicted in **Table 6.4.4**. 4,6-O-Furylidene-d-glucopyranose had the highest maximum human tolerated dose with a value of 22.18 mg/kg/day, while hexadecanoic acid had the least (0.19 mg/kg/day). Hexadecanoic acid; and 2,3,4-trimethyl-1-pentanol were predicted to have the least oral LD50 values for 130 and 200 mg/kg respectively. This was followed by nitric acid, decyl ester with a value of 505 mg/kg. While 4,6-O-Furylidene-d-glucopyranose was the highest (4000 mg/kg). The toxicity class for hexadecanoic acid; and 2,3,4-trimethyl-1-pentanol was predicted to be class 3; nitric acid, decyl ester as class 4; while the others were class 5.

Table 6.4.4: Predicted metabolism and tolerable dose of GC-MS identified compounds in *C. volubile*

Compounds	**GI Absorption**	**CYP Substrate**	**CYP Inhibition**	**Max. tolerated dose (Human) (mg/kg/day)**	**Predicted LD_{50} (mg/kg)**	**Predicted Toxicity Class**
Hexadecanoic acid	High	CYP3A4	CYP1A2, CYP2C9	0.19	130	3
Oxalic acid, allyl pentadecyl ester	High	CYP3A4	CYP2C9	4.14	3300	5
1,1-Dodecanediol, diacetate	High	CYP3A4	–	3.18	3000	5
Nitric acid, decyl ester	High	CYP3A4	–	1.88	505	4
4,6-O-Furylidene-d-glucopyranose	High	–	–	22.18	4000	5
2,3,4-trimethyl-1-pentanol	High	–	–	6.33	200	3

6.4.5 Discussion

The cost of treating diabetes and its complications remain a huge bane in sub-Saharan Africa, where the burden of other non-communicable diseases such as HIV, hypertension, cardiovascular diseases and stroke as well as poor health infrastructures is a drain on the poor economies of her countries (Beran and Yudkin 2006). This has led to the search of alternative therapies from natural products, with plants being the major source (Chikezie et al. 2015; Farzaei et al. 2017; Mohammed et al. 2014). Though natural, there are increasing concerns about toxicity and safety of medicinal plants. In this study, the antidiabetic properties of the DCM fraction of *C. volubile* leaves methanolic extract and its cytotoxic effect on HEK-293 cells were investigated.

The role of antioxidants in the management of type 2 diabetes and its complication is well documented (Bajaj and Khan 2012; Maritim et al. 2003). Oxidative stress plays an influential role in the pathogenesis of type 2 diabetes and its complication. This has been attributed to excessive levels of free radicals arising from oxidation of glucose to an enediol radical anion, which is converted into reactive ketoaldehydes and superoxide anion radicals (Maritim et al. 2003). Antioxidants protects against these free radicals by scavenging them, thus preventing them from attacking the cell proteins and membrane lipids (Rahimi et al. 2005). The free radical scavenging and FRAP activities (**Figure 6.4.1**) of the fraction therefore portrays an antioxidative protective potential against hyperglycemia–induced oxidative stress. These activities can be attributed to the identified functional groups particularly the N–H, C–H and =C–H bends, N–H, C–N and C=O stretches (**Figure 6.4.9** and **Table 6.4.3**). Their electron – deficient moiety designates them as potent antioxidants owing to their electron-withdrawing ability (Harrold, 2013). The presence of ether and esters also portrays an antioxidant activity, as their oxygen atom are potent hydrogen acceptor (Berg and Tymoczko 2002; Harrold 2013).

Some antidiabetic drugs function by inhibiting key enzymes linked to type 2 diabetes. These enzymes play a major role in the digestion of dietary carbohydrate and lipids to glucose and fatty acids respectively (Oboh et al. 2017b). The inhibitory effect by the fraction on α-glucosidase and pancreatic lipase activities (**Figures 6.4.2**A and **6.4.2C**) portrays an antidiabetic and anti-obesogenic potential. This corroborates previous reports on the inhibitory effect of the aqueous extract of the leaves on α-glucosidase and α-amylase (Adefegha and Oboh 2016). The inhibition of these enzymes by the fraction indicates its ability to slow down the digestion of dietary

carbohydrate and lipids, thus limiting blood glucose and fatty acids level. These activities can be attributed to the identified secondary metabolites (**Figure 6.4.8** and **Table 6.4.2**), particularly hexadecanoic acid which has been reported as constituent of some plant extracts with antidiabetic properties (Ezuruike and Prieto 2014; Li et al. 2012)

The cytotoxic effect of the fraction on HEK-293 cells as depicted by its inhibitory activity on cell viability (**Figure 6.4.3**) further brings to question the safety of medicinal plants. The cytotoxic effect of the fraction has earlier been reported against normal rat (Wistar) liver cell (CC-1) lines (Erukainure et al. 2017b). However, isolated compounds from the fraction had no cytotoxic activity thus suggesting the cytotoxicity may be synergistic effect of the chemical constituents (Erukainure et al. 2017b). Similar effect was also reported for the DCM fraction of the methanolic extract of the flower on CC-1 cell lines (Erukainure et al. 2016a). Dietary fatty acids from the leaves had little or no cytotoxic effect on CC-1 cell lines (Erukainure et al. 2016b). These effects correspond with reports that solvents play crucial roles in the toxicity and safety of medicinal plants (Ezuruike and Prieto 2014). The cytotoxic activity of the fraction may be attributed to the identified phytoconstituents (**Figure 6.4.8** and **Table 2**), particularly hexadecanoic acid and Oxalic acid, allyl pentadecyl ester with reported cytotoxic activities (Manilal et al. 2011; Moravcova et al. 2015; Ravi and Krishnan 2017).

Oxidative stress and proinflammation are well-known toxicity mechanisms in numerous cells often associated with the pathogenesis and complications of most diseases (Kim et al. 2010; McKim et al. 2016; Suliman et al. 2015). Adverse oxidative stress has been implicated in cytotoxicity and employed as distinct therapeutic mechanism in the treatment of certain diseases like cancer (Carrasco-Torres et al. 2017; Han and Chen 2012). Oxidative stress occurs when there is a depletion in the body's antioxidant system, favoring increased production of free radicals. The depleted GSH level, SOD and catalase activities with concomitant increased MDA level (**Figures 6.4.4A – 6.4.4B**) in cells treated with the fraction indicates an occurrence of oxidative stress. Increased oxidative stress in normal cells portrays a toxic effect as it has been linked to cell membrane rupture and increased DNA fragmentation (Aponte and Agarwal 2013; Dutta et al. 2015).

The increased myeloperoxidase activity and NO (**Figure 6.4.5**) level indicate proinflammation. The high NO level and decreased SOD activity portrays an increased concentration of

peroxynitrate due to the availability of $O_2^{\cdot-}$ to react with the excess NO (Jourd'heuil et al. 2001). Likewise, the decreased catalase activity and GSH level portrays increased availability of H_2O_2 for myeloperoxidase to convert to $HOCl^-$ (Furtmüller et al. 2000). Accumulation of these free radicals has been implicated in oxidative injury leading to cytotoxicity (Chen and Sulik 1996; Rankin et al. 2017). The depleted antioxidative (**Figure 6.4.4**) and increased proinflammatory activities (**Figure 6.4.5**) thus corresponds with the cytotoxic effect of the fraction (**Figure 6.4.4**).

Impaired ATPase activity has been implicated in cytotoxicity as depleted ATP level triggers apoptotic processes (Hernández et al. 2017; Wang et al. 2003). The decreased ATPase activity (**Figure 6.4.6**) in the fraction treated cells therefore portrays a depleted ATP level, which may be responsible for apoptosis of the cells (**Figures 6.4.7A** and **6.4.7B**). The high apoptotic index of the treated cells (**Figure 6.4.7B**) can also be attributed to the excess reactive oxygen species (ROS) production owing to the depleted antioxidative activities and increased lipid peroxidation (**Figure 6.4.3**). ROS have been shown to induce mitochondrial Ca^{2+} cycling which leads to loss of membrane potential and ATP depletion (Nikoletopoulou et al. 2013; Richter et al. 1996). In addition, both NO and $ONOO^-$ have been implicated in induction of apoptosis. While the former induces apoptosis via expression of the tumor suppression gene p53, the latter induces by triggering mitochondrial Ca^{2+} (Meßmer et al. 1994; Richter et al. 1996). Thus, the morphological changes in the cells indicating apoptosis can be attributted to the depleted antioxidative and increased proinflammatory biomarkers.

Induction and inhibition of cytochromes (CYPs) can have severe drug–drug, food – drug and herbal – drug interactions with tremendous health complications. Most of the identified compounds were predicted to be CYP3A4 substrates (**Table 6.4.4**), indicating their potentials to activate the enzyme. CYP3A is the most abundantl CYP in the human hepatic tissue (Li et al. 1995). Mizuno et al. (Mizuno et al. 2009) implicated the hepatic cytotoxic effect of benzodiazepines to metabolic activation by CYP3A4. They attributted the activation to the presence of nitro group in the side chain of benzodiazepines (Mizuno et al. 2009). The presence of amines in the fraction (**Figure 6.4.9** and **Table 6.4.2**) may thus be responsible for its metabolic activation by CYP3A4 (**Table 6.4.4**), which may lead to production of cytotoxic metabolites. Inhibition of CYP1A2 and CYP2C9 by hexadecanoic acid and oxalic acid, allyl pentadecyl ester may pose a toxic effect when the fraction is ingested with drugs metabolized by these enymes

particularly caffeine (Urry et al. 2016). The low maximum tolerated human dose (**Table 6.4.4**) by most of the compounds also portrays a toxic potential, particularly hexadecanoic acid with reported cytotoxic activity (Manilal et al. 2011; Moravcova et al. 2015; Ravi and Krishnan 2017). This also correlates with its predicted low oral LD_{50} and toxicity class (**Table 6.4.4**).

6.4.6 Conclusion

These results suggests further portrays the antidiabetic properties of the leaves of *C. volubile* but also indicates its toxic potential against normal cells. The toxicity can be attributted to the ability of its DCM fraction to induce oxidative stress and proinflammation with conconmittant depletion of ATP level leading to apoptosis of the cells

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CHAPTER 7

THE ANTI-OXIDATIVE AND ANTI-DIABETIC ACTIVITIES OF *COLA NITIDA* (KOLA NUT) WINE *IN VITRO, EX VIVO,* AND *IN VIVO*

7.1 Cola nitida

Figure 7.1.1*:* *Cola nitida.* Common names: Kola nut (English); *Obi* (Yoruba, Nigeria); *Gworo* (Hausa, Nigeria); *Oji* (Igbo, Nigeria); *Colatier* (French)*; kolabaum* (German); and *colatero* (Spanish). Photo: https://www.herbazest.com/herbs/cola-nut (accessed on November 11, 2018 without permission).

7.1.2 Background

Cola nitida from the genus, *Cola* is an evergreen tree which is native to the tropical rain forest of West Africa particularly, Ghana, Nigeria, Ghana, Leone, Liberia and Ivory Coast (Burdock et al. 2009). It often attains a height of 12 to 20 m, with a trunk of 1.5 m in diameter and a thick fibrous bark (Tachie-Obeng and Brown 2004). The leaves are alternate, glabrous, oblong, leathery and touch, with stocks and waxy margins (FAO 1995; Tachie-Obeng and Brown 2004). The flowers grow from the leaf axils in panicles, without petals. The seeds are contained in pods, which splits when ripe (Tachie-Obeng and Brown 2004).

7.1.3 Ethnopharmacological uses

In folkloric medicine, the seeds are used in treating asthma, stomach ulcers, headache, depression, whooping coughs, piles, and rheumatism (Asogwa et al. 2011; Dorathy et al. 2014; Ndagi et al. 2012; Orwa et al. 2009). They are also used as aphrodisiac, appetite suppressants and anti-parasitic (Ezuruike and Prieto 2014).

7.1.4 Biological activities

C. nitida seeds have been reported for their ability to reduce blood sugar level in alloxan – induced diabetic rats (Dorathy et al. 2014). The ability of the aqueous and polyphenolic – rich extracts to scavenge free radicals *in vitro* have also been reported (Ayebe et al. 2012; Oboh et al. 2014b). Its antidiabetic and antioxidant properties have been demonstrated *in vitro* and *in vivo* (Ayebe et al. 2012; Erukainure et al. 2017b; Oboh et al. 2014b). Its consumption with alcohol has been reported to modify neuronal processes (Obochi et al. 2009). Its influence on the etiology of malaria-morbidity have been reported (Alaribe et al. 2003). Its chronic consumption has been shown to have little or no significant effect on locomotor activities (Umoren et al. 2009).

7.1.6 Phytochemistry

C. nitida seeds have been reported to be rich in caffeine, theobromine, kolatine, d-catechin, and L-epicatechin (Lowor et al. 2010).

7.1.7 Aims and objective

The aim of this study was to investigate the antioxidative, antidiabetic, and neuroprotective activities of the hot infusion of C. *nitida*, using *in vitro, ex vivo, in silico* and *in vivo* models. Its modulatory effect on dysregulated metabolic pathways in oxidative hepatic injury was also investigated *ex vivo*.

7.2 Caffeine – Rich Infusion from *Cola nitida* (Kola Nut) inhibits Major Carbohydrate Catabolic Enzymes; Abates Redox Imbalance; and Modulates Oxidative Dysregulated Metabolic Pathways and Metabolites in Fe^{2+} - induced Hepatic Toxicity

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Preface: This article investigated the *in vitro* antioxidant and enzyme inhibitory activities of *C. nitida* hot infusion, as well as its modulatory effect on redox imbalance and dysregulated metabolic pathways in oxidative hepatic pathways *ex vivo*. The phytochemical constituents were also investigated using HPLC and LC-MS. It has already been published in Biomedicine and Pharmacotherapy (**Erukainure OL**, Oyebode OA, Sokhela MK, Koorbanally NA, Islam MS. (2017). Caffeine – Rich Infusion from *Cola nitida* (Kola Nut) inhibits Major Carbohydrate Catabolic Enzymes; Abates Redox Imbalance; and Modulates Oxidative Dysregulated Metabolic Pathways and Metabolites in Fe^{2+} - induced Hepatic Toxicity. Biomedicine and Pharmacotherapy, 96: 1065–1074).

7.2.1 Abstract

The antioxidative, antidiabetic and toxicity of caffeine-rich infusion of *cola nitida* were investigated using *in vitro*, *ex vivo* and *in silico* models. *C. nitida* was infused in boiling water and allowed to cool before concentrating at $\geq 50^{o}$C. HPLC analysis of the infusion revealed a caffeine content of 80.08%. The infusion showed potent *in vitro* antioxidant activity by significantly ($p<0.05$) scavenging 2,2'-diphenyl-1-picrylhydrazyl (DPPH). It significantly ($p<0.05$) inhibited α-

glucosidase and α-amylase activities. Treatment of Fe^{2+} induced oxidative hepatic tissues with the infusion led to increased Superoxide Dismutase (SOD) and catalase activities, and glutathione (GSH) level as well as decreased malondialdehyde (MDA) level. FTIR spectroscopy of hepatic metabolite revealed restoration of oxidative–induced depleted functional groups by the infusion. LC-MS analysis of the metabolite also revealed restoration of most depleted metabolites with concomitant generation of 4-O-Methylgallic, (-)-Epicatechin sulfate, L-Arginine, L-yrosine, Citric acid and Decanoic acid in infusion-treated tissues. Pathway analysis of the identified metabolites revealed the presence of 21 metabolic pathways involved in normal hepatic tissues, 12 in oxidative injured tissues and 17 in the treated tissues. Treatment with the infusion restored 4 metabolic pathways common to the normal tissue and further activated 4 additional pathways. Prediction of oral toxicity of caffeine showed it to belong to class 3, with a LD_{50} of 127 mg/kg. Its toxicity target was predicted as Adenosine Receptor A2a. It was also predicted to be an inhibitor of CYP1A2. These results suggest the antioxidative and antidiabetic properties of *C. nitida* infusion, with caffeine as the major constituent.

Keywords: Antioxidative; Kola nuts; Metabolomics; Type 2 diabetes

7.2.2 Introduction

Diabetes mellitus (DM) remains one of the fast growing and unabated scourge in the world. It is a metabolic disorder affecting carbohydrate, protein and lipid metabolism (Maritim et al. 2003a). It is characterized by dearth of insulin secretion as seen in type 1 diabetes or inability to utilize insulin secreted in the case of type 2 diabetes. Of these two types, type 2 diabetes has been recognized as a major contributor to global mortality and morbidity (IDF 2015). It accounted for 12% of global health expenditure and five million deaths in 2015 (IDF 2015). Insulin resistance and pancreatic β-cell dysfunction leading to hyperglycemia have been implicated in the progression of type 2 diabetes (Evans et al. 2003). Increased hyperglycemia elevates the production of reactive oxygen species (ROS) which triggers an imbalance in the endogenous antioxidant system resulting to oxidative stress (Tiwari et al. 2013). Oxidative stress plays an influential role in the chronic pathogenic micro- and macro-vascular complications associated with type 2 diabetes such as nephropathy, microangiopathy, retinopathy and death (Barar 2000; Constantino et al. 2013).

The economic cost of diabetes remains a huge burden to most developing countries with low Gross Domestic Product (GDP). The cost of synthesized drugs coupled to its side effects have led to a paradigm shift to medicinal plants (Erukainure et al. 2015). Their folkloric use dates back time immemorial and can be attributed to their phytochemical constituents (Erukainure et al. 2016). Amongst such plants is the kola nut fruit, *Cola nitida*.

This study aims to report the antioxidative effect of *C. nitida* infusion on oxidative imbalance and metabolites in hepatic injury; its inhibitory effect on key carbohydrate digestive enzymes linked to T2D; as well as phytochemical constituents.

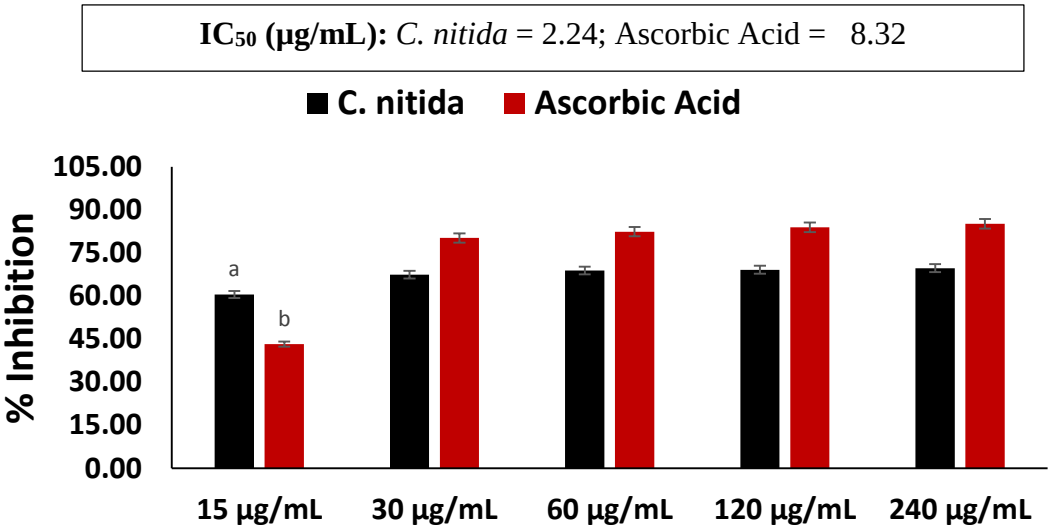
7.2.3 Materials and Methods

Kindly refer to chapter 2, subsections: 2.1.1, 2.1.4, 2.1.6, 2.3-2.4, 3.6, 2.8, 2.10.2 – 2.10.4, 2.11, and 2.13 for details.

7.2.4 Results

Total phenol analysis of the infusion revealed a total phenolic content of 23.30 ± 0.01 µg/g Gallic acid equivalent.

C. nitida infusion showed significant ($p<0.05$) dose – dependent DPPH scavenging and FRAP activities as depicted in **Figures 7.2.1A** and **7.2.1B,** respectively. The low IC_{50} value of 2.24 µg/mL portrays a strong scavenging and reducing potential compared to ascorbic acid (8.32 µg/mL).



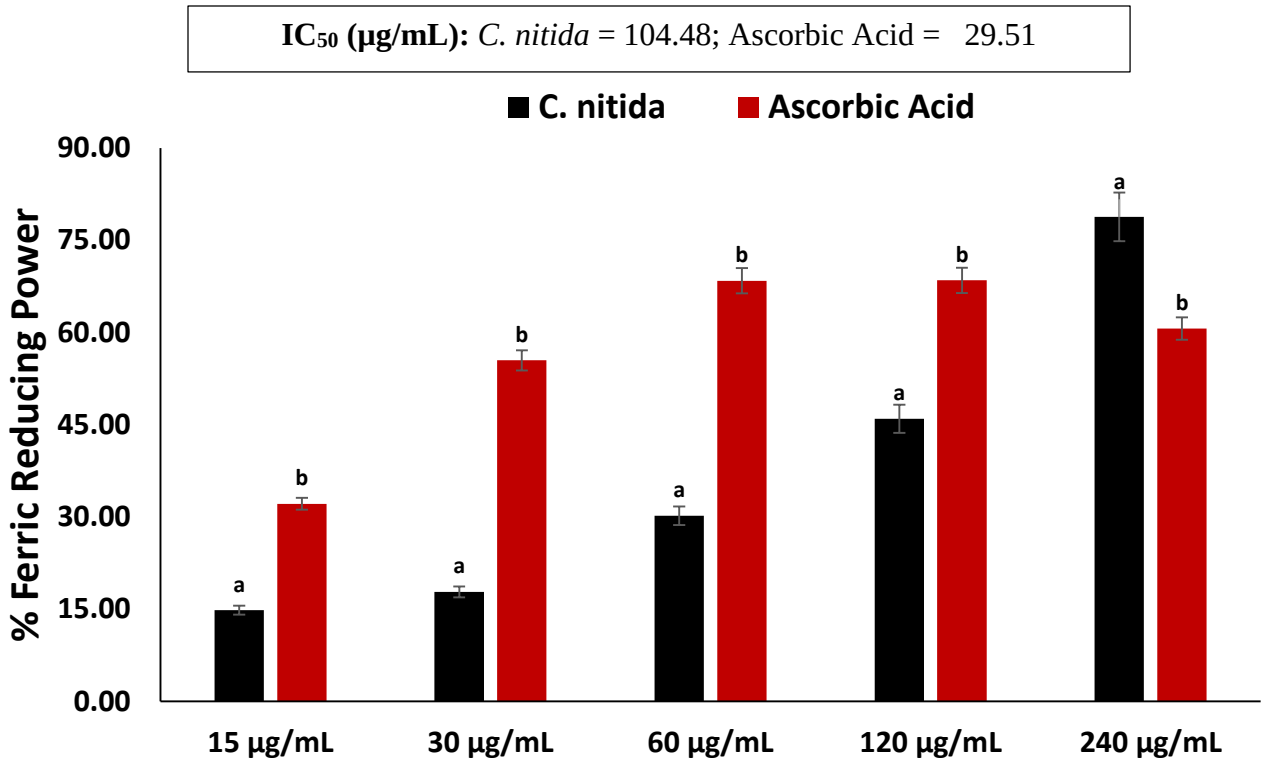
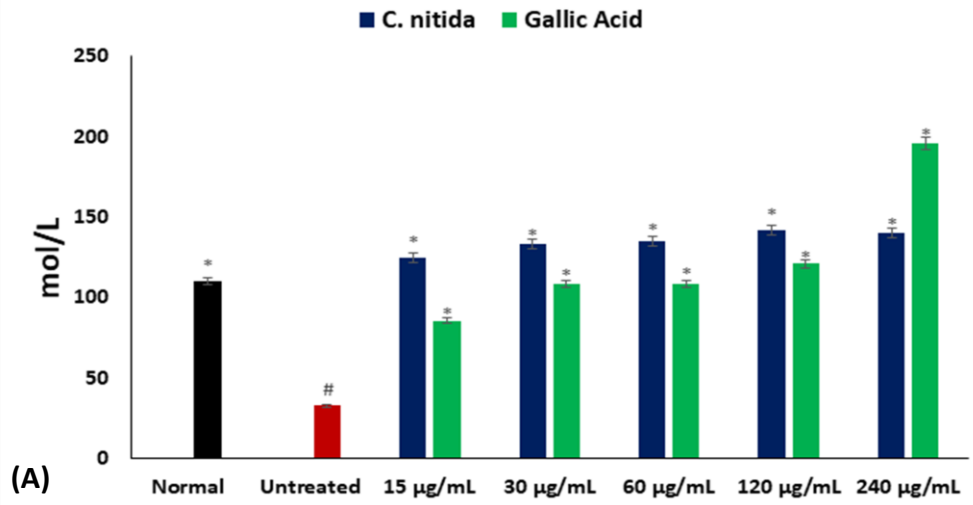


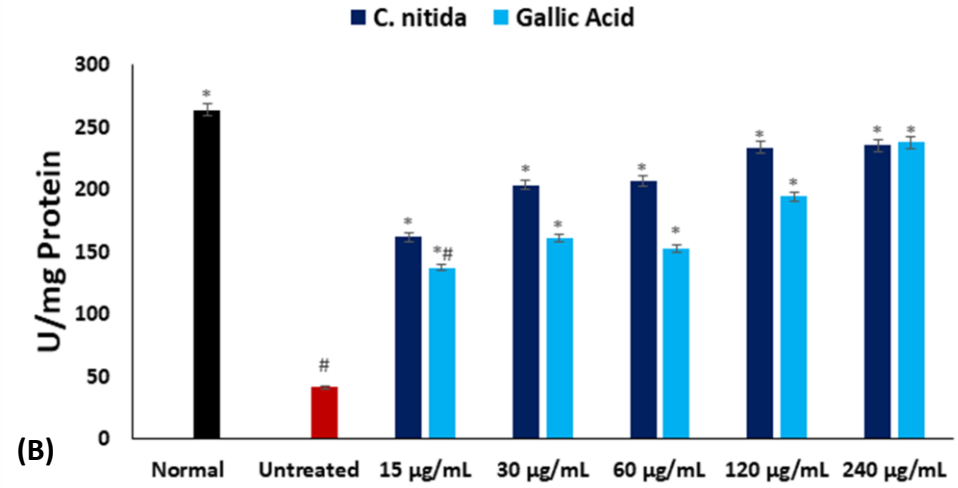
Figure 7.2.1: (A) DPPH scavenging activity; and **(B)** Ferric reducing antioxidant power (FRAP) activity of *C. nitida* infusion. Data are presented as mean ± SD. [ab]Values with different letters over the bars for a given concentration are significantly different from each other ($p < 0.05$, Tukey's HSD-multiple range post-hoc test, IBM SPSS for Windows).

The GSH level was significantly ($p<0.05$) depleted on incubation with $FeSO_4$ indicating an occurrence of oxidative stress as shown in **Figure 7.2.2A**. Incubation with $FeSO_4$ also led to decreased SOD and catalase activities as depicted in **Figures 7.2.2B** and 7.2.2C, further corroborating an occurrence of oxidative imbalance. These were significantly ($p<0.05$) reversed with increasing concentration of the infusion, indicating an antioxidative activity in a dose – dependent manner (**Figures 7.2.2A – 7.2.2C**).

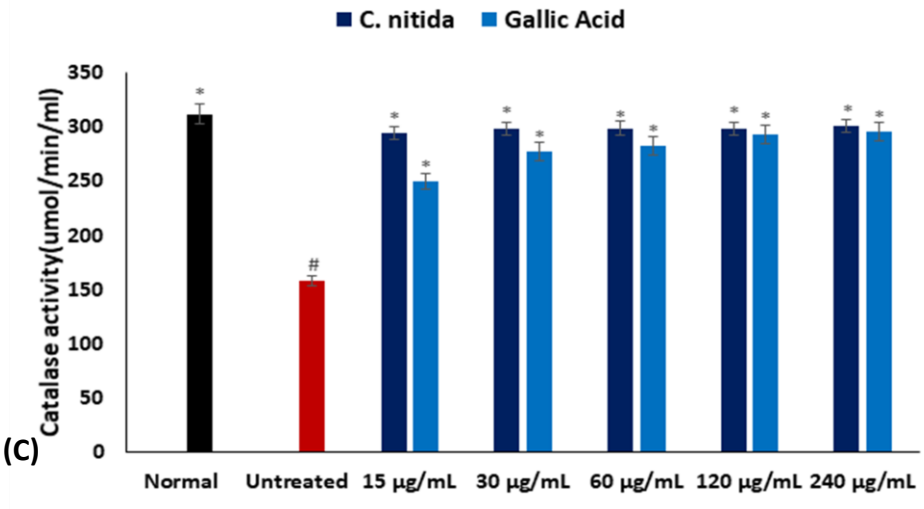
IC_{50} (µg/mL): *C. nitida* = 10.62; Gallic Acid = 17.37



IC_{50} (µg/mL): *C. nitida* = 17.33; Gallic Acid = 54.55



IC_{50} (µg/mL): *C. nitida* = 14.12; Gallic Acid = 13.33



IC_{50} (µg/mL): *C. nitida* = 29.51; Gallic Acid = 17.34

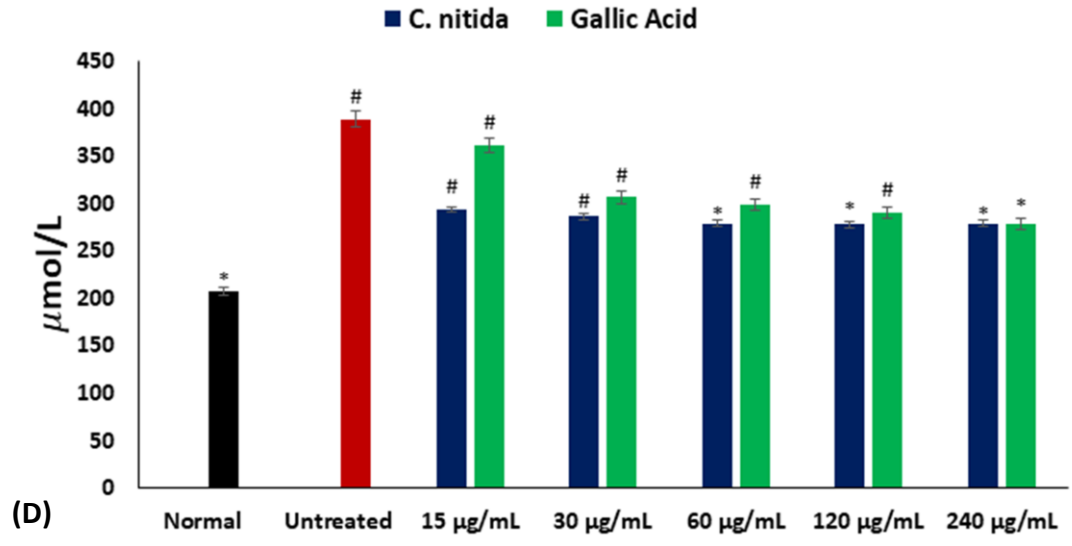


Figure 7.2.2: Effect of *C. nitida* infusion on (A) GSH level; (B) SOD and (C) catalase activities; and (D) MDA level in oxidative hepatic injury. Data are presented as mean ± SD. *Significantly different from untreated sample and #Significantly different from normal sample ($p < 0.05$, Tukey's HSD-multiple range post-hoc test, IBM SPSS for Windows).

Hepatic MDA level was significantly ($p<0.05$) increased on incubation with $FeSO_4$, depicting an occurrence of lipid peroxidation as shown in **Figure 7.2.2D**. Incubation with the infusion led to a dose – dependent reduction, indicating an anti-peroxidative effect ($p<0.05$).

The infusion showed significant ($p<0.05$) inhibitory activity against alpha – glucosidase as depicted in **Figure 7.2.3a**. A similar effect was also observed against pancreatic alpha – amylase (**Figure 7.2.3B**). However, the infusion showed a rather poor inhibitory activity against pancreatic lipase (**Figure 7.2.3C**). The activities were dose – dependent with increasing concentration.

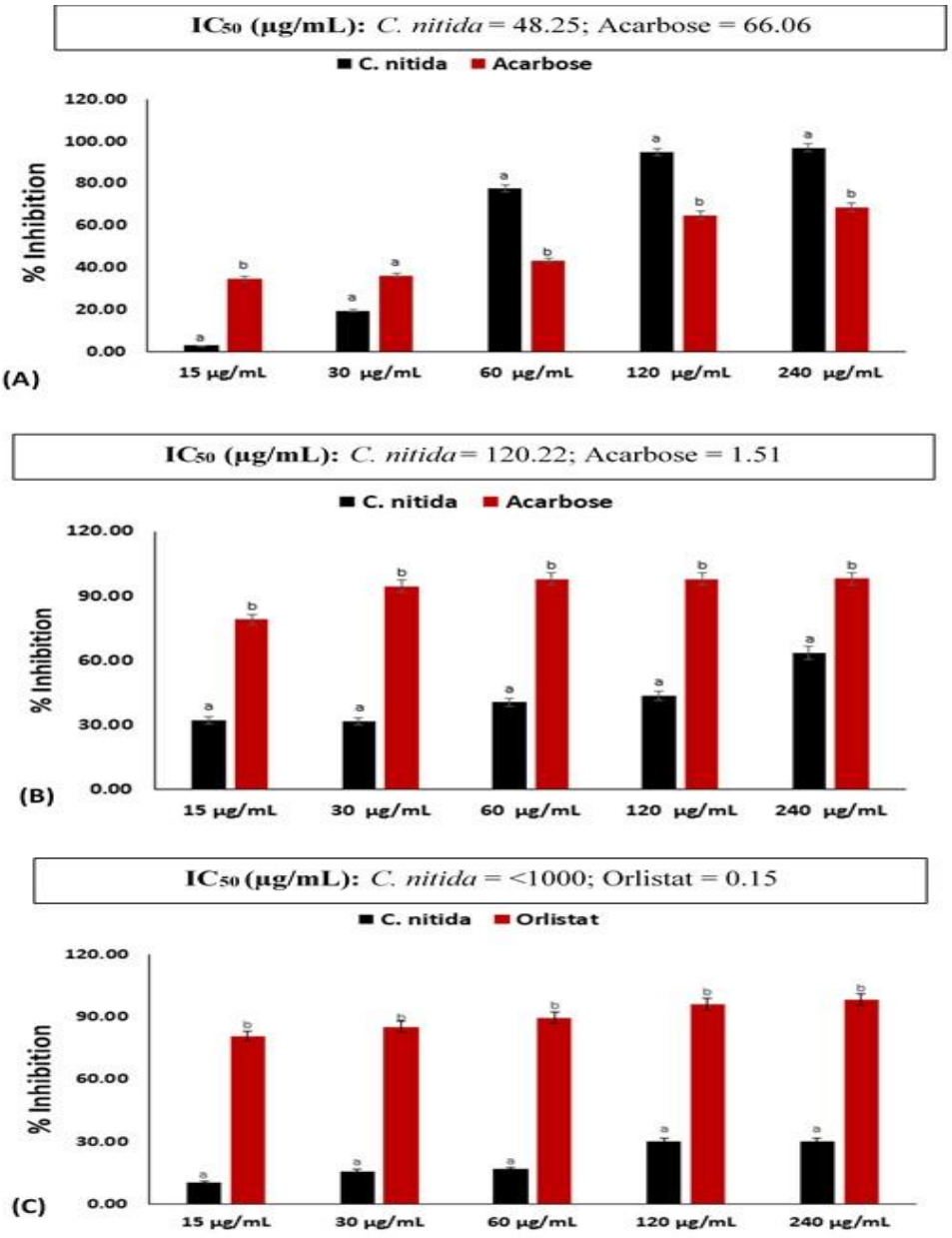


Figure 7.2.3: (A) α-glucosidase; (B) α-amylase; and (C) pancreatic lipase inhibitory activities of *C. nitida* infusion. Data are presented as mean ± SD. [ab]Values with different letters over the bars for a given concentration are significantly different from each other ($p < 0.05$, Tukey's HSD-multiple range post-hoc test, IBM SPSS for Windows).

Induction of hepatic oxidative imbalance caused a significant ($p<0.05$) increase in DNA fragmentation as depicted in **Figure 7.2.4**. Incubation with the infusion reduced the fragmentation dose – dependently with no statistical significance.

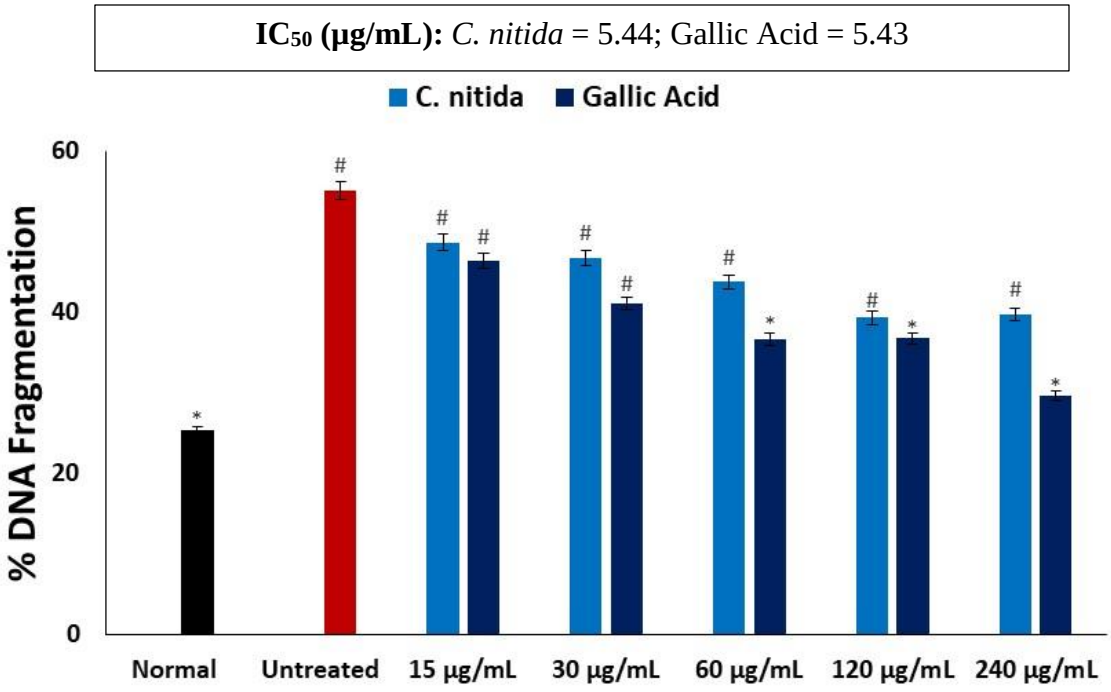


Figure 7.2.4: Effect of *C. nitida* infusion on DNA fragmentation in oxidative hepatic injury. Data are presented as mean ± SD. *Significantly different from untreated sample and #Significantly different from normal sample ($p < 0.05$, Tukey's HSD-multiple range post-hoc test, IBM SPSS for Windows).

HPLC analysis of caffeine content of the infusion revealed a high caffeine content of 80.08% as shown in **Figure 7.2.5**.

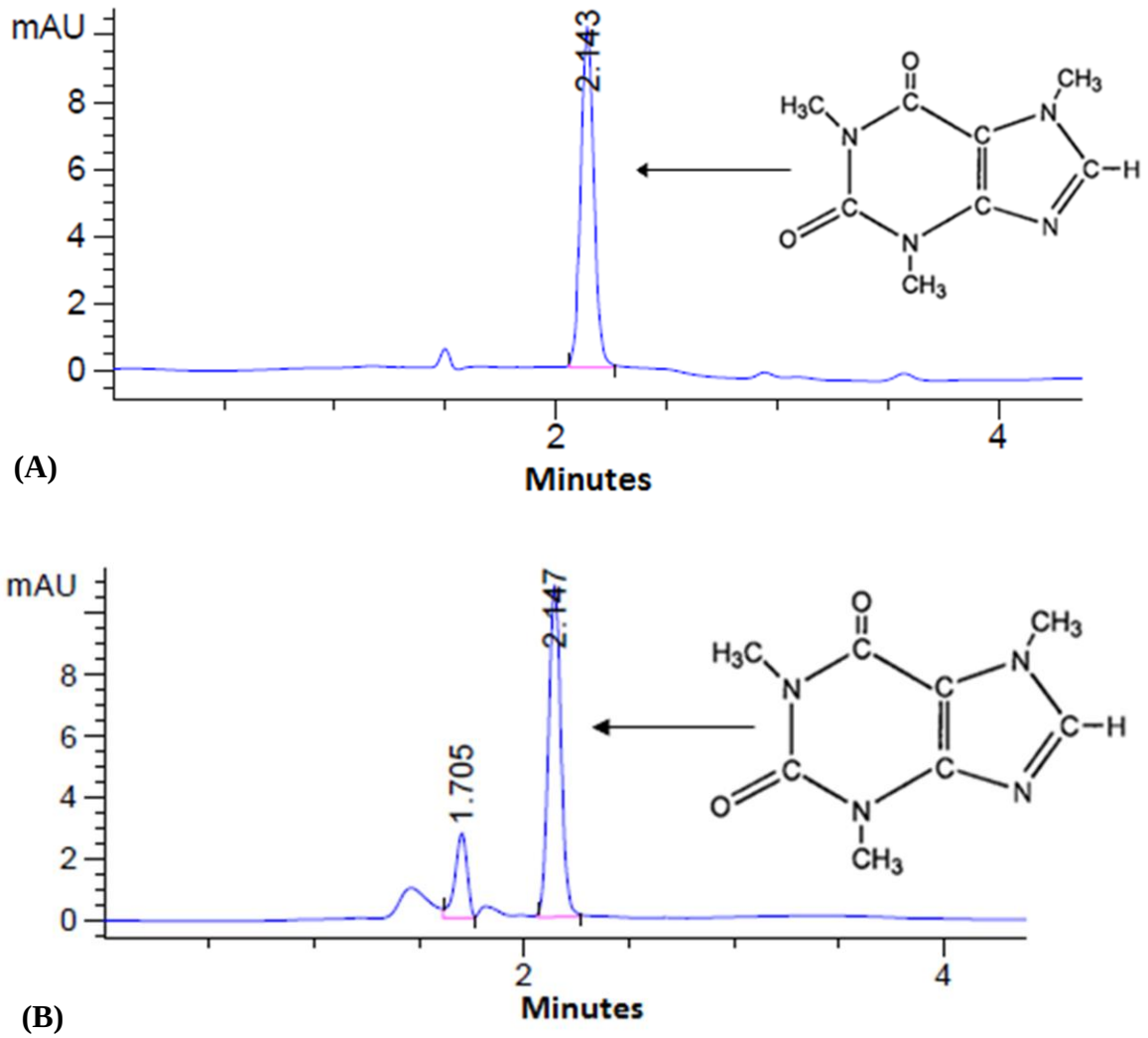
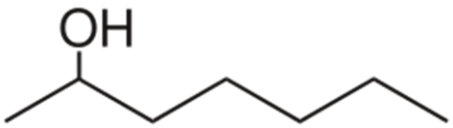
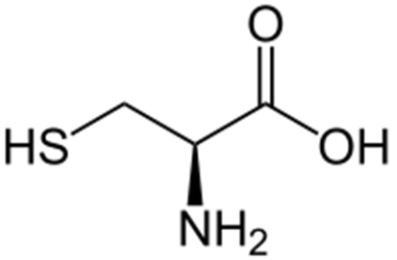


Figure 7.2.5: HPLC chromatogram of (A) caffeine standard and (B) caffeine in C. nitida infusion

LC – MS analysis of the infusion also revealed the presence of other alkaloids namely: 2-Heptanol (7.04%); L-Cysteine (10.56%); trans-2-Decen-1-ol (11.27%); Spilanthol (40.85%); L-Methionine, N-(2-thienylcarbonyl)-, methyl ester (26.76); and Cinchonine (3.52) as shown in **Figure 7.2.6** and **Table 7.2.1**.

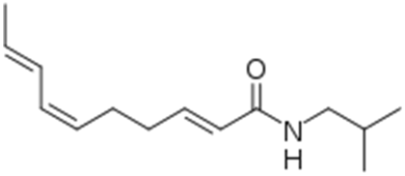


2-Heptanol

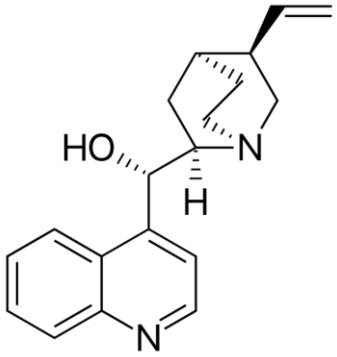


L-Cysteine

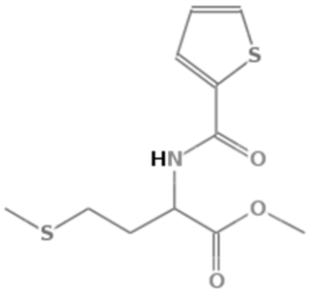
trans-2-Decen-1-ol



Spilanthol



Cinchonine



L-Methionine, N-(2-thienylcarbonyl)-, methyl ester

Figure 7.2.6: LC-MS identified compounds of *C. nitida* infusion

Table 7.2. 1: Identified compounds of *C. nitida* infusion by LC-MS

Compounds	Molecular mass	Relative abundance (%)
2-Heptanol	116	7.04
L-Cysteine	121	10.56
trans-2-Decen-1-ol	156	11.27
Spilanthol	221	40.85
L-Methionine, N-(2-thienylcarbonyl)-, methyl ester	273	26.76
Cinchonine	297	3.52

FTIR spectroscopy of the hepatic metabolite revealed that induction of oxidative injury led to an absence of functional groups at 1619.15 (Amide II) and 2852.23 (Lipid (CH_2)) cm^{-1} regions, with additions at 921.30 (nucleic acid), 1515.64 (amide II) and 1581.82 (amide I (αβ – unsaturated)) cm^{-1} regions when compared to normal tissues as depicted in **Figure 7.2.7** and **Table 7.2.2**. Treatment with *C. nitida* infusion led to restoration of the depleted functional groups. It also removed the oxidative induced added functional groups except amide I (αβ – unsaturated).

Table 7.2.2: Quantitative analysis of FTIR spectra of hepatic metabolites

Regions	Normal (cm^{-1})	Untreated (cm^{-1})	Treated (cm^{-1})	Assignment
1200 – 900 cm^{-1}	-	921.30	-	Nucleic Acid
	1044.45	1075.00	1047.38	Nucleic Acid
1500 – 1200 cm^{-1}	1222.49	1352.60	1351.07	Amide I
	1408.42	1403.79	1403.50	Amine I; Alcohol
	1456.16	1456.76	1456.31	Amide II
1800 – 1500 cm^{-1}	-	1515.64	1516.17	Amide II
	-	1581.82	-	Amide I (αβ – unsaturated)
	1619.15	-	1602.97	Amide II
3000 – 2800 cm^{-1}	2852.23	-	2853.27	Lipid (CH_2)
	2921.28	2922.47	2921.96	Lipid (CH_2)

- = not present

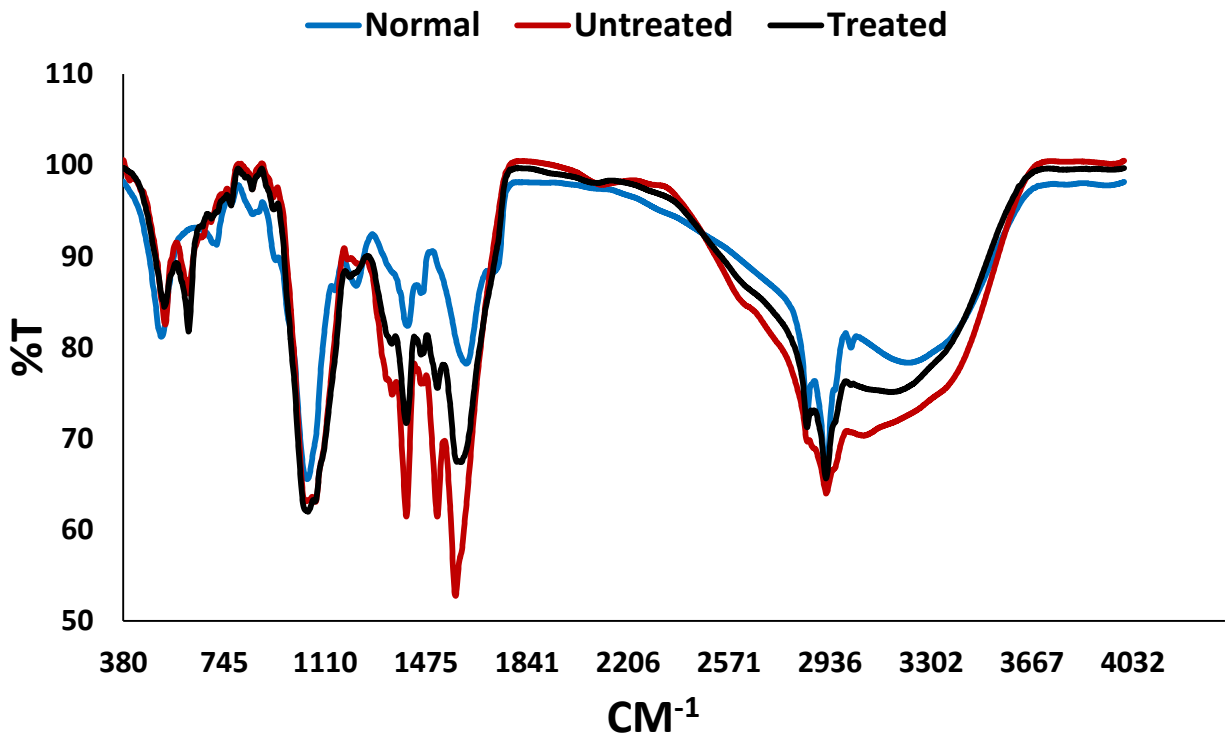


Figure 7.2.7: FTIR spectra of hepatic metabolite

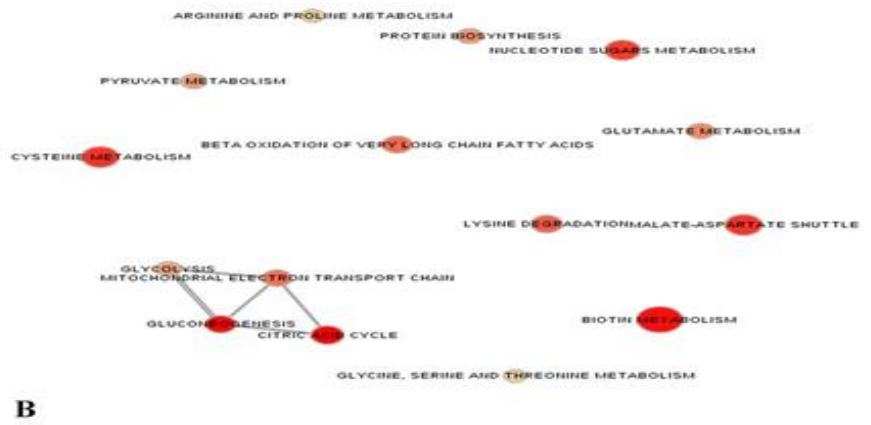
LC – MS analysis of the extracted hepatic metabolites revealed the presence of amino acid, fatty acid, carbohydrate and Krebs cycle metabolites in normal hepatic tissues as shown in **Table 7.2.3**. Induction of oxidative injury led to a depletion of these metabolites with concomitant generation of the glycolytic deviated metabolite, 2,3-Diphosphoglyceric acid and cysteine metabolic intermediate, 3-Mercaptopyruvic acid **(Table 7.2.2)**. Treatment with the infusion led to restoration of most of the depleted metabolites with concomitant generation of 4-O-Methylgallic, (-)-Epicatechin sulfate, L – Arginine, L – Tyrosine, Citric acid and Decanoic acid (**Table 7.2.3**).

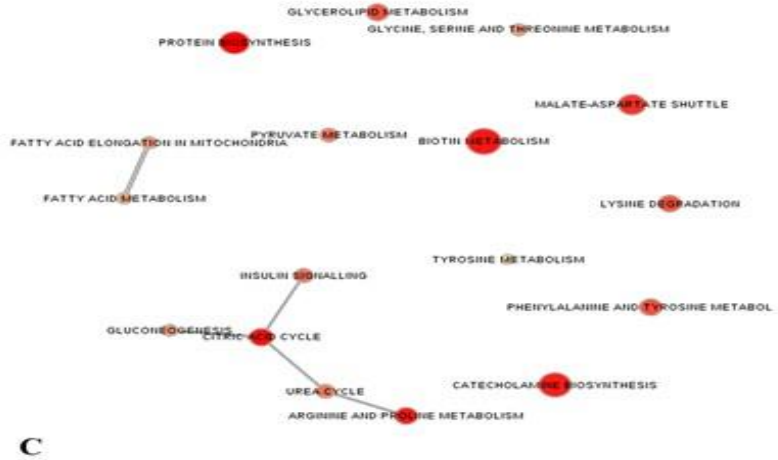
Table 7.2.3: Identified hepatic metabolites by LC-MS

Metabolites	Accession number	Normal	Untreated	Treated
4-O-Methylgallic	HMDB13198	–	–	X
Hexandecanoic Acid	HMDB00535	X	X	–
Succinate	HMDB00254	X	X	–
Adenosine 2',3'-cyclic Phosphate	HMDB11616	X	–	X
Creatinine	HMDB00064	X	X	X
L – Malate	HMDB00156	X	X	X
Fucose 1-phosphate	HMDB01265	X	–	–
(-)-Epicatechin sulfate	HMDB12467	–	–	X
L – Lysine	HMDB00182	X	X	X
Uridine 2',3'-cyclic phosphate	HMDB11640	X	X	X
L – Arginine	HMDB00517	–	–	X
L – Tyrosine	HMDB00158	–	–	X
Indole-3-propanoite	HMDB02302	X	–	X
Citric acid	HMDB00094	–	–	X
Decanoic acid	HMDB00638	–	–	X
2,3-Diphosphoglyceric acid	HMDB01294	–	X	–
Cytidine triphosphate	HMDB00082	X	–	–
Palmitic acid	HMDB00220	X	–	X
3-Mercaptopyruvic acid	HMDB01368	–	X	–
UDP-D-Xylose	HMDB01018	X	X	–
Trans-Vaccenic acid	HMDB03231	X	–	X

Key: X = Present; – = Absent

Pathway analysis of the identified metabolites revealed the presence of 21 metabolic pathways in normal hepatic tissues, 12 in oxidative injured hepatic tissues and 17 in *C. nitida* treated hepatic tissues as depicted in **Figure 7.2.8** and **Table 7.2.4**. Metabolic pathways common to normal and oxidative injured hepatic tissues were only 4: Beta oxidation of very long chain fatty acids; Glutamate metabolism; Mitochondrial electron transport chain; and Nucleotide sugars metabolism. Induction of oxidative injury led to activation of glycolytic and cysteine metabolism pathways. There were no common metabolic pathways between oxidative injured and *C. nitida* treated hepatic tissues, indicating a therapeutic effect of the infusion. Treatment with *C. nitida* restored 4 metabolic pathways common to the normal hepatic tissue namely: fatty acid elongation in mitochondria, fatty acid metabolism, glycerolipid metabolism, insulin signaling, and lysine degradation. The infusion further activated 4 additional pathways: catecholamine biosynthesis, phenylalanine and tyrosine metabolism, tyrosine metabolism, and urea cycle.





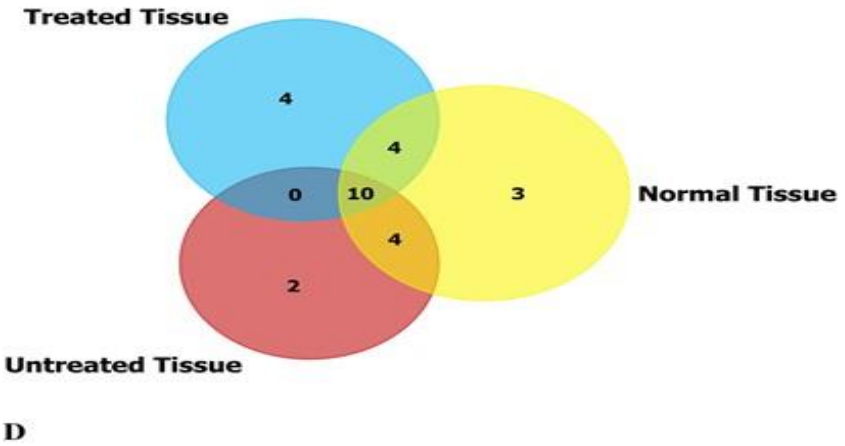


Figure 7.2.8: Identified metabolic pathways of (A) normal hepatic tissue; (B) oxidative injured hepatic tissue; and (C) treated hepatic tissue.

Table 7.2.4: Identified metabolic pathways of hepatic tissues

Metabolic Pathways	Normal	Untreated	Treated
Arginine and proline metabolism	**X**	**X**	**X**
Beta oxidation of very long chain fatty acids	**X**	**X**	**–**
Biotin metabolism	**X**	**X**	**X**
Catecholamine biosynthesis	**–**	**–**	**X**
Citric acid cycle	**X**	**X**	**X**
Cysteine metabolism	**–**	**X**	**–**
Fatty acid elongation in mitochondria	**X**	**–**	**X**
Fatty acid metabolism	**X**	**–**	**X**
Fructose and mannose degradation	**X**	**–**	**–**
Gluconeogenesis	**X**	**X**	**X**
Glutamate metabolism	**X**	**X**	**–**
Glycerolipid metabolism	**X**	**–**	**X**
Glycine, serine and threonine metabolism	**X**	**X**	**X**
Glycolysis	**–**	**X**	**–**
Insulin signaling	**X**	**–**	**X**
Lysine degradation	**X**	**–**	**X**
Malate-aspartate shuttle	**X**	**X**	**X**
Mitochondrial electron transport chain	**X**	**X**	**–**
Nucleotide sugars metabolism	**X**	**X**	**–**
Phenylalanine and tyrosine metabolism	**-**	**-**	**X**
Protein biosynthesis	**X**	**X**	**X**
Pyrimidine metabolism	**X**	**–**	**–**
Pyruvate metabolism	**X**	**–**	**X**
RNA transcription	**X**	**–**	**–**
Tyrosine metabolism	**-**	**-**	**X**
Urea cycle	**-**	**-**	**X**

Key: X = Present; – = Absent

Prediction of oral toxicity of caffeine on ProTox (tox.charite.de) web-based server showed it to belong to class 3, with a predicted LD_{50} of 127 mg/kg and an accuracy of 100% as shown in Table **7.2.5**. The predicted toxicity target was identified as Adenosine Receptor A2a with an average similarity known ligands value of 96.8%. The intestinal absorption value was 100%, with a total clearance of 0.239 log ml/min/kg. It is hepatotoxic, with the maximum tolerated dose for human predicted to be 0.536 log mg/kg/day. It was also predicted to be a substrate for P-glycoprotein and an inhibitor of CYP1A2.

Table 7.2.5: Predicted toxicity of caffeine

Parameters	Predicted Values
Intestinal absorption (human)	100%
P-glycoprotein substrate	Yes
CYP2D6 substrate	No
CYP3A4 substrate	No
CYP1A2 Inhibitor	Yes
CYP2C19 Inhibitor	No
CYP2C9 Inhibitor	No
CYP2D6 Inhibitor	No
CYP3A4 Inhibitor	No
Total Clearance	0.239 log ml/min/kg
Max. tolerated dose (human)	0.536 log mg/kg/day
Hepatotoxicity	Yes
Oral toxicity LD_{50} (rats)	127 mg/kg
Toxicity Class	3
Toxicity Target	Adenosine Receptor A2a

7.2.5 Discussion

The role of medicinal plants in the treatment and management of type 2 diabetes is well documented (Erukainure et al. 2017a; Ezuruike and Prieto 2014; Mohammed et al. 2014) and dates back to time immemorial before the advent of orthodox medicine. These antidiabetic properties

have been attributed to the phytochemical and nutritional constituents of the plants (Erukainure et al. 2016). However, there are few reports on the metabolic pathways through which these plants and their constituents bring about their biological activities. In this study, the antidiabetic and antioxidative properties of caffeine – rich infusion from *Cola nitida* as well as the metabolic pathways involved were investigated.

The role of oxidative stress in the pathogenesis and progression of type 2 diabetes and its complications are well documented (Pi et al. 2010; Pitocco et al. 2013). This has been attributed to increased hyperglycemia with concomitant suppression of the body antioxidant system, thereby leading to dysregulated cellular metabolism (Pitocco et al. 2013). The suppressed hepatic SOD and catalase activities as well as depleted GSH level on incubation with $FeSO_4$ indicates an occurrence of oxidative stress (**Figure 7.2.2A – 7.2.2C**). This is also evidenced by the increased MDA level, signifying lipid peroxidation (Fig. 2d). SOD dismutates $O_2^{\cdot -}$ to H_2O_2 which is broken down by catalase to O_2 and H_2O_2. If not broken down by catalase, H_2O_2 would further react with Fe^{3+} to generate $^{\bullet}OH$ which attacks the lipid membrane leading to lipid peroxidation (Aslan et al. 2000). Interestingly, the additional metabolites identified in the oxidative tissues (2,3-Diphosphoglyceric acid) have been recognized as biomarkers of oxidative stress (Richards et al. 2007). 2,3-Diphosphoglyceric acid is a three-carbon isomer of the glycolytic intermediate, 1,3-bisphosphoglyceric acid which reflects the activated glycolytic pathway in the oxidative injured tissue (**Figure 7.2.8B** and **Table 7.2.4**). The other metabolite, 3-Mercaptopyruvic acid (**Figure 7.2.8B** and **Table 7.2.4**) is an intermediate of cysteine metabolism and involved in oxidoreductase activity (Módis et al. 2013; Rose et al. 2017). The reversed antioxidant activities, GSH and MDA levels after treatment with the infusion (**Figure 7.2.2A** – **7.2.2D**), demonstrates an antioxidative activity. This is also reflected in its free radical scavenging and FRAP activities (Figs. 1a and 1b). These activities can be attributed to the high caffeine content and other identified alkaloids of the infusion with reported antioxidative activities (**Figures 7.2.5** and **7.2.6, Table 7.2.1**). Caffeine and alkaloids have been reported for their antioxidative properties (Azam et al. 2003; Jasiewicz et al. 2016). The absence of 2,3-Diphosphoglyceric and 3-Mercaptopyruvic acids as well as deactivation of their pathways in the treated tissue, further portrays the antioxidant therapeutic effect of the infusion. Interestingly, 4-O-Methylgallic and (-)-epicatechin sulfate (**Table 7.2.3**) are metabolic intermediates of gallic acid and (-)-epicatechin metabolism with reported antioxidative activities (Actis-Goretta et al. 2012; Ottaviani et al. 2012). Although not detected in the infusion, *cola nitida*

has been reported for its high content of catechins and gallic acid (Niemenak et al. 2008; Umoren et al. 2009). Thus, responsible for the identified metabolites.

The inhibitory effect of the infusion on α – glucosidase and amylase activities (Figs. 3a and 3b) portrays an antidiabetic potential as both enzymes play major roles in carbohydrate catabolism and have been implicated in the progression of type 2 diabetes (Oboh et al. 2017). This correlates with the reactivated insulin signaling and deactivated glycolytic pathways in the infusion – treated hepatic tissues (**Figure 7.2.8C** and **Table 7.2.4**). The reactivated insulin signaling pathway can be attributed to catecholamine biosynthesis in the treated tissue (**Figure 7.2.8C** and **Table 7.2.4**). Catecholamines play a major role in insulin release via the α_2 and β_2-adrenergic receptors. Stimulation of α_2-adrenergic receptors causes an inhibition of insulin release, leading to increase in blood glucose level (Cawston and Miller 2010). While stimulation of β_2-adrenergic receptors causes insulin release, leading to decreased blood glucose level (Barth et al. 2007). The activation of the catecholamine biosynthetic pathways also correlates with the activated phenylalanine and tyrosine, and tyrosine metabolic pathways in the treated tissue (**Figure 7.2.8C** and **Table 7.2.4**). This is also portrayed by the metabolite, L – Tyrosine (**Table 7.2.3**) as it is a major amino acid in the biosynthesis of catecholamines (Fernstrom and Fernstrom 2007). Caffeine has also been demonstrated to inhibit to monoamine oxidases (MAO), a major degrader of catecholamines (Akomolafe et al. 2017). Thus, inferring that the high level of caffeine implies maintenance of high catecholamine levels. The urea cycle in the treated tissue (**Figure 7.2.8C** and **Table 7.2.4**) can be attributed to the breakdown of catecholamines (Kuntz and Kuntz 2009).

The safety and toxicity of herbal medicine and their constituents are of major concern, as studies have reported complications arising from herbal – drugs and herbal – food interactions (Fugh-Berman and Ernst 2001; Posadzki et al. 2013). The predicted toxicity class and hepatotoxicity of caffeine (**Table 7.2.5**) indicates that caution should be considered when using the infusion. CYP1A2 plays a major role in the metabolism of caffeine (Urry et al. 2016). Its presence has been reported in type 2 diabetics on caffeine therapy and/or consumes coffee (Urry et al. 2016). Its predicted inhibition by caffeine portrays a low metabolism thereby leading to high dose consequences because of high concentration of caffeine, which corroborates its low total clearance (**Table 7.2.5**). The predicted toxicity target, adenosine A_{2a} receptor (A2AR) has been recognized to be non-selectively blocked by caffeine (Beukers et al. 2006). Adenosine play major roles on

glucose and homeostasis via the activation of A_1, A_2 or A_3 receptors (Koupenova and Ravid 2013), thus its blockade could lead to unfavorable metabolic consequences.

7.2.6 Conclusion

These results suggest the antioxidative and antidiabetic properties of *C. nitida* infusion, with caffeine as the major constituent. This can be attributed to the ability of the infusion to reactivate the insulin signaling and pyruvate metabolic pathways as well as activation of catecholamine biosynthesis, phenylalanine and tyrosine metabolism, and tyrosine metabolism pathways in oxidative injured hepatic tissues. However, the predicted toxicity class and target of caffeine may require caution in its utilization.

7.3 *Cola nitida* Modulates Glucose Homeostasis in Type 2 Diabetes by Inducing Insulin Secretion; Abating Redox Imbalance and Dyslipidemia; Inhibiting Glycolytic and Cholinergic Enzymes Activities; and Downregulating Nrf2 Expression

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Preface: This article investigated the antidiabetic effect of *Cola nitida* hot water infusion in type 2 diabetic rats. Its antioxidative, anti-proinflammatory, antilipemic and insulin stimulatory effects were investigated. Its inhibitory effect on glycolytic, cholinergic and carbohydrate digesting enzymes were also investigated. Its ability to inhibit Nrf2 expression was also investigated *in vivo* and *in silico*

7.3.1 Abstract

The antidiabetic properties of the hot infusion of *Cola nitida* was investigated in type 2 diabetic rats for its effects on hyperglycemia, insulin level, lipid profile, pancreatic β-cell function, perfusion and morphology, redox imbalance, glycolytic and cholinergic enzymes, as well as of caspase-3 and Nrf2 expressions. Treatment with the infusion led to significant depleted levels of blood glucose, triglycerides, LDL-c, fructosamine, ALT, and uric acids, while elevating serum insulin and HDL-c levels. The infusion also significantly ($p<0,05$) elevated the GSH, SOD, catalase, α-amylase, and ATPase, with concomitant depletion of myeloperoxidase activity, NO and MDA levels in the serum and pancreas. There were significantly ($p<0.05$) improved pancreatic

β-cell function and morphology in rats treated with *C. nitida*, while restoring pancreatic capillary networks. *C. nitida* inhibited the activities of glycogen phosphorylase, fructose-1,6-biphosphatase, glucose-6-phosphatase, and acetylcholinesterase, while downregulating Nrf2 expression. NMR analysis of the infusion revealed the presence of caffeine and theobromine. The identified compounds displayed strong molecular interactions when molecularly docked with caspase-3 and Nrf2. These results insinuate the antidiabetic activities of *C. nitida* hot infusion and may be attributed to the NMR-identified compounds.

Keywords: Anti-hyperglycemia; *Cola nitida*; Glycolytic enzymes; Nrf2; Type 2 diabetes

7.3.2 Introduction

There have been increasing interest in the use of plants in treating various diseases and ailments. The efficacies of these plants have been attributed to their phytochemical and nutritional constituents. This can be attributed to the quest of natural products with little or no side effects, coupled with affordability and availability, which have also led to a paradigm shift from commercial drugs to medicinal plants (Erukainure et al. 2013). The use of medicinal plants remains the oldest health system and are mostly rooted in the traditions and cultural beliefs of most communities. From time immemorial, medicinal plants have been employed in the folkloric treatment of various diseases and ailments such as dysentery, cancers, and diabetes. Amongst these plants is *Cola nitida* from the genus, *Cola*.

Diabetes mellitus (DM) is amongst the common ailments reported to be treated with *C. nitida*. It is regarded as the fastest growing epidemy, with over 425 million people estimated to be diabetic in 2017 (Cho et al. 2018; I.D.F. 2018). These figures have been projected to increase by 35.35% in 2045 (Cho et al. 2018; I.D.F. 2018). DM is a metabolic disease affecting carbohydrate, protein and fatty acid metabolism leading to increased blood glucose level. Of the 2 common types of DM, type 2 diabetes (T2D) is most prevalent as it accounts for more than 90% of all morbidity and mortality attributed to DM (I.D.F. 2016; I.D.F. 2018). It occurs owing to inability of the cells to utilize the insulin secreted by the pancreatic β-cells. It is often characterized by insulin resistance and pancreatic β-cell dysfunctions, which leads to hyperglycemia. Chronic hyperglycemia has been recognized as the hallmark of T2D as it leads to generation of free radicals. Continuous production of these free radicals would lead to redox imbalance, with concomitant depletion of the

endogenous antioxidant system. Thereby leading to oxidative stress. Oxidative stress has been implicated as the main trigger of T2D micro and macro – vascular complications such as nephropathy, microangiopathy, retinopathy and death (Barar 2000; Constantino et al. 2013).

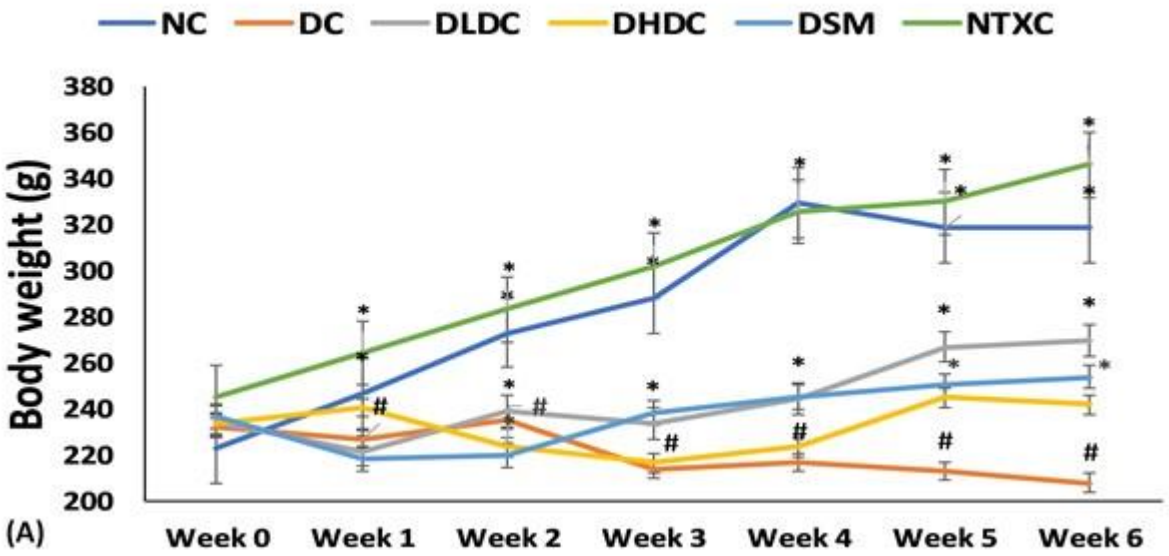
The aim of this study was to investigate the antidiabetic effect of the hot aqueous infusion of *C. nitida* in fructose-streptozotocin (STZ) induced T2D rats. Its modulatory effect on pancreatic redox imbalance, and morphology as well its inhibitory activities on cholinergic, glycolytic and carbohydrate hydrolyzing enzymes were also investigated. The ability of the infusion to suppress the expressions of caspase-3 and nuclear factor erythroid 2–related factor 2 (Nrf2) were investigated as well.

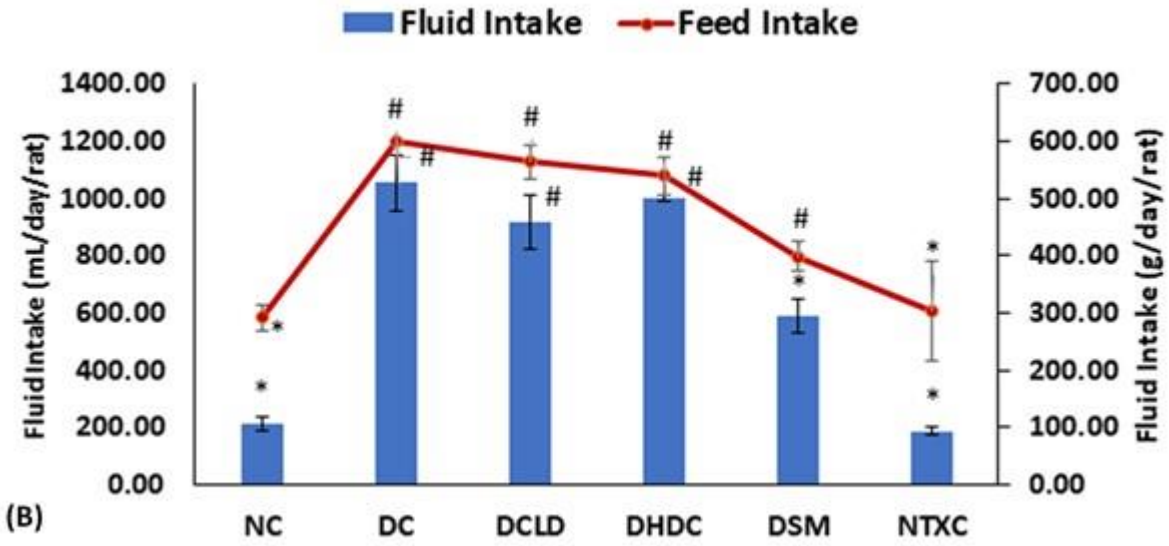
7.3.3 Materials and Methods

Kindly refer to chapter 2, subsections 2.1.1, 2.1.4, 2.10.5; 2.11.2, 2.12 – 2.13 for details materials and methods.

7.3.4 Results

Induction of T2D led to significant ($p<0.05$) body weight loss as shown in **Figure 7.3.1A**. As the intervention period proceeded, there were significant ($p<0.05$) weight gain in all treatment groups, with rats treated with low dose of *C. nitida* having the highest gain. Induction of T2D also led to significant ($p<0.05$) feed and water intake (**Figure 7.3.1B**). Treatment with both doses of the infusion led to a slight decrease in fluid and food intake but these were significantly ($p<0.05$) decreased in rats treated with metformin. Additionally, the weight and relative weight of the pancreas were significantly ($p<0.05$) deceased in untreated diabetic rats (DC) as depicted in **Figure 7.3.1C**. These were significantly increased in all treatment groups except the metformin-treated group.


NC
DC
DLDC
DHDC
DSM
NTXC
Body weight (g)
380
360
340
320
300
280
260
240
220
200
(A)
Week 0
Week 1
Week 2
Week 3
Week 4
Week 5
Week 6


Fluid Intake
Feed Intake
Fluid Intake (mL/day/rat)
1400.00
1200.00
1000.00
800.00
600.00
400.00
200.00
0.00
700.00
600.00
500.00
400.00
300.00
200.00
100.00
0.00
Fluid Intake (g/day/rat)
(B)
NC
DC
DCLD
DHDC
DSM
NTXC

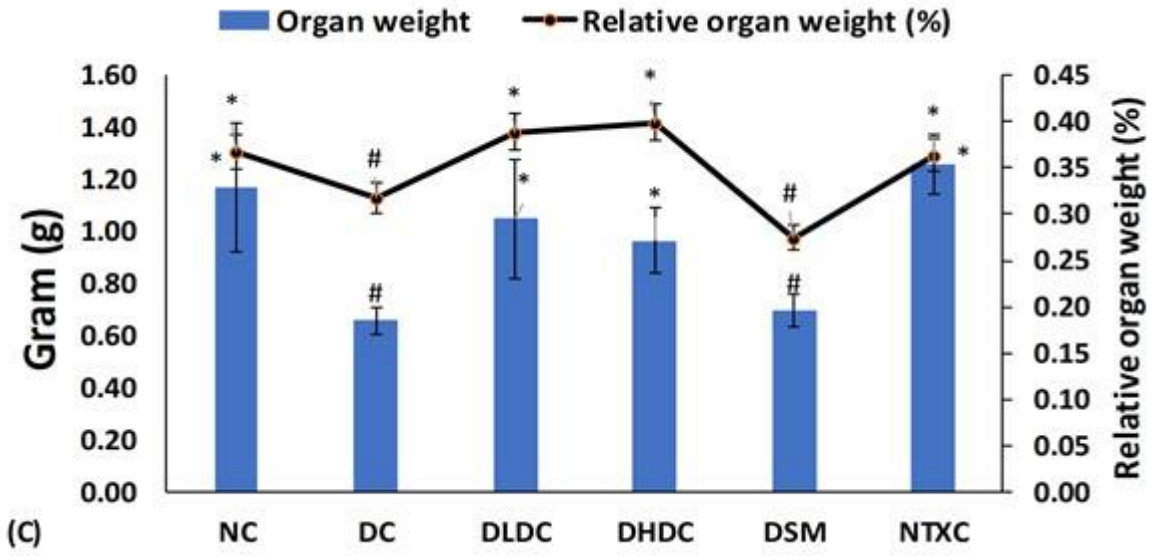


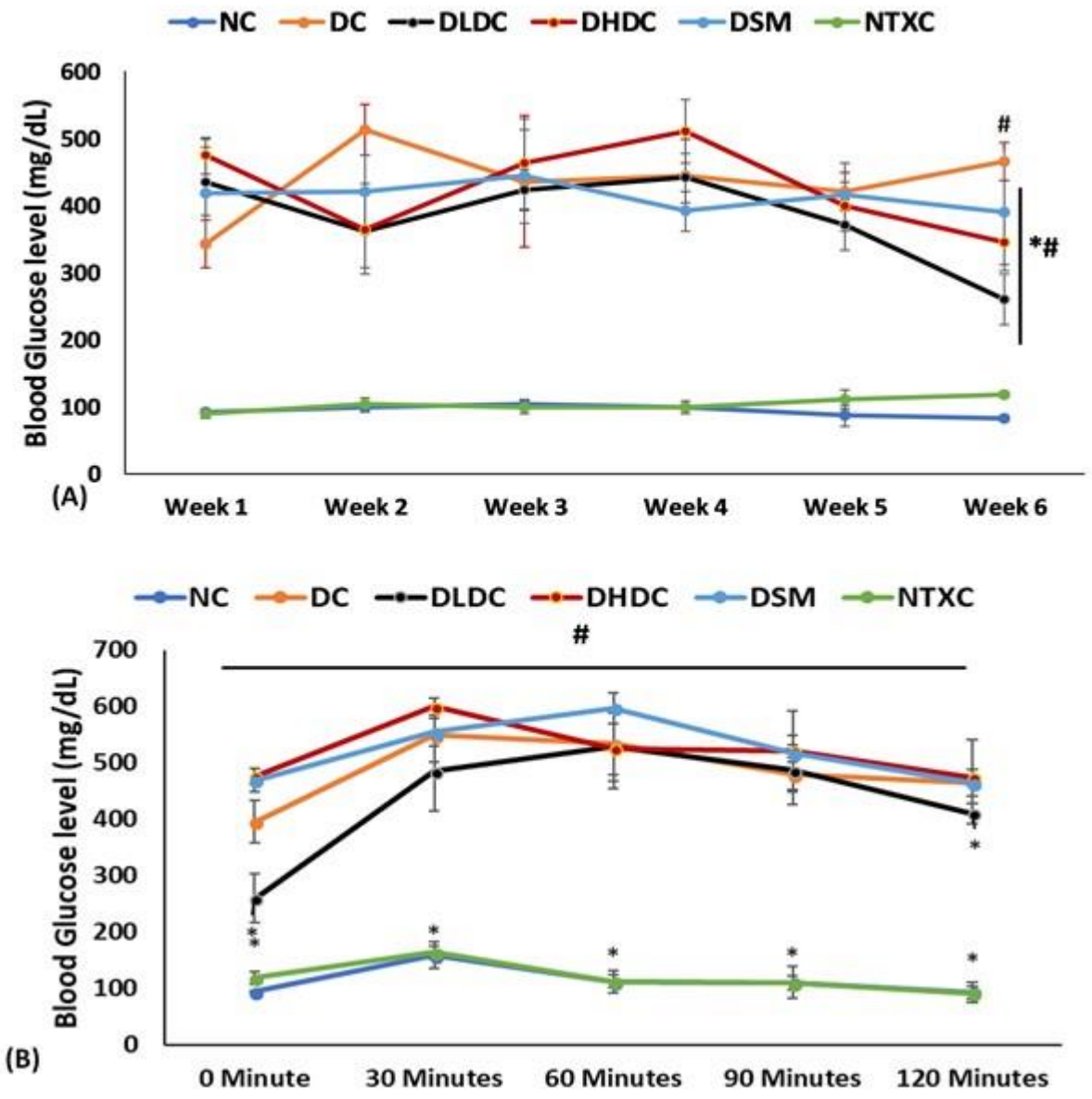
Figure 7.3.1: (A) Body weight; (B) feed and fluid intake; and (C) weight and relative weight of pancreas of experimental groups. Values = mean ± SD; n = 5 (NC and NTXC) and 7 (DC, DLDC, DHDC, and DSM). *Statistically ($p<0.05$) significant to DC, #statistically significant ($p<0.05$) to NC. **NC** = normal rats, **DC** = diabetic control, **DLDC** = diabetic rats + *C. nitida* (150 g/kg bw), **DHDC** = diabetic rats + *C. nitida* (300 g/kg bw), **DSM** = diabetic rats + metformin (200 g/kg bw), and **NTXC** = Normal rats + *C. nitida* (300 g/kg bw).

Induction of T2D led to significant ($p<0.05$) increase in blood glucose level as depicted in **Figure 7.3.2A**. As the intervention proceeded, the blood glucose level peaked for all treatments at the 4th week and started sliding till the end of the experiment. Treatment with low and high doses of *C. nitida* led to 40.48 and 27.38% reduction, respectively (**Figure 7.3.2A**).

As shown in **Figure 7.3.2B**, oral administration of glucose (2 g/kg bw) led to significant ($p<0.05$) increase in blood glucose level at 30 min for all experimental animals. Except for DLDC and DSM, all groups peaked at this time and started declining with increasing time. The blood glucose level peaked at 60 min for DLDC and DSM, and started declining with increasing time. The lowest levels were recorded at 120 min for all groups.

The serum insulin level was significantly ($p<0.05$) reduced after the induction of T2D, with concomitant reduction of pancreatic β-cell function as depicted in **Figure 7.3**.2C. Treatment with

low dose of *C. nitida* had little or no significant effect on the serum insulin level. However, treatment with high dose of *C. nitida* and metformin led to significant ($p<0.05$) increased levels and β-cell functions (**Figure 7.3.2C**).



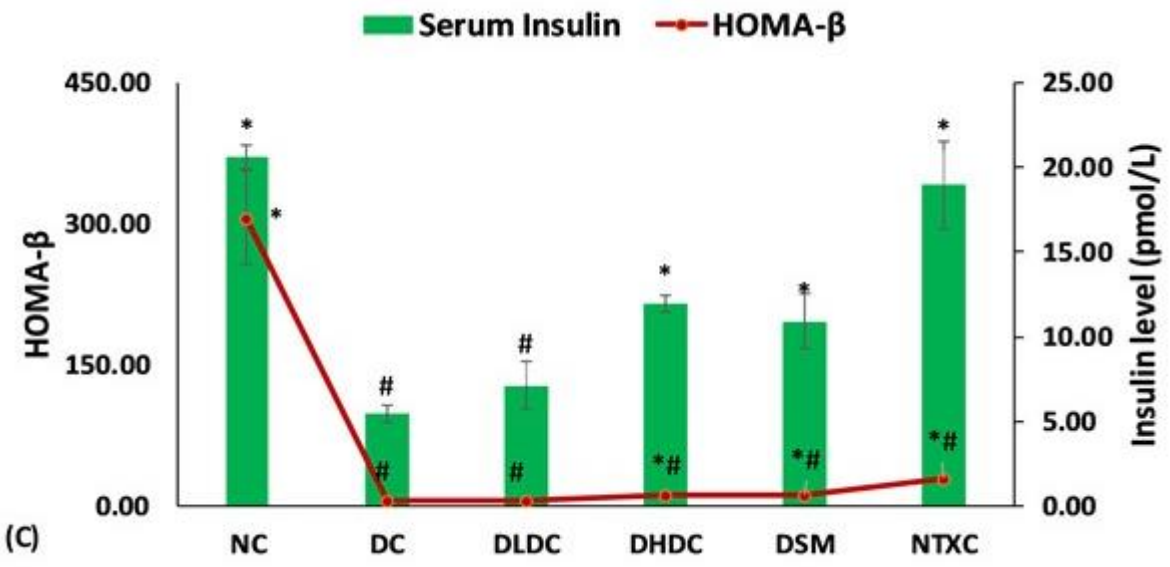


Figure 7.3.2: (A) Blood glucose level; (B) oral glucose tolerance; and (C) serum insulin and β-cell function of experimental groups. Values = mean ± SD; n = 5 (NC and NTXC) and 7 (DC, DLDC, DHDC, and DSM). *Statistically ($p<0.05$) significant to DC, #statistically significant ($p<0.05$) to NC. NC = normal rats, DC = diabetic control, DLDC = diabetic rats + *C. nitida* (150 g/kg bw), DHDC = diabetic rats + *C. nitida* (300 g/kg bw), DSM = diabetic rats + metformin (200 g/kg bw), and NTXC = Normal rats + C. nitida (300 g/kg bw).

There was no significant difference in the serum total cholesterol levels of all the experimental groups as shown in **Figure 7.3.3.** However, induction of T2D led to significant ($p<0.05$) increased levels of triglycerides and LDL-c with concomitant reduction in HDL-c level. These were significantly reversed in the treatment groups, *C. nitida* showing an appreciable antilipemic activity compared to the standard drug, metformin (**Figure 7.3.3**).

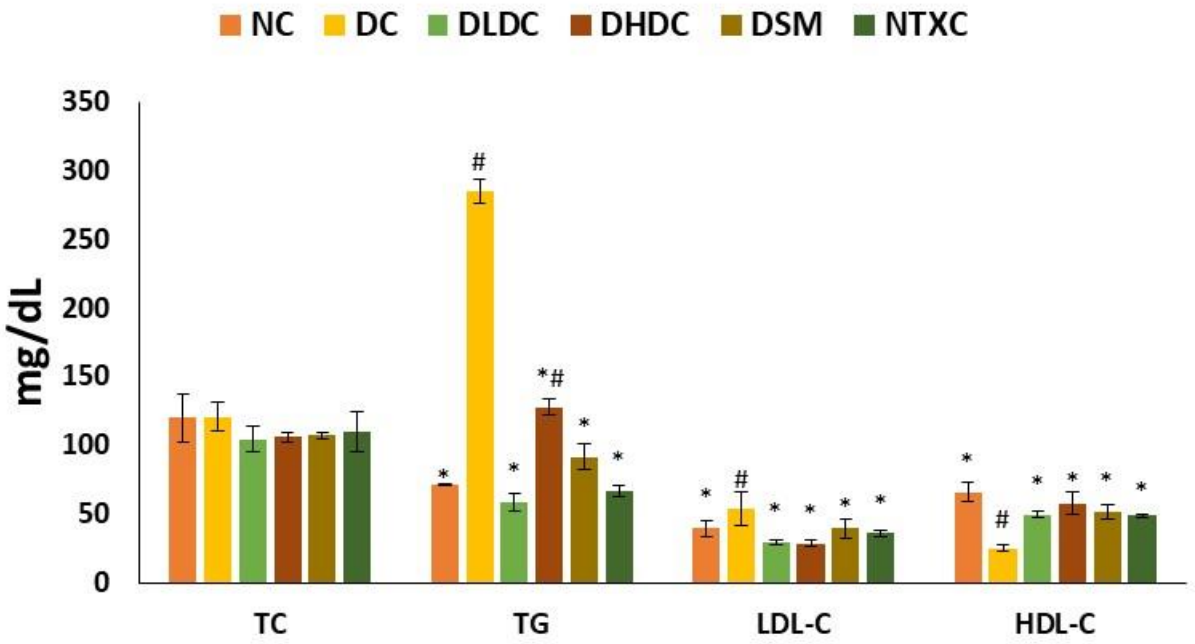


Figure 7.3.3: Serum lipid profile of experimental groups. Values = mean ± SD; n = 5 (NC and NTXC) and 7 (DC, DLDC, DHDC, and DSM). *Statistically ($p<0.05$) significant to DC, #statistically significant ($p<0.05$) to NC. **NC** = normal rats, **DC** = diabetic control, **DLDC** = diabetic rats + *C. nitida* (150 g/kg bw), **DHDC** = diabetic rats + *C. nitida* (300 g/kg bw), **DSM** = diabetic rats + metformin (200 g/kg bw), and **NTXC** = Normal rats + *C. nitida* (300 g/kg bw).

As shown in **Table 7.3.1**, α-amylase activity was significantly ($p<0.05$) increased in both pancreatic tissues and serum after the induction of T2D. This was significantly ($p<0.05$) decreased in all treatment groups, with rats treated with low dose of *C. nitida* and metformin showing the best activities respectively.

Table 7.3.1: Serum enzyme activities of experimental groups

Enzyme Activities	**NC**	**DC**	**DLDC**	**DHDC**	**DSM**	**NTXC**
Serum α-amylase	25.38±3.50*	72.36±4.25$^{\#}$	44.89±5.56$^{\#}$	54.88±2.80$^{\#}$	41.57±9.53*	29.71±2.40*
Pancreatic α-amylase	46.90±4.25*	89.72±4.21$^{\#}$	39.56±5.70*	46.97±7.18*	28.02±3.37*	52.20±9.06*
Serum ATPase	279.18±5.60*	201.24±17.89$^{\#}$	271.32±17.06*	273.45±13.31*	241.92±12.42*	232.49±2.88*
Pancreatic ATPase	144.35±21.59*	234.49±7.781$^{\#}$	162.11±2.48*	153.14±13.57$^{\#}$	103.95±14.28*	95.57±13.88*$^{\#}$
Acetylcholinesterase	29.56±3.24*	44.20±3.24$^{\#}$	25.83±3.76*	22.58±3.65*	22.96±3.76*	20.09±4.87*
Glycogen Phosphorylase	174.22±9.98*	209.89±17.50$^{\#}$	179.89±1.99*	155.09±6.31*	169.35±24.65*	167.53±10.00*
Fructose 1,6 Biphosphatase	177.19±7.93*	247.46±9.90$^{\#}$	187.59±5.68*	180.97±6.93*	191.78±19.49*	184.76±8.18*
Glucose 6 Phosphatase	153.68±3.06*	223.14±12.03$^{\#}$	154.69±7.97*	171.96±6.76*	176.92±23.96*	173.14±6.95*

Values = mean ± SD; n = n = 5 (NC and NTXD) and 7 (DC, DLDC, DHDC and DSM). *Statistically ($p<0.05$) significant to DC, $^{\#}$Statistically significant ($p<0.05$) to NC. **NC** = normal rats, **DC** = diabetic control, **DLDC** = diabetic rats + *C. nitida* (150 g/kg bw), **DHDC** = diabetic rats + *C. nitida* (300 g/kg bw), **DSM** = diabetic rats + metformin (200 g/kg bw), and **NTXC** = Normal rats + *C. nitida* (300 g/kg bw).

There was significant ($p<0.05$) decrease in serum ATPase activity, with concomitant increased in pancreatic ATPase activity untreated T2D rats (DC) as depicted in **Table 7.3.1**. These were significantly ($p<0.05$) reversed in all the treatment groups, with metformin and high dose of *C. nitida* showing the best inhibitory activities (**Table 7.3.1**).

Induction of T2D also led to significant ($p<0.05$) increase in pancreatic acetylcholinesterase activity as shown in **Table 7.3.1**. This was significantly ($p<0.05$) reduced in all treatment group, with high dose of *C. nitida* and metformin showing the best inhibitory activities (**Table 7.3.1**).

The increased glycolytic enzymes activities on induction of T2D, were significantly ($p<0.05$) depleted in all treatment groups as depicted in Table 1. Rats treated with high dose of *C. nitida* (DHDC) revealed the best inhibitory activities on glycogen phosphorylase and fructose 1,6 biphosphatase, while the low dose was best for glucose-6-phosphatase (**Table 7.3.1**).

As shown in **Table 7.3.2**, there was a significant ($p<0.05$) depletion in GSH level, SOD and catalase activities, with concomitant increased MDA level in both serum and pancreatic tissues on induction of T2D, which suggests the induction of oxidative stress. The increased NO level and myeloperoxidase activity on induction of T2D, also portrays proinflammation on induction of T2D. These activities and levels were significantly ($p<0.05$) reversed after the treatment with *C. nitida*, with the low dose showing the better activities (**Table 7.3.2**).

Table 7.3.2: Antioxidant status of (A) serum and (B) pancreas of experimental groups

(A)

	GSH (mol/L)	SOD (U/mg protein)	Catalase (U/mg protein)	MDA (mol/L)	NO (µml)	Myeloperoxidase (U/mg protein)
NC	64.28±11.90*	86.21±8.13*	636.37±29.48*	63.09±4.57*	0.075±0.006*	100.27±9.68*
DC	36.65±2.47$^{\#}$	40.23±3.32$^{\#}$	490.64±42.75$^{\#}$	71.19±4.52$^{\#}$	0.102±0.007$^{\#}$	121.08±18.63$^{\#}$
DLDC	56.75±3.27*	177.20±10.36*$^{\#}$	643.89±26.30*	46.19±9.50*	0.020±0.005*	55.45±6.46*
DHDC	54.39±6.77*	99.14±8.78*	598.81±15.21*	61.30±11.59*	0.019±0.003*	67.10±10.49*
DSM	53.31±2.27*	68.97±8.78*	650.50±4.82*	50.09±9.53*	0.053±0.008*	47.44±1.53*
NTXC	50.30±8.04*	246.65±17.95*$^{\#}$	664.29±24.19*	51.27±4.64*	0.041±0.009$^{\#}$	72.77±16.10*

(B)

	GSH (mol/L)	SOD (U/mg protein)	Catalase (U/mg protein)	MDA (mol/L)	NO (µml)	Myeloperoxidase (U/mg protein)
NC	104.87±3.09*	659.96±57.9*	783.32±28.21*	40.96±5.79*	0.24±0.02*	20.31±5.75*
DC	54.06±5.01$^{\#}$	150.86±10.6$^{\#}$	711.48±34.46$^{\#}$	71.75±3.60$^{\#}$	0.70±0.02$^{\#}$	35.35±3.61#
DLDC	90.84±2.87*	573.28±4.06*	780.64±24.02*	48.31±3.21*	0.17±0.01*	16.55±0.65*
DHDC	110.19±2.74*	674.57±15.67*	788.25±46.87*	57.34±7.15*	0.40±0.01*	16.11±1.80*
DSM	51.80±6.38$^{\#}$	583.33±31.8*	754.29±40.8*	55.08±5.99*	0.34±0.03*	17.85±1.81*
NTXC	67.29±6.33$^{\#}$	466.24±10.45*	754.81±25.91*	54.24±2.78*	0.25±0.01*	16.71±4.41*

Values = mean ± SD; n = n = 5 (NC and NTXD) and 7 (DC, DLDC, DHDC and DSM). *Statistically ($p<0.05$) significant to DC, #statistically significant ($p<0.05$) to NC. **NC** = normal rats, **DC** = diabetic control, **DLDC** = diabetic rats + *C. nitida* (150 g/kg bw), **DHDC** = diabetic rats + *C. nitida* (300 g/kg bw), **DSM** = diabetic rats + metformin (200 g/kg bw), and **NTXC** = Normal rats + *C. nitida* (300 g/kg bw).

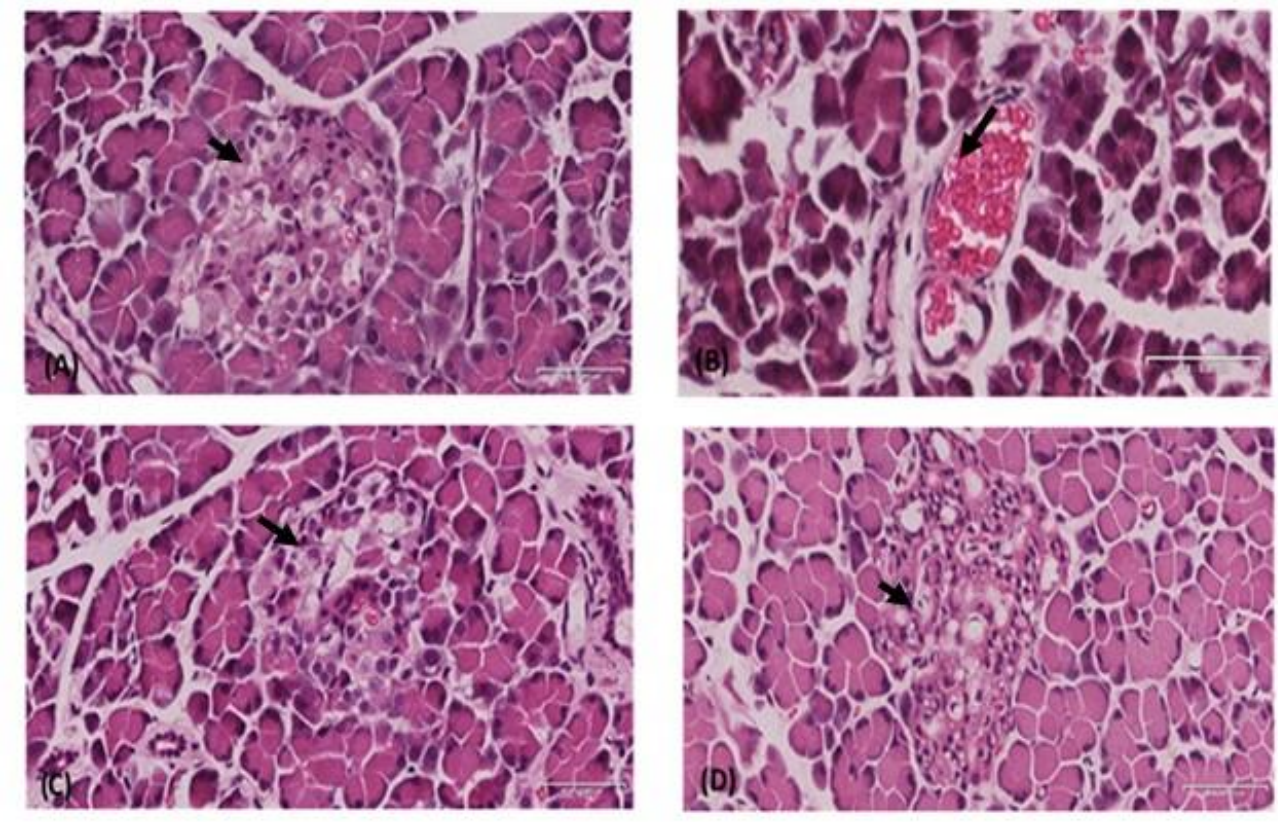
Table 7.3.3: Organ function biomarkers of experimental groups

Enzyme Activities	**NC**	**DC**	**DLDC**	**DHDC**	**DSM**	**NTXC**
ALP U/L	101.00±5.55*	1235.00±29.88#	459.75±2.83*	540.00±9.90*	582.33±10.67*	93.00±5.56*
ALT U/L	91.00±4.24	87.50±0.71	87.5±0.58	88.00±1.41	88.00±3.20	87.33±1.53
CK-MB U/L	625.00±15.80*	959.00±25.10#	815.3±68.59	684.3±13.65#	739.40±12.89#	484.50±15.84*
Fructosamine mg/dL	656.00±12.6*	743.00±15.56#	700.3±68.59*	695.00±32.39#	658.50±12.02*	573.00±15.56*
Urea mg/dL	58.00±0.71	46.00±5.67	37.00±2.31#	28.00±2.65#	31.50±6.81#	55.33±7.66
Uric acid mg/dL	2.46±0.24	3.48±0.70	2.035±0.39	2.04±0.03	1.60±0.23*#	2.02±0.31

Values = mean ± SD; n = n = 5 (NC and NTXD) and 7 (DC, DLDC, DHDC and DSM). *Statistically ($p<0.05$) significant to DC, #statistically significant ($p<0.05$) to NC. **NC** = normal rats, **DC** = diabetic control, **DLDC** = diabetic rats + *C. nitida* (150 g/kg bw), **DHDC** = diabetic rats + *C. nitida* (300 g/kg bw), **DSM** = diabetic rats + metformin (200 g/kg bw), and **NTXC** = Normal rats + *C. nitida* (300 g/kg bw).

There were significant ($p<0.05$) increase in the serum levels of ALP, CK-MB, fructosamine and uric acid after the induction of T2D as shown in **Table 7.3.3**. These levels were significantly ($p<0.05$) reduced in all the treatment groups, with rats treated with high dose of *C. nitida* ($p<0.05$) having the most reduced levels. Although induction of T2D had little or no significant effect on uric acid level, it was however significantly ($p<0.05$) reduced in all treatment groups compared to the diabetic control group (**Table 7.3.3**).

As shown in **Figure 7.3.4**, induction of T2D led to depletion and inflammation of the pancreatic β-cells, with concomitant alteration in the acinar cell population (**Figure 7.3.4B**). This contrasts the intact pancreatic β- and acinar cells and intralobular ducts of that of the normal control (NC) group. The altered morphology was restored to near normal in all treatment groups as evident by the replenished β- and acinar cells (**Figures 7.3.4C – 7.3.4E**).



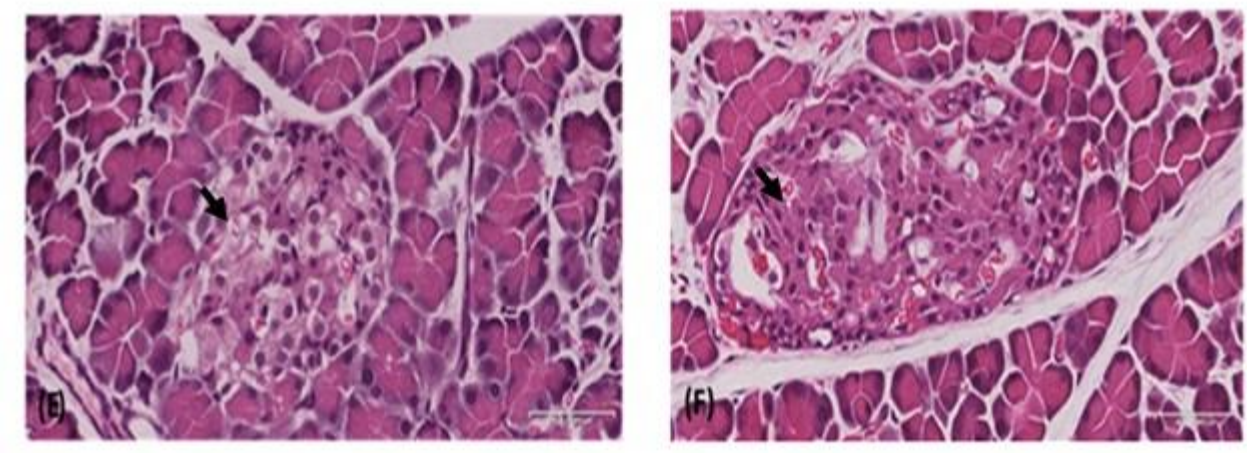


Figure 7.3.4: Morphological changes in pancreatic tissues of experimental groups. Magnification: 10x. (A) = NC, (B) = DC, (C) = DLDC, (D) = DHDC, (E) = DSM, and (F) = NTXC. NC = normal rats, DC = diabetic control, DLDC = diabetic rats + *C. nitida* (150 g/kg bw), DHDC = diabetic rats + *C. nitida* (300 g/kg bw), DSM = diabetic rats + metformin (200 g/kg bw), and NTXC = Normal rats + *C. nitida* (300 g/kg bw). Arrow: β-cells

As revealed in **Figure 7.3.5**, induction of T2D also led to the alteration in the clusters of exocrine cells and acini in the pancreatic tissues, with concomitant disruption of the surface capillary network (**Figure 7.3.**5B) as compared to NC (**Figure 7.3.5A**). Treatment with *C. nitida* improved the capillary network, while restoring the clusters of exocrine cells and acini (**Figures 7.3.5C** and **7.3.5D**). Metformin-treated rats exhibited similar recovery as well (**Figure 7.3.5E**).

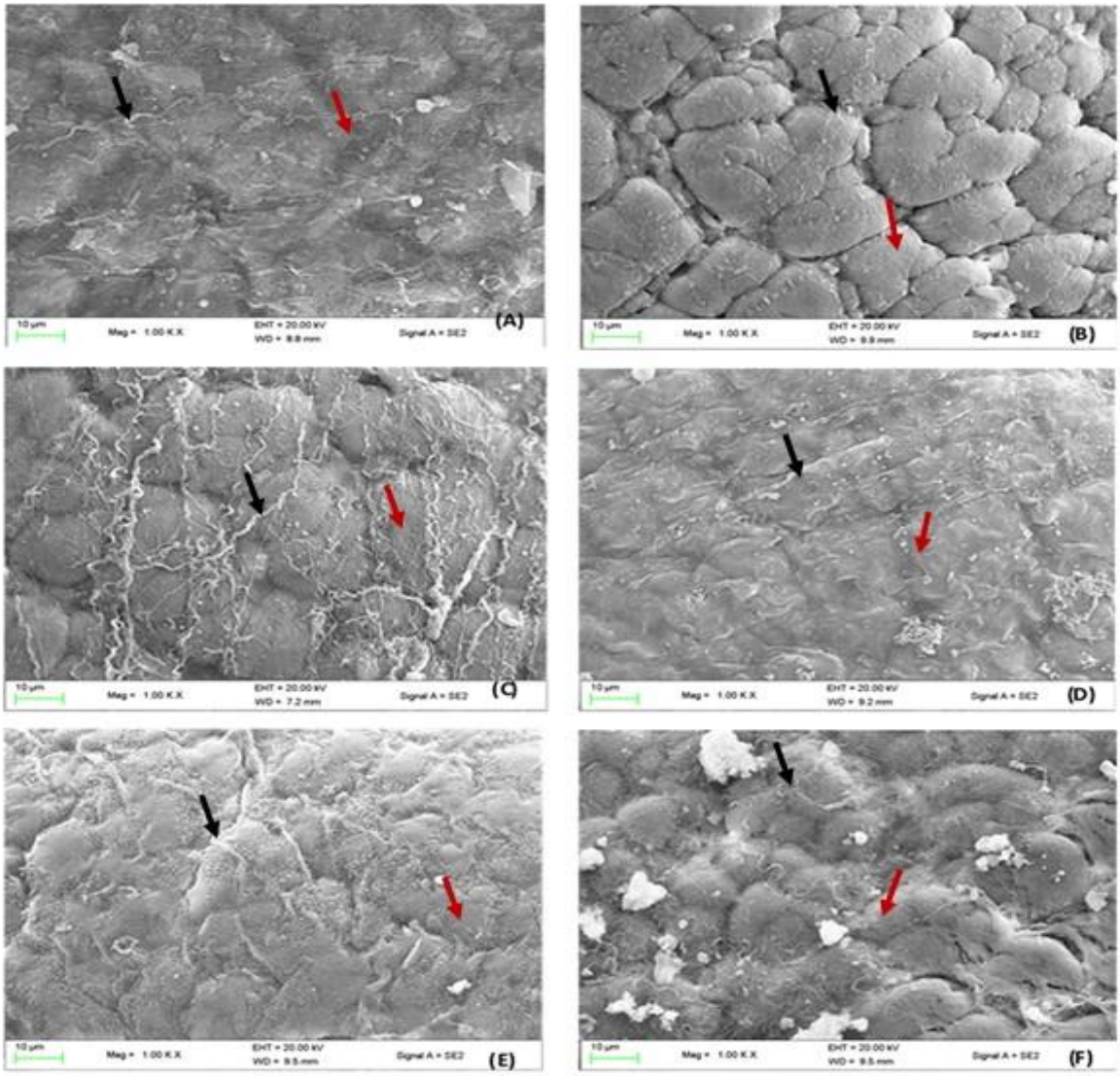


Figure 7.3.5: Electron microscopy of pancreatic tissues of experimental groups. Magnification = 1000x. (A) = NC, (B) = DC, (C) = DLDC, (D) = DHDC, (E) = DSM, and (F) = NTXC. **NC** = normal rats, **DC** = diabetic control, **DLDC** = diabetic rats + *C. nitida* (150 g/kg bw), **DHDC** = diabetic rats + *C. nitida* (300 g/kg bw), **DSM** = diabetic rats + metformin (200 g/kg bw), and **NTXC** = Normal rats + *C. nitida* (300 g/kg bw). Arrow: Black = blood vessels; Red = acini

There was an insignificant upregulation of caspase-3 expression in the pancreatic tissues of untreated diabetic rats (DC) as shown in Fig. 6A. Treatments with *C. nitida* and metformin had little or no significant effect on the upregulated expression (**Figure 7.3.6C – 7.3.6E**).

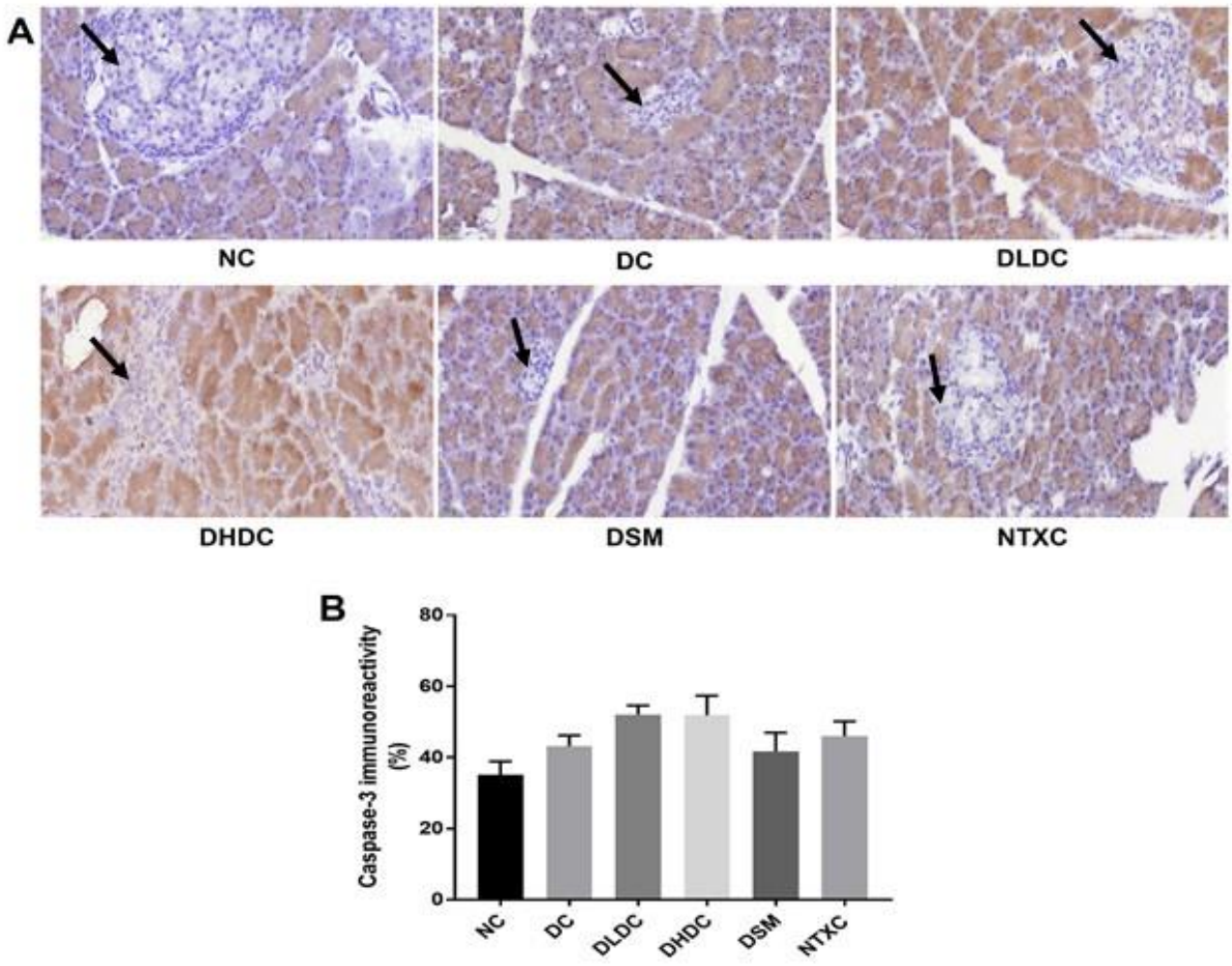


Figure 7.3.6: (A): Immunohistochemistry photomicrographs and (B): immunoreactivity of caspase3 expression in pancreatic tissues of experimental groups. Magnification = 200x; Values = mean ± SD; n = 5 (NC and NTXD) and 7 (DC, DLDC, DHDC and DSM). *Statistically ($p<0.05$) significant to DC, #statistically significant ($p<0.05$) to NC. **NC** = normal rats, **DC** = diabetic control, **DLDC** = diabetic rats + *C. nitida* (150 g/kg bw), **DHDC** = diabetic rats + *C. nitida* (300 g/kg bw), **DSM** = diabetic rats + metformin (200 g/kg bw), and **NTXC** = Normal rats + *C. nitida* (300 g/kg bw). Arrow: β-cells

Similarly, induction of T2D led to upregulation of Nrf2 in the pancreatic tissues as depicted in **Figure 7.3.7A** and **7.3.7B**. Treatment with *C. nitida* significantly ($p<0.05$) downregulated the expressions in diabetic rats, suggesting an inhibitory effect.

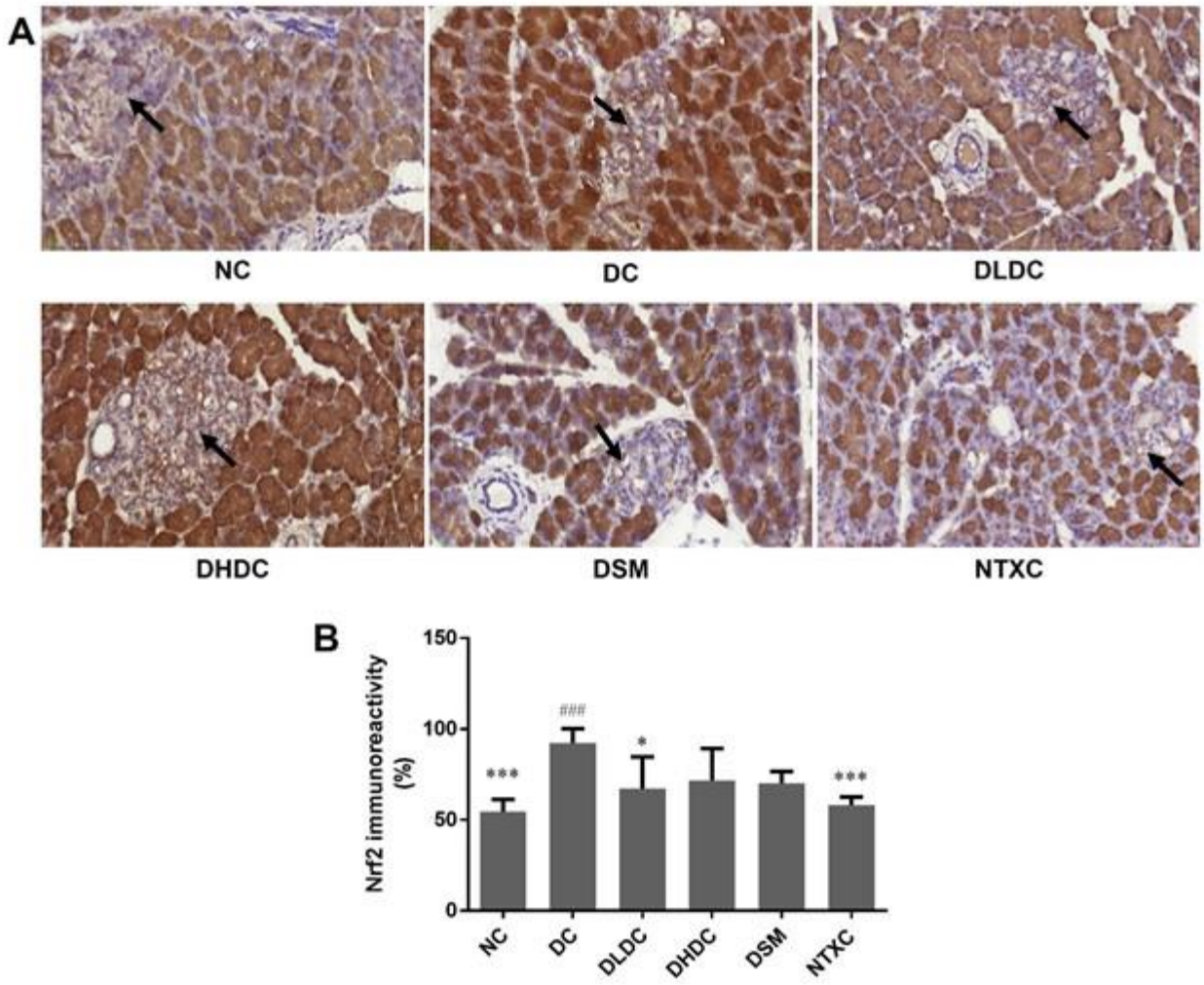


Figure 7.3.7: (A): Immunohistochemistry photomicrographs and (B): immunoreactivity of Nrf2 expression in pancreatic tissues of experimental groups. Magnification = 200x; Values = mean ± SD; n = n = 5 (NC and NTXD) and 7 (DC, DLDC, DHDC and DSM). *Statistically ($p<0.05$) significant to DC, #statistically significant ($p<0.05$) to NC. **NC** = normal rats, **DC** = diabetic control, **DLDC** = diabetic rats + *C. nitida* (150 g/kg bw), **DHDC** = diabetic rats + *C. nitida* (300 g/kg bw), **DSM** = diabetic rats + metformin (200 g/kg bw), and **NTXC** = Normal rats + *C. nitida* (300 g/kg bw). Arrow: β-cells

^{1}H NMR analysis of *C. nitida* infusion revealed the presence of the alkaloids, caffeine [^{1}H NMR (600 MHz, DMSO, δ, ppm) 7.98 (s, H, ArA), 3.88 (s, 3H, CH_3), 3.22 (s, 3H, CH_3), 3.14 (s, 3H, CH_3)] and theobromine [^{1}H NMR (600 MHz, DMSO, δ, ppm) 8.75 (s, br, 1H, NH), 7.95 (s, 1H,

ArA), 3.84 (s, 3H, CH_3)] as depicted in **Figure 7.3.8**. The data were compared with literature (Ohsaki et al. 1986).

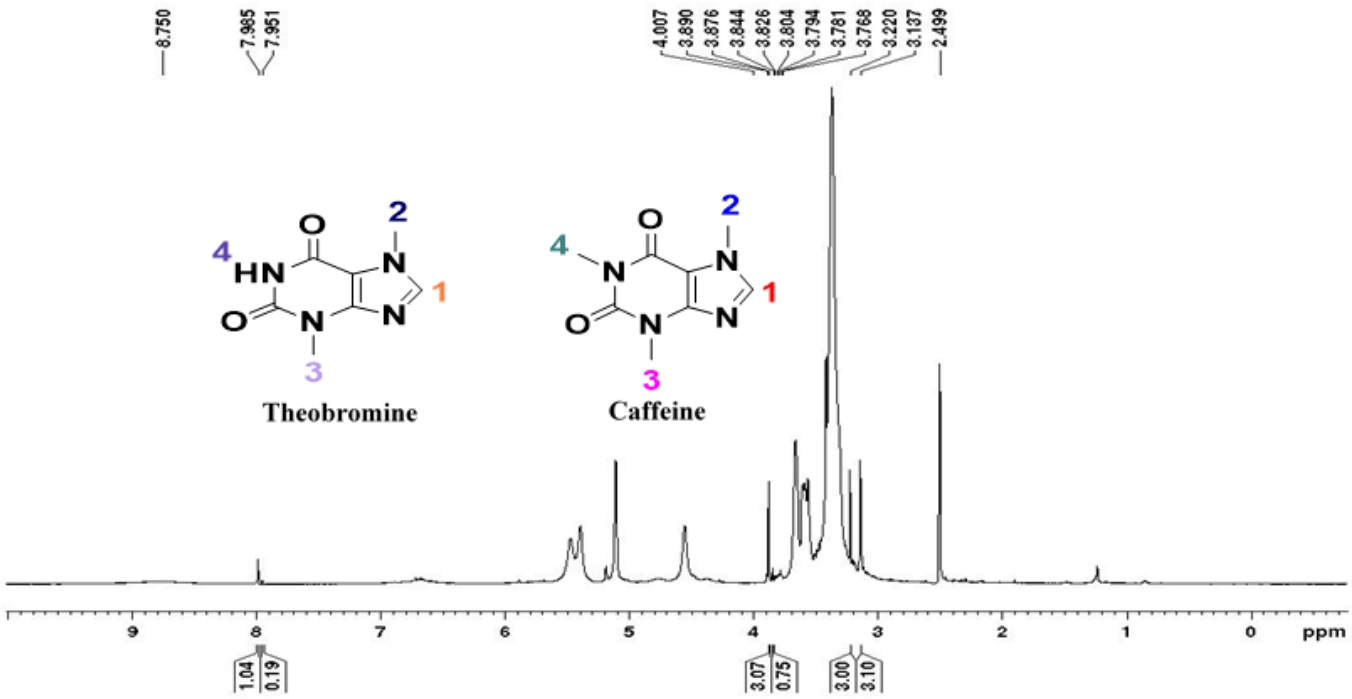


Figure 7.3.8: NMR chromatogram and identified compounds of *C. nitida* hot infusion

The 3D structure of caspase-3-caffeine (highest binding affinity) complex and Nrf2-theobromine complex are shown in **Figure 7.3.9.** The active site residues are represented in CPK format; 2-D representations of docked complex of caspase-3-caffeine (highest binding affinity) complex and Nrf2-complex theobromine showing the different interactions with amino acid residues are also displayed in **Figure 7.3.9**.

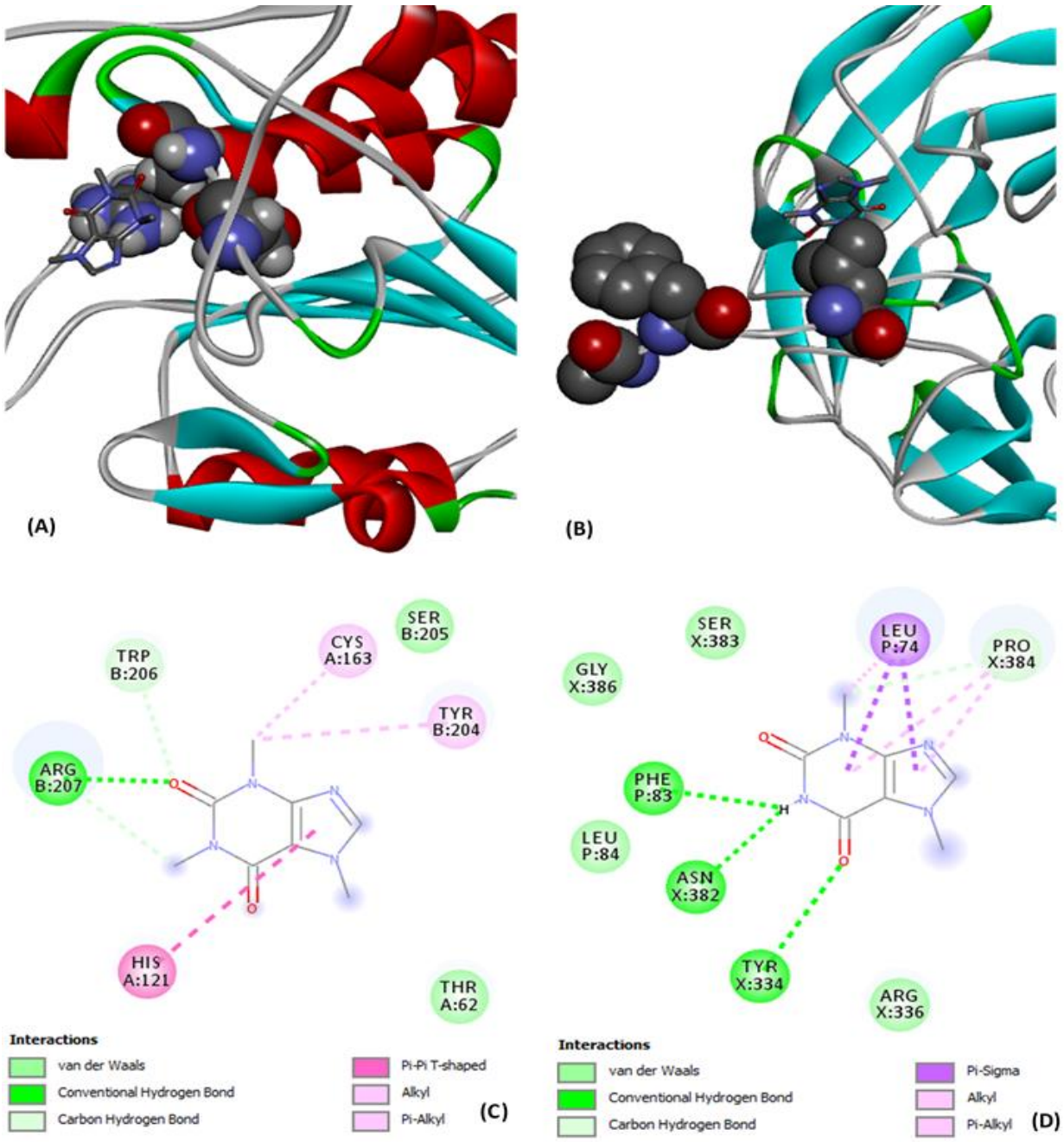


Figure 7.3.9: 3D structure of (A) caspase-3 and caffeine (highest binding affinity) and (B) Nrf2 and theobromine. The active site residues are represented in CPK format; 2-D representations of docked complex of (C) caspase-3 and caffeine (highest binding affinity) and (D) Nrf2 and theobromine showing the different interactions with amino acid residues.

The Binding affinities in kcal mol $^{-1}$ of compounds with caspase3 and Nrf2 are presented in **Table 7.3.4**. Results revealed that Caffeine binds with caspase3 with a higher binding affinity -5.0

compared to Theobromine while the binding of theobromine to Nrf2 gave higher binding energy compared to caffeine-Nrf2 complex

Table 7.3. 4: Binding affinities of NMR-identified compounds with caspase3 and Nrf2

Compounds	**Binding affinity (kcal mol^{-1})**	
	Caspase-3	**Nrf2**
Theobromine	4.8	4.9
Caffeine	5.0	4.3

7.3.5 Discussion

The quest for affordable medicine to augment the cost of treating DM have led to a paradigm shift to medicinal plants with little or no side effects. This have also led to the search of the active phytochemical constituents that mainly influence the medicinal properties of the plants. In this study, the antidiabetic properties of *C. nitida* as well as possible underlying mechanisms were investigated in T2D rats. Its phytochemical constituents were also investigated.

The reduced bodyweights of the untreated diabetic rats (**Figure 7.3.1A**) is consistent with previous studies in STZ-induced diabetes models (Gajdosik et al. 1999; Ibrahim et al. 2016). This also correlates with the increased food and fluid intake (**Figure 7.3**.1B), which depicts an occurrence of polyphagia and polydipsia (Ibrahim et al. 2016). Although treatment with *C. nitida* did not significantly suppress polyphagia and polydipsia, the increased weight gains in DLDC and DHDC groups (**Figure 7.3.1A**) may indicate a therapeutic effect against weight loss in T2D.

Chronic hyperglycemia resulting from consistent hyperglycemia is amongst the major features of T2D (Monnier et al. 2006). Studies have attributed the hyperglycemia which is consistent to insulin insufficiency arising from depleted pancreatic β-cell mass and dysfunction (Donath and Halban 2004). This is in consent with the elevated blood glucose level (**Figure 7.**3.2A), depleted serum insulin level and pancreatic β-cell function (Fig. 2C) and pancreatic weight loss (**Figure 7.3**.1C) in the untreated diabetic rats. These can be attributed to the depleted and inflamed pancreatic β-

cells (**Figure 7.3**.4B), as inflammation of the β-cells have been implicated its dysfunction and failure, which impairs insulin secretion and utilization, and thereby leading to hyperglycemia (Donath et al. 2009; Newsholme et al. 2013). The ability of *C. nitida* to reduce blood glucose level (**Figure 7.3.2A**), with concomitant elevated pancreatic β-cell function and serum insulin level (**Figures 7.3.2C** and **7.3.2D**) indicates an antidiabetic effect. This is also evident by the regenerated β-cells and improved morphology (**Figure 7.3.4C** and **7.3.4D**) and increased pancreatic weight (**Figure 7.3.1C**). The improved glucose tolerance in diabetic rats treated with *C. nitida* (**Figure 7.3**.2B) further portrays the anti-hyperglycemic potential of the infusion.

Activation of key enzymes involved in glycogenolytic, gluconeogenic and glycolytic pathways have been implicated in increased glycogenolysis, gluconeogenesis and glycolysis in T2D, respectively (Clore et al. 2000; Guo et al. 2012; Wu et al. 2005). Thus, the increased glycogen phosphorylase and fructose-1,6-biphosphatase activities in the untreated diabetic rats (**Table 7.3.1**), implies the activation of glycogenolysis and gluconeogenesis respectively after the induction of T2D. Activation of these enzymes may contribute to the elevated blood glucose level (**Figure 7.3**.2A) as increased glycogenolysis and gluconeogenesis will lead to increase endogenous glucose output (EGO) in T2D and suppressed insulin functions, thereby exacerbating hyperglycemia (Basu et al. 2005; Gastaldelli et al. 2000). The increased glucose-6-phosphatase activity in the untreated diabetic rats (**Table 7.3.1**) indicates an activation of the glycolytic pathway, which has been linked to the pathogenesis of T2D owing to the generated metabolites for polyol, AGEs, protein kinase C, and hexosamine pathways (Luo et al. 2016; Rolo and Palmeira 2006). The reduced activities of the enzymes in diabetic rats treated with *C. nitida* (**Table 7.3.1**), therefore insinuates a suppressive effect by the infusion on glycogenolysis, gluconeogenesis and glycolysis in T2D. This also correlates with the depleted blood glucose and elevated serum insulin levels (**Figures 7.3.2A** and **7.3.2C**).

The pancreatic α-amylase play an essential role in the elevation of postprandial glucose level as it involved in the breakdown of dietary carbohydrate to glucose (Adisakwattana et al. 2011; Kim et al. 2005). Therefore, its higher activity in the untreated diabetic rats (**Table 7.3.1**) may also contribute to their elevated blood glucose level (**Figure 7.3.2A**). The higher activity can be attributed to the distorted pancreatic acinar cells (**Figure 7.3.4B**) as these cells are responsible for the secretion of digestive enzymes from pancreas (Muniraj et al. 2015; Williams 2010). The

reduced activities in diabetic rats treated with *C. nitida* (**Table 7.3.1**), therefore indicates an inhibitory effect by the infusion which also correlates with their improved acinar cell morphology (**Figures 7.3.4C** and **7.3.4D**). The inhibitory activity of *C. nitida* may validate previous studies that reported the ability of its hot infusion and phenolic extract to inhibit the activities of α-amylase *in vitro* (Erukainure et al. 2017b; Oboh et al. 2014a). This is of great advantage as inhibition of α-amylase activities are the main mechanism of some commercial antidiabetic drugs such as miglitol and acarbose (Chelladurai and Chinnachamy 2018; Rahimzadeh et al. 2014)

Decreased ATPase activity with concomitant influx of Ca^{2+} leading to depolarization of the pancreatic β-cell membrane has been reported as one of the major mechanism by which glucose stimulates insulin secretion (Owada et al. 1999). Thus, implying that the decreased ATPase activity in diabetic rats treated with *C. nitida* (**Table 7.3**.1) reflects an increased stimulation of insulin secretion. This is consistent with the increased serum insulin level of the groups (**Figure 7.3.2C**). Decreased serum ATPase activity has however been reported in T2D (Kiziltunç et al. 1997; Zadhoush et al. 2015), which corroborates with the decreased activity of the untreated diabetic rats (**Table 7.3.1**) in the present study. The increased activity in the treatment groups, thus further indicates an antihyperglycemic potential of *C. nitida*.

Persistent hyperglycemia and insulin resistance in T2D have been implicated in the alteration of lipid metabolism, leading to diabetic dyslipidemia (Parhofer 2015; Vijayaraghavan 2010). It is often characterized by elevated levels of total cholesterol, LDL-cholesterol, triglycerides, and depleted level of HDL-c (Boden and Laakso 2004). This correlates with the elevated levels of triglycerides, LDL-c and depleted HDL-c level in the untreated diabetic rats (**Figure 7.3**.3), thus indicating an occurrence of diabetic dyslipidemia. The normal level of total cholesterol in all the experimental groups is in consent with previous reports that total cholesterol level may not be altered in dyslipidemia (Boden and Laakso 2004; Parhofer 2015; Sugden and Holness 2011). The reversed levels in the treatment groups therefore indicates an antilipemic activity of *C. nitida*. This corroborates previous studies which reported the ability of *C. nitida* methanolic extract to suppress hyperlipidemia in alloxan-induced diabetic rats (Dorathy et al. 2014)

Oxidative stress and proinflammation have been recognized as the major mechanism of hyperglycemia-induced pancreatic injury leading to destruction of the pancreatic β-cells, which in turn alters insulin secretion and β-cell function. This can be attributed to the extremely low levels

of endogenous antioxidant enzymes the pancreas, thus making the organ highly susceptible to oxidative damage (Acharya and Ghaskadbi 2010; Donath 2014). This is evident in the present study by the depleted levels of GSH, SOD and catalase activities, and concomitant elevated levels of MDA, NO, and myeloperoxidase activity in both serum and pancreatic tissues of the untreated diabetic rats (**Table 7.3.2**). The depleted GSH level, SOD and catalase activities indicates an occurrence of oxidative stress on induction of T2D. The role of these antioxidant markers in the maintenance of redox balance have been reported in several studies, as they protect the cells against the harmful effects of free radicals particularly superoxide anion ($O_2^{\bullet-}$). SOD dismutates $O_2^{\bullet-}$ to hydrogen peroxide (H_2O_2), which in turn is converted by catalase to water (H_2O) and oxygen (O_2). If not dismutated by SOD, $O_2^{\bullet-}$ can react with NO to produce the potent nitric radical peroxynitrite ($ONOO^-$). If the generated H_2O_2 from SOD dismutation is not acted on by catalase, it will be broken down to hydroxyl radical (•OH), a potent radical implicated in lipid peroxidation. H_2O_2 can also be converted to hypochlorous acid (HOCl) in the presence of hydrochloric acid, catalyzed by myeloperoxidase. The high level of NO and myeloperoxidase activity indicates proinflammation, which also corroborates the increased acetylcholinesterase activity (**Table 7.3.1**). Increased activity of pancreatic acetylcholinesterase has been implicated in inflammation of the tissue (Zhang et al. 2012). The high MDA level reflects an occurrence of lipid peroxidation, which can be attributed to the reduced catalase activity. The reversion of these levels and activities of these biomarkers in the treatment groups therefore indicates the antioxidative and anti-proinflammatory protective potentials of *C. nitida* in T2D, which may be portrayed by the improved β-cell function (**Figure 7.3.2C**) and attenuation of inflamed β-cells (**Figures 7.3.4C** and **7.3.4D**). This corroborates previous studies which reported the antioxidant activities of *C. nitida* infusion (Erukainure et al. 2017b; Oboh et al. 2014a).

Alteration in pancreatic perfusion have been reported in T2D, and has been linked to β-cell dysfunction (Honka et al. 2014). Pancreatic perfusion occurs via multiple arteries resulting from the abdominal aorta and contributes to the physiological maintenance of the islets and normal endocrine (Honka et al. 2014; Jansson et al. 2016). The decreased capillary network on the pancreatic tissue of the untreated diabetic rats (**Figure 7.3.5B**) may thus reflect an altered perfusion and may be attributed to the β-cell dysfunction (**Figure 7.3.2C**). An increased perfusion on

treatment with *C. nitida* is portrayed by the increased network on the pancreatic surfaces of the treatment groups (**Figures 7.3.5C – 7.3.5F**).

The pancreatic β-cell apoptosis often arising from hyperglycemia-induced oxidative stress and inflammation have been implicated in the pathogenesis and progression of T2D and its complications (Acharya and Ghaskadbi 2010; Rojas et al. 2018; Tomita 2016). Caspases have been vastly studied for their orchestrating effect on apoptosis. The rather insignificant effect of *C. nitida* on the increased expression of caspase3 in diabetic rats (**Figures 7.3.6C** and **7.3.6D**), implies that the antidiabetic mechanism of the infusion does not involve the caspase cascade. This however contrasts the molecular interaction of the identified active constituents of the infusion, caffeine and theobromine with caspase3 (**Figures 7.3.9A** and **7.3.9C**, and **Table 7.3.4**). The molecular interactions may rather reflect an exacerbative effect than suppressive.

Although exacerbated Nrf2 expression have linked with improved antioxidant status in some studies (David et al. 2017; Dieter 2014), its upregulation has however been reported in the onset of T2D (He et al. 2012; Miao et al. 2012). This reflected by the increased expression in pancreatic tissues of the untreated diabetic rats, thereby contradicting some reports on upregulation of Nrf2 as possible therapeutic target in T2D (David et al. 2017; Dieter 2014). Its activation of oxidative stress has also been reported, which also correlates with the exacerbated pancreatic redox imbalance (**Table 7.3.2**). Downregulation of its expression in diabetic rats treated with *C. nitida* (**Figure 7.3.7C** and **7.3.7D**) as well as the other treatment groups, depicts a suppressive effect by the infusion. The downregulation may also contribute to the improved pancreatic antioxidative status (**Table 7.3.2**). The down regulatory effect of the infusion is also portrayed by the molecular interaction of its phytochemical constituents (**Figure 7.3.9B** and **7.3.9D**, and **Table 7.3.4**).

The increased fructosamine level of the untreated diabetic rats (**Table 7.3.3**) portrays an activation of the glycation cascade, as it has been reported as an early stage glycated protein product oxidatively sliced to form advanced glycation end (AGE) products (Ibrahim et al. 2016; Sen et al. 2011). It is a glycemic biomarker for diabetes (Malmström et al. 2014). The reduced level in the treatment groups therefore indicate a disruption in the cascade by *C. nitida*.

The increased ALP, CK-MB and uric acids levels have been reported in T2D (Čaušević et al. 2010; Erukainure et al. 2017a; Fazel et al. 2005), which correlates with the elevated levels in the

untreated diabetic rats in the present study (**Table 7.3.4**). These elevations portray hepato-, cardio-, and nephro-toxicities (Erukainure et al. 2018; Mortazavi et al. 2016) and can be attributed to leakage into the blood stream due to inflammation of the liver, heart, and kidney respectively (Giordano et al. 2015; Kim et al. 2008; Peppes et al. 2008). The depleted levels of these biomarkers in rats treated with *C. nitida*, portrays a protective effect of the infusion against hyperglycemia-induced hepato-, cardio- and nephro-toxicities. This correlates with previous studies that reported the ability of the methanolic extract of *C. nitida* to suppress the elevation of serum liver and kidney biomarkers in diabetic rats (Adeosun et al. 2017).

The NMR-identified phytochemical constituents of *C. nitida* infusion (**Figure 7.3.8**), caffeine and theobromine is in consent with previous reports on the phytochemical properties of *C. nitida* (Erukainure et al. 2017b; Lowor et al. 2010). These alkaloids have been reported for their biological activities. Caffeine has been recognized to exert its effects via antagonism of the adenosine receptors (Kerr et al. 1993). Its coadministration have been reported to improve the hypoglycemic activities of gliclazide and metformin (Mohiuddin et al. 2009). Theobromine has been reported for its antioxidant, anti-hyperlipidemic and antidiabetic activities (Martínez-Pinilla et al. 2015; Mubarak and Yiquen 2015; Ramos et al. 2017). Thus, the antidiabetic effect of *C. nitida* infusion can be attributed to the synergetic activities of the identified alkaloids.

7.3.6 Conclusion

These results indicate the therapeutic potentials of *C. nitida* infusion against T2D, which may be attributed to the synergetic effect of caffeine and theobromine. This gives credence to the folkloric claims and use of the plant in the treatment and management of DM and its complications.

7.4 Hyperglycemia-Induced Oxidative Brain Injury: Therapeutic Effects of *Cola nitida* Infusion against Redox Imbalance, Cerebellar Neuronal Insults, and Upregulated Nrf2 Expression in type 2 diabetes Rats

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Preface: This article investigated the antioxidative protective effect of *Cola nitida* on hyperglycemia-induced brain injury in a type 2 diabetes model of rats. Its antioxidative and anti-proinflammatory effects were investigated. Its inhibitory effect on cholinergic enzyme activity and Nrf2 expression were investigated. Its ability to protect against neuronal damages and elemental dysregulation was also investigated.

7.4.1 Abstract

Neurodegeneration owing to persistent hyperglycemia has been associated with type 2 diabetes (T2D), with oxidative stress playing an influential role. In this study, the therapeutic effect of the hot water infusion of *Cola nitida* against hyperglycemia-induced neurotoxicity, cerebellar neurodegeneration and elemental dysregulation was investigated in fructose-streptozotocin induced T2D rats. A diabetic group was administered distilled water and served as a negative control, two other diabetic groups were treated with *C. nitida* infusion at 150 and 300 mg/kg body weight respectively, while another was administered a standard anti-diabetic drug, metformin (200 mg/kg bodyweight). Two other groups consisting of normal rats, were administered distilled water (normal control) and *C. nitida* infusion (300 mg/kg bodyweight). After 6 weeks of treatment, their

brains were collected. Treatment with *C. nitida* led to the suppression of oxidative stress by significantly ($p<0.05$) elevating reduced glutathione (GSH) level, superoxide dismutase and catalase activities, with concomitant depletion of malondialdehyde level. Acetylcholinesterase and ATPase activities were significantly ($p<0.05$) inhibited in *C. nitida*-treated diabetic rats. Histological and microscopic analysis also revealed a restorative effect of *C. nitida* on T2D-altered distribution of elements, neurons and axonal nodes. Treatment with *C. nitida* also led to significant inhibition of Nrf2 expression in the cerebellar cortex. These results suggest the therapeutic effects of *C. nitida* in maintenance of the neuronal integrity and antioxidant status of the brain in T2D. These neuroprotective activities can be attributed to the GC-MS identified alkaloid, caffeine in the infusion.

Keywords: *Cola nitida*; SEM-EDX; Neurodegeneration; Nrf2; and Type 2 diabetes

7.4.2 Introduction

Neurodegeneration have been recognized amongst the many complications of type 2 diabetes (T2D), with the brain being the most affected (Gejl et al. 2017; Wrighten et al. 2009). The susceptibility of the brain to T2D can attributed to its polyunsaturated fatty acids (PUFAs) constituents, which are the target of peroxidative attack in oxidative stress. Oxidative stress arises in T2D owing to increase production of free radicals outweighing the body's endogenous antioxidant system (Maritim et al. 2003a). This increase has been attributed to chronic hyperglycemia arising from persistent high blood glucose level due to insulin resistance and pancreatic β-cell dysfunction (Maritim et al. 2003a). Insulin resistance and pancreatic β-cell dysfunction are the main features of T2D and distinct it from type 1 diabetes (T1D), which occurs owing to the inability of the pancreatic β-cell to secrete insulin (Erukainure et al. 2018).

In spite of high blood glucose level, glucose uptake to the brain have been reported to be altered in T2D owing to downregulation of glucose transporters at the blood brain barrier (BBB) (Gejl et al. 2017; Hwang et al. 2017; Pardridge et al. 1990). This alteration can also be a major contributor to brain damage in T2D, as the brain is glucose-dependent for its functions.

The cost of treating diabetes mellitus (DM) and its complications are of tremendous concern to health practitioners, particularly in developing countries with poor health infrastructure and very low gross domestic products (GDP). The search for cheaper alternatives with little or no side

effects have led a paradigm shift to natural medicines, with medicinal plants playing an influential role. Medicinal plants have been reported for their roles in the folkloric treatment of several ailments such as DM, malaria, tuberculosis and neurodegeneration.

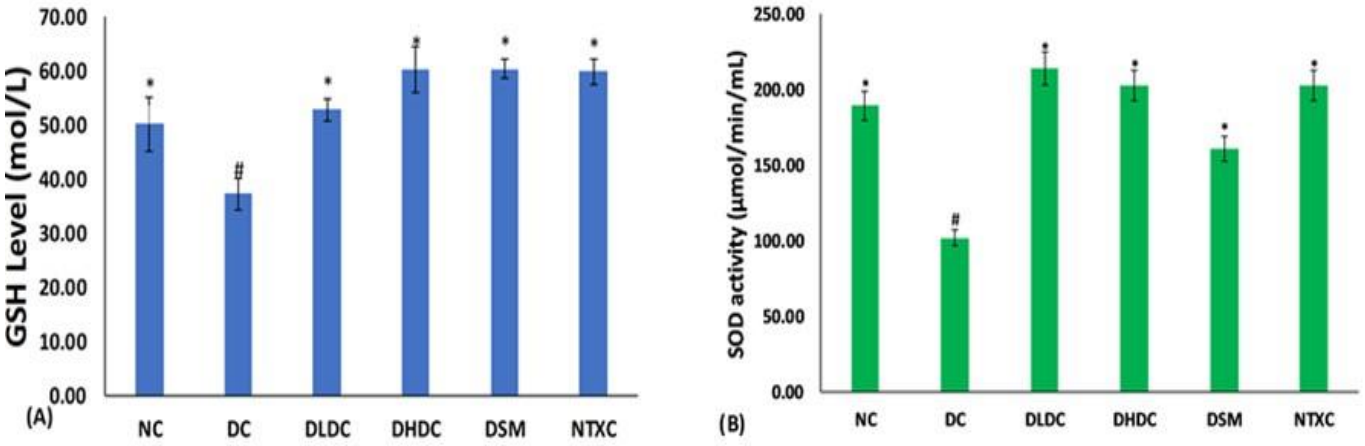
This study aims at investigating the therapeutic effect of *C. nitida* hot infusion on oxidative imbalance, ATPase and cholinergic enzyme activities in whole brain of T2D rats. The cerebellar cortexes were also investigated for morphological changes, neuronal and elemental distribution, as well as Nrf2 expression.

7.4.3 Materials and Methods

Kindly refer to Chapter 2, subsections 2.1.1; 2.1.4; 2.10.1; 2.12 – 2.13 for details materials and methods.

7.4.4 Results

As depicted in Fig. 1, there was a significant ($p<0.05$) decrease in GSH level (**Figure 7.4.1A**), SOD and catalase activities (**Figures 7.4.1B** and **7.4.1C**), with concomitant elevation of MDA level (**Figure 7.4.1D**) on induction of T2D. These were significantly ($p<0.05$) reversed in the treatment groups, with *C. nitida* displaying a favourable antioxidant activity when compared to metformin.



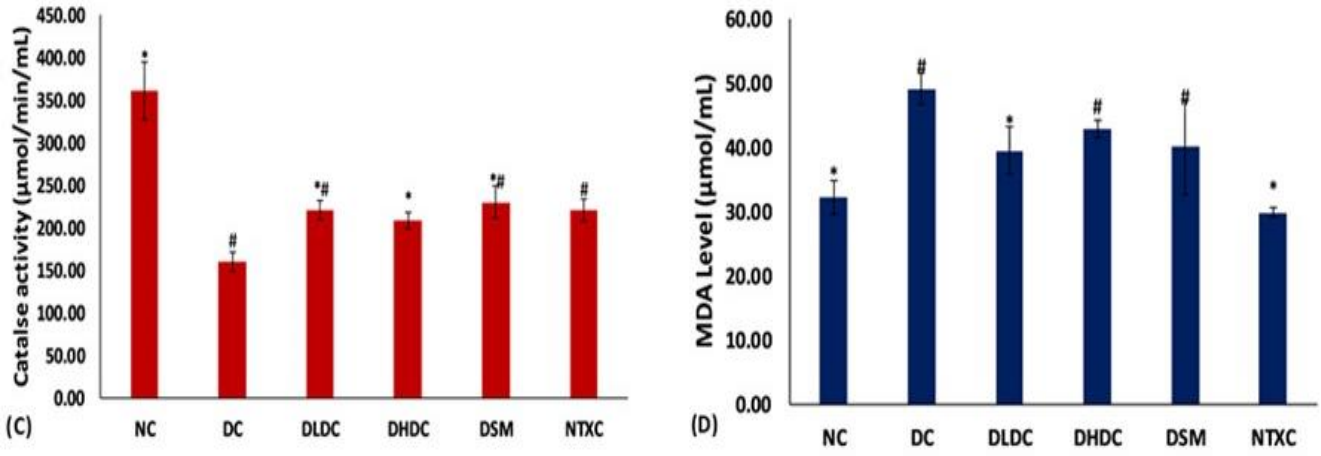


Figure 7.4.1: Antioxidant status of experimental groups. Values = mean ± SD; n = 5 (NC and NTXC) and 7 (DC, DLDC, DHDC, and DSM). *Statistically ($p<0.05$) significant to DC, #statistically significant ($p<0.05$) to NC. **NC** = normal rats, **DC** = diabetic control, **DLDC** = diabetic rats + *C. nitida* (150 g/kg bw), **DHDC** = diabetic rats + *C. nitida* (300 g/kg bw), **DSM** = diabetic rats + metformin (200 g/kg bw), and **NTXC** = Normal rats + *C. nitida* (300 g/kg bw).

Induction of T2D led to an increased myeloperoxidase activity as shown in **Figure 7.4.2**, depicting a proinflammatory effect. Treatment with both doses of *C. nitida* led to significant ($p<0.05$) reduction reaching levels indistinguishable from the normal controls (NC).

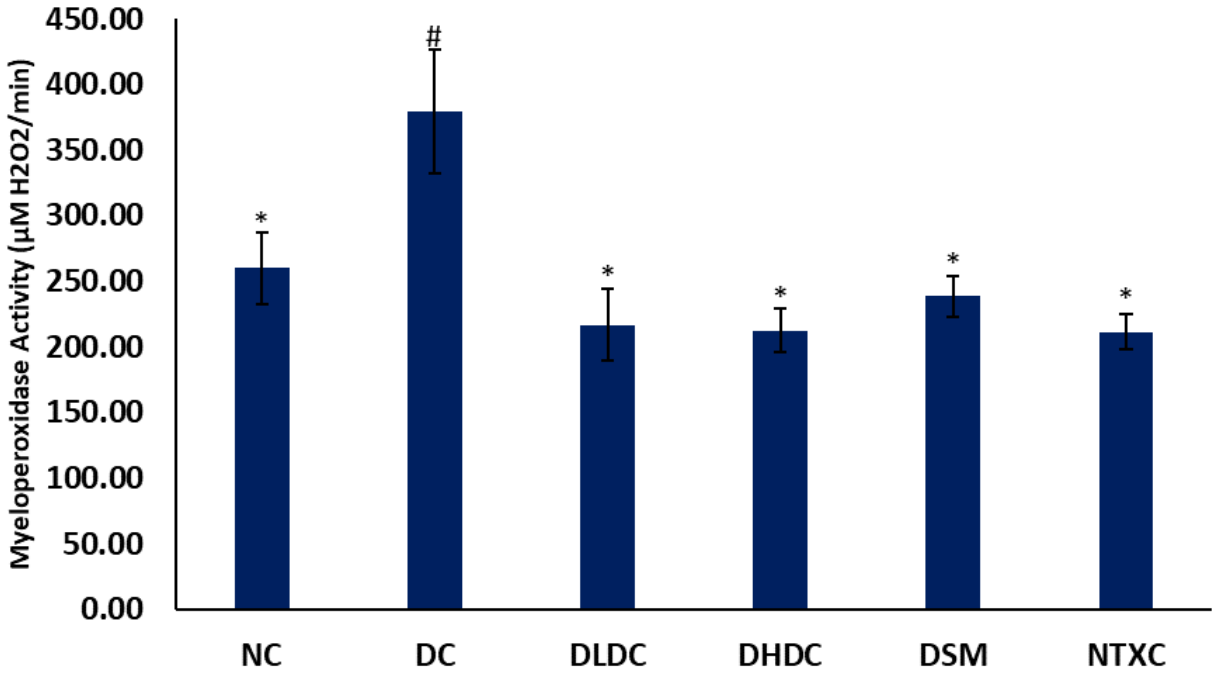


Figure 7.4.2: Myeloperoxidase activities of experimental groups. Values = mean ± SD; n = 5 (NC and NTXC) and 7 (DC, DLDC, DHDC, and DSM). *Statistically ($p<0.05$) significant to DC, #statistically significant ($p<0.05$) to NC. **NC** = normal rats, **DC** = diabetic control, **DLDC** = diabetic rats + *C. nitida* (150 g/kg bw), **DHDC** = diabetic rats + *C. nitida* (300 g/kg bw), **DSM** = diabetic rats + metformin (200 g/kg bw), and **NTXC** = Normal rats + *C. nitida* (300 g/kg bw).

The ATPase activity was significantly ($p<0.05$) elevated on induction of T2D as shown in **Figure 7.4.3**. This was significantly ($p<0.05$) reduced in rats treated with *C. nitida* to levels insignificant from the normal control.

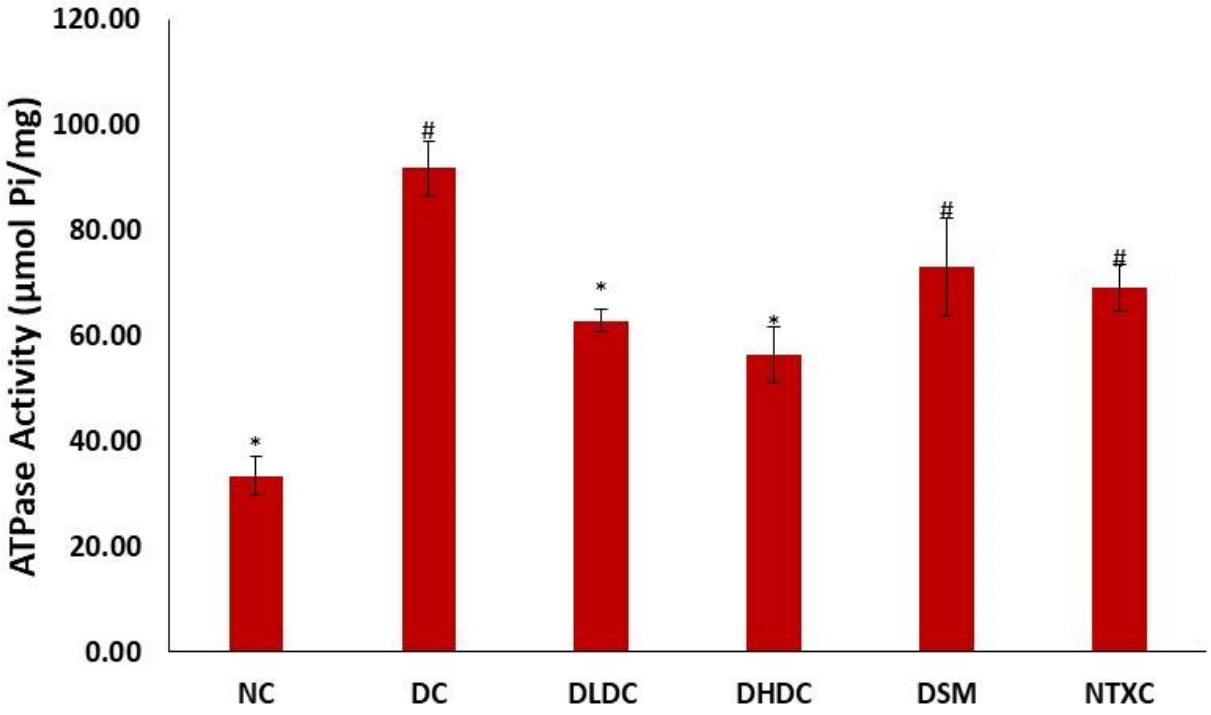


Figure 7.4.3: ATPase activities of experimental groups. Values = mean ± SD; n = 5 (NC and NTXC) and 7 (DC, DLDC, DHDC, and DSM). *Statistically ($p<0.05$) significant to DC, #statistically significant ($p<0.05$) to NC. **NC** = normal rats, **DC** = diabetic control, **DLDC** = diabetic rats + *C. nitida* (150 g/kg bw), **DHDC** = diabetic rats + *C. nitida* (300 g/kg bw), **DSM** = diabetic rats + metformin (200 g/kg bw), and **NTXC** = Normal rats + *C. nitida* (300 g/kg bw).

There was a significant ($p<0.05$) elevation of acetylcholinesterase activity on induction of T2D as depicted in **Figure 7.4.4**. This was significantly ($p<0.05$) reduced in all treatment groups to levels indistinguishable from the normal controls, with *C. nitida*-treated rats showing more inhibitory effect compared to metformin.

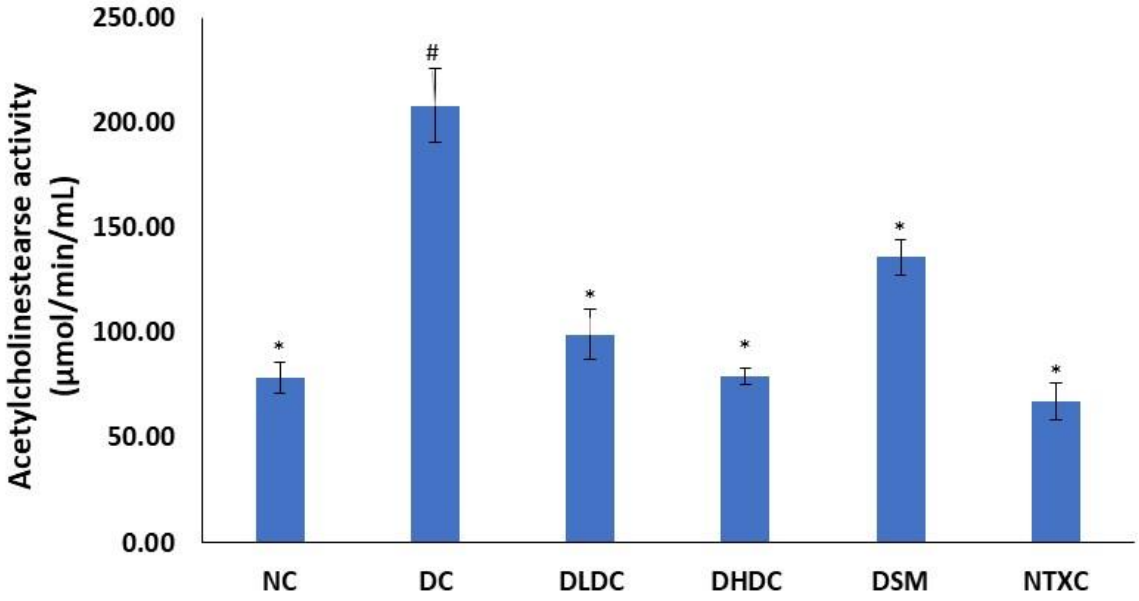


Figure 7.4.4: Acetylcholinesterase activities of experimental groups. Values = mean ± SD; n = 5 (NC and NTXC) and 7 (DC, DLDC, DHDC, and DSM). *Statistically ($p<0.05$) significant to DC, #statistically significant ($p<0.05$) to NC. **NC** = normal rats, **DC** = diabetic control, **DLDC** = diabetic rats + *C. nitida* (150 g/kg bw), **DHDC** = diabetic rats + *C. nitida* (300 g/kg bw), **DSM** = diabetic rats + metformin (200 g/kg bw), and **NTXC** = Normal rats + *C. nitida* (300 g/kg bw).

As shown in **Figure 7.4.5**, induction of T2D led to an altered morphology of the cerebellar cortex as portrayed by inflamed and distorted Purkinje and granular cell layers (**Figure 7.4.5 [2]**) indicating a neuronal insult and compromised integrity when compared to the control (**Figure 7.4.5 [1]**). An intact morphology indistinguishable from the normal control (NC) were observed for all treatment groups, with rats treated with high dose of *C. nitida* showing a more intact morphology (**Figure 7.4.5[4]**).

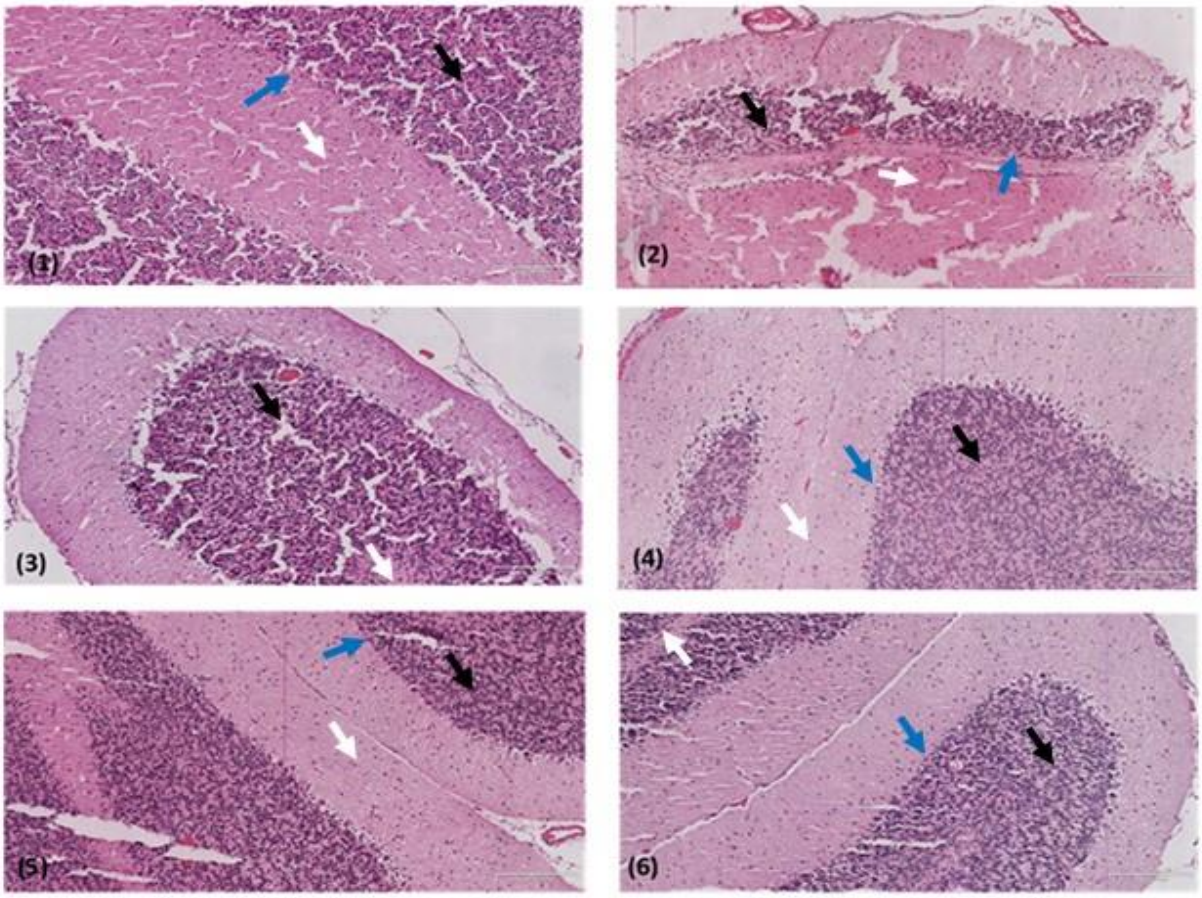


Figure 7.4.5: Morphological changes in cerebellar tissues of experimental groups. Magnification: 10x. (1) = NC, (2) = DC, (3) = DSG-LD, (4) = DSG, (5) = DSM, and (6) = PHD. **NC** = normal rats, **DC** = diabetic control, **DLDC** = diabetic rats + *C. nitida* (150 g/kg bw), **DHDC** = diabetic rats + *C. nitida* (300 g/kg bw), **DSM** = diabetic rats + metformin (200 g/kg bw), and **NTXC** = Normal rats + *C. nitida* (300 g/kg bw). Arrows: Blue = Purkinje cell layers; Red: Granule cell layer; White = White matter

As shown in **Figure 7.4.6**, the cerebellar cortex of the normal rats consists of intact Purkinje and glial cells, with vast networks of dendrites and axons (**Figure 7.4.6A**) as revealed by SEM analysis. This intactness was however distorted on induction of T2D as depicted by the depleted number of Purkinje and glial cells, with a concomitant altered dendrites and axons network (**Figure 7.4.6B**). Treatment with low dose of *C. nitida* led to improved network of dendrites and axons but showed little or no improvement on the numbers of Purkinje and glial cells (**Figure 7.4.6C**). These were more improved in rats treated with high dose of *C. nitida* (**Figure 7.4.6D**). Administration of *C.*

nitida to normal rats increased the population of Purkinje and glial cells in the cerebral cortex, with discernible effect on the dendrites and axons networks (**Figure 7.4.6F**).

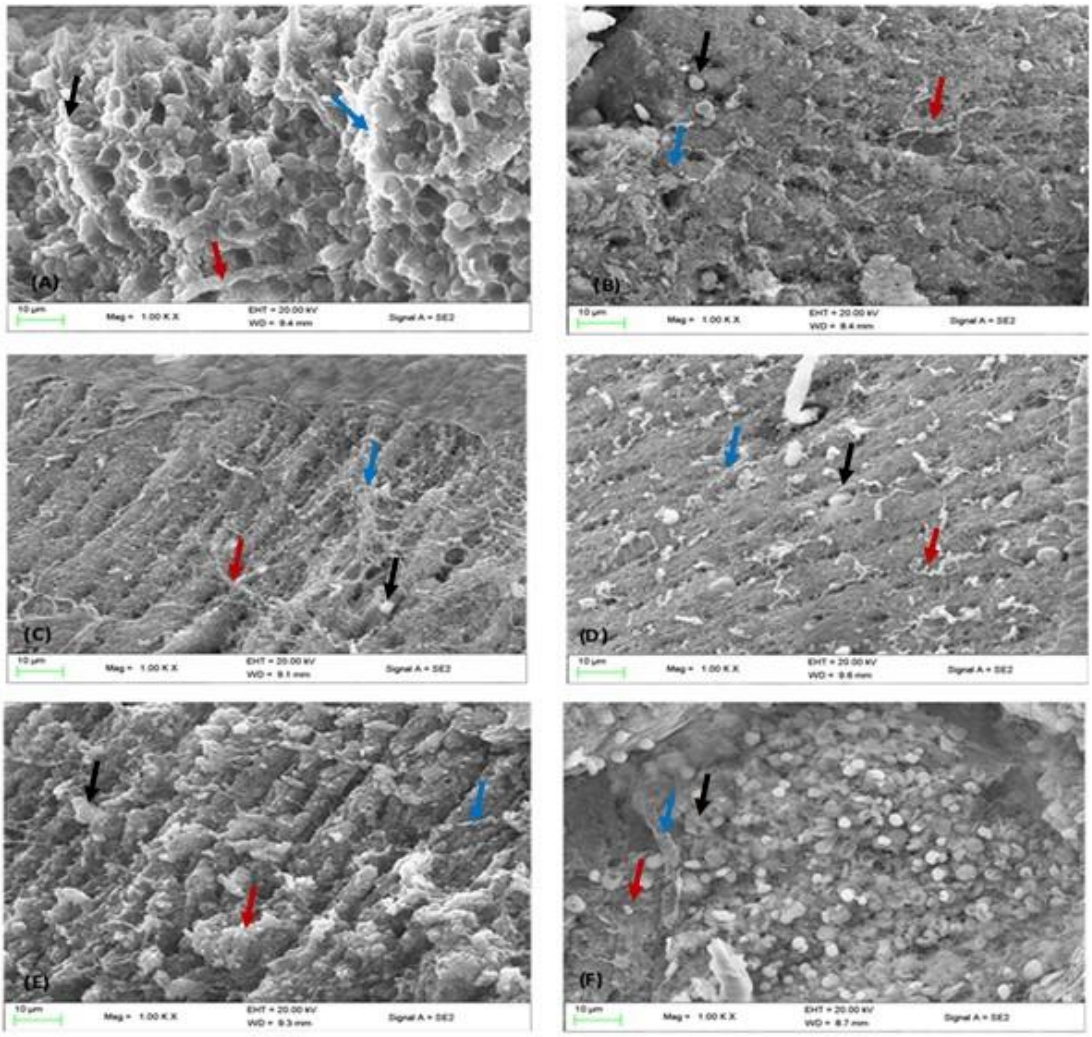
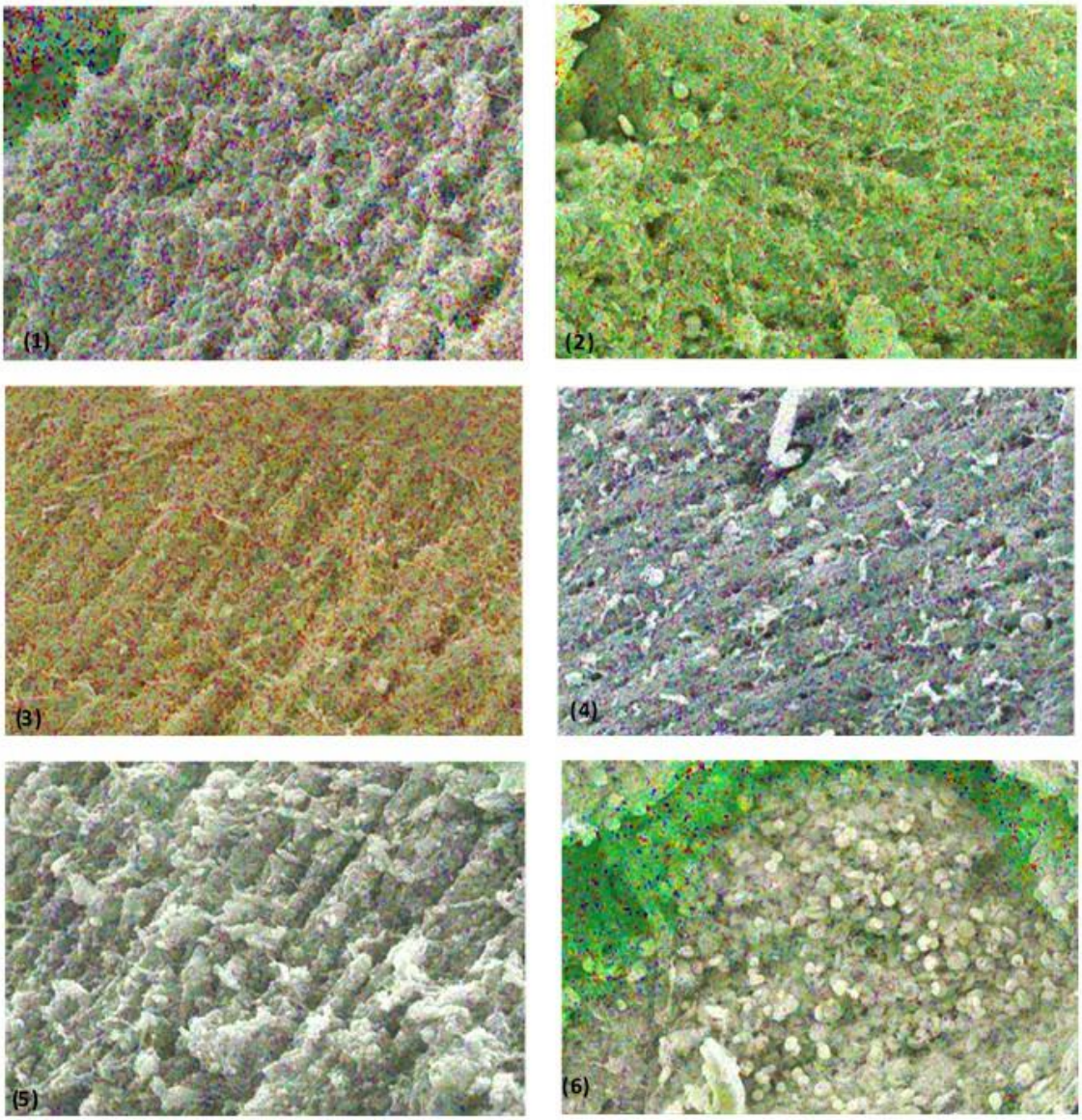


Figure 7.4.6: SEM analysis of cerebellar tissues of experimental groups. Magnification = 1000x. (A) = NC, (B) = DC, (C) = DSG-LD, (D) = DSG, (E) = DSM, and (F) = PHD. **NC** = normal rats, **DC** = diabetic control, **DLDC** = diabetic rats + *C. nitida* (150 g/kg bw), **DHDC** = diabetic rats + *C. nitida* (300 g/kg bw), **DSM** = diabetic rats + metformin (200 g/kg bw), and **NTXC** = Normal rats + *C. nitida* (300 g/kg bw). Arrows: Black = Purkinje cells; Red = dendrite network; Blue = axon

Quantitative analysis of the of EDX mapping of the cerebellar cortex revealed depleted levels of carbon, nitrogen, iron, and potassium with concomitantly elevated levels of oxygen, sodium,

aluminum and calcium as depicted in **Figure 7.4.7A** and **7.4.7B**. Treatment with C. nitida had no discernible effects on the carbon and oxygen levels, but led to the reversal of the other elements to levels indistinguishable from the normal controls. Treatment with metformin led to the presence of cobalt in cerebellar cortex of T2D rats (**Figure 7.4.7A** and **7.4.7B**).



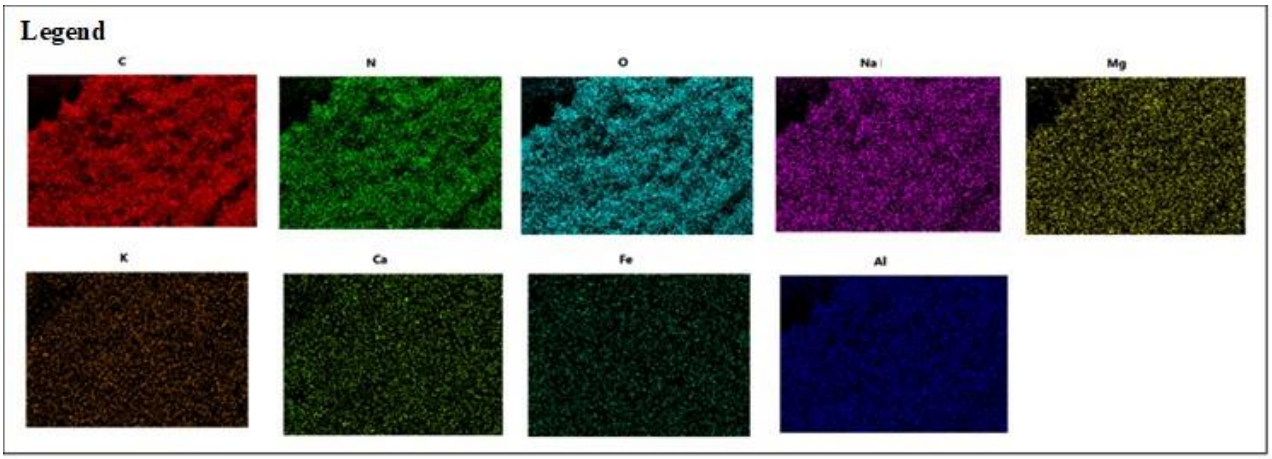
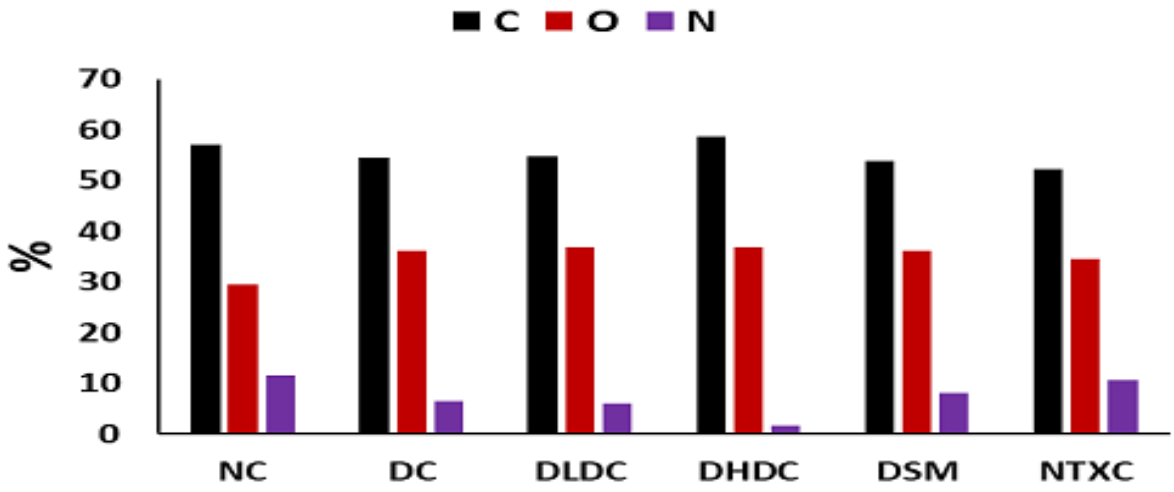


Figure 7.4.7A: EDX mapping of cerebellar tissues of experimental groups. Magnification = 1000x. (1) = NC, (2) = DC, (3) = DLDC, (4) = DHDC, (5) = DSM, and (6) = NTXC. Legends: C = carbon, N = nitrogen, O = oxygen, Na = sodium, Mg = magnesium, K = potassium, Ca = calcium, Fe = iron, and Al = aluminum. **NC** = normal rats, **DC** = diabetic control, **DLDC** = diabetic rats + *C. nitida* (150 g/kg bw), **DHDC** = diabetic rats + *C. nitida* (300 g/kg bw), **DSM** = diabetic rats + metformin (200 g/kg bw), and **NTXC** = Normal rats + *C. nitida* (300 g/kg bw).



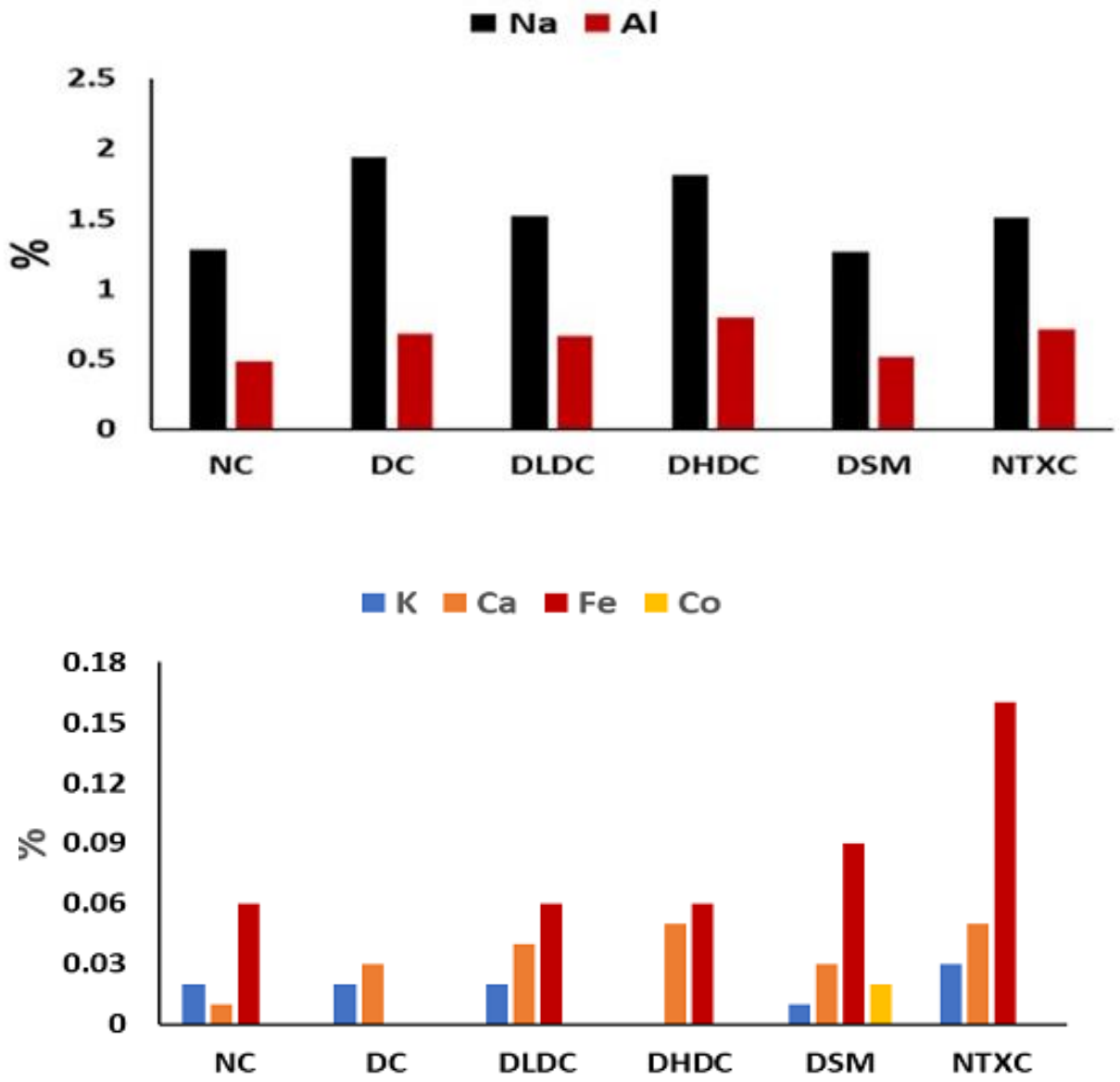


Figure 7.4.7B: Quantitative analysis of EDX mapping of cerebellar tissues of experimental groups. Legends: C = carbon, N = nitrogen, O = oxygen, Na = sodium, Co = cobalt, K = potassium, Ca = calcium, Fe = iron, and Al = aluminum. **NC** = normal rats, **DC** = diabetic control, **DLDC** = diabetic rats + *C. nitida* (150 g/kg bw), **DHDC** = diabetic rats + *C. nitida* (300 g/kg bw), **DSM** = diabetic rats + metformin (200 g/kg bw), and **NTXC** = Normal rats + C. nitida (300 g/kg bw).

There was a significantly ($p<0.05$) exacerbated expression of Nrf2 in the cerebellar cortex on induction of T2D as depicted in **Figure 7.4.8**. The expressions were significantly ($p<0.05$)

attenuated in the cerebellar cortexes of the treatment groups to levels indistinguishable from the normal control.

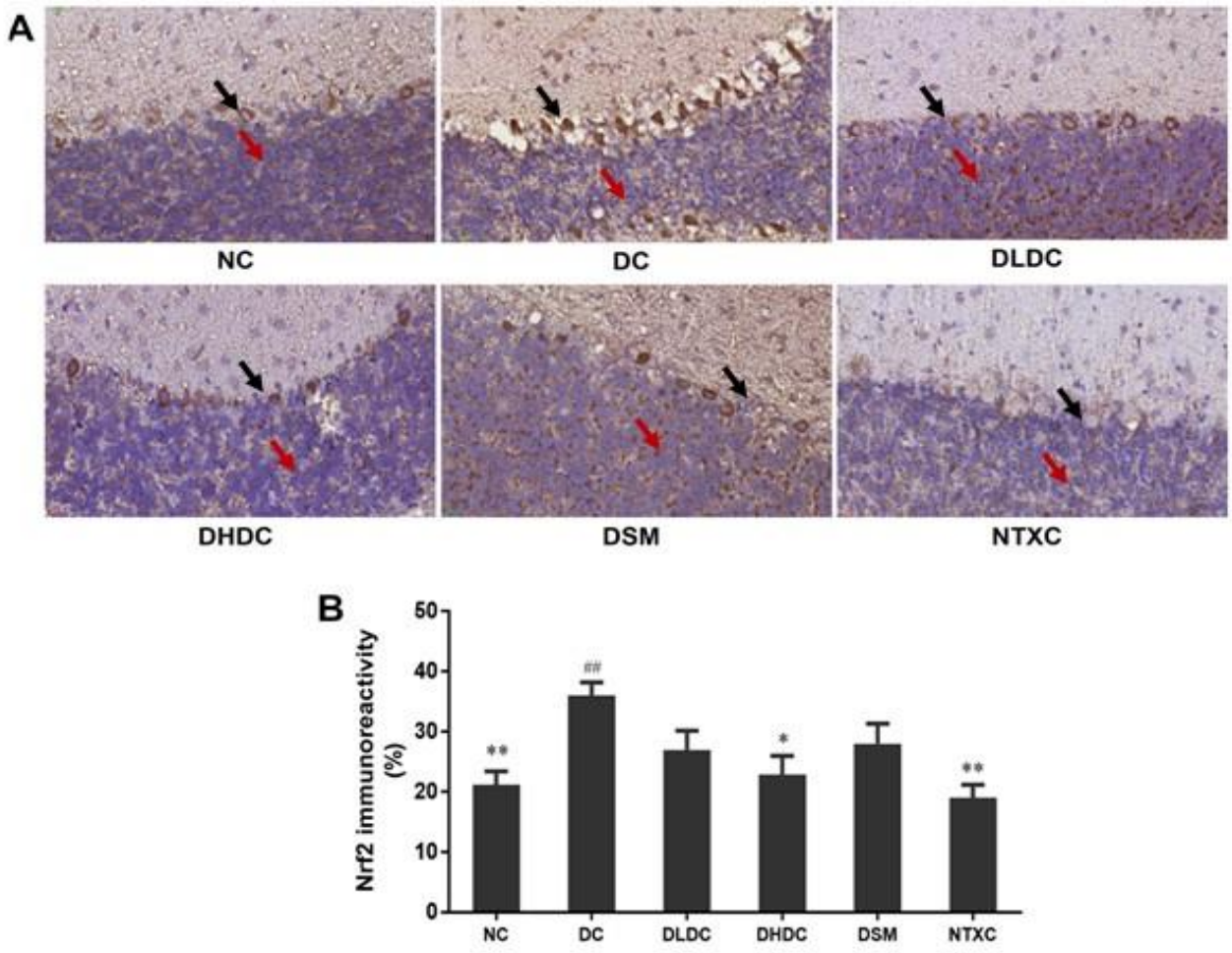


Figure 7.4.8: (A): Immunohistochemistry photomicrographs and (B): immunoreactivity of Nrf2 expression in cerebellar cortex of experimental groups. Magnification = 200x; Values = mean ± SD; n = n = 5 (NC and NTXD) and 7 (DC, DLDC, DHDC and DSM). *Statistically ($p<0.05$) significant to DC, #statistically significant ($p<0.05$) to NC. **NC** = normal rats, **DC** = diabetic control, **DLDC** = diabetic rats + *C. nitida* (150 g/kg bw), **DHDC** = diabetic rats + *C. nitida* (300 g/kg bw), **DSM** = diabetic rats + metformin (200 g/kg bw), and **NTXC** = Normal rats + *C. nitida* (300 g/kg bw).

GC-MS analysis of *C. nitida* hot infusion revealed the presence of caffeine only as shown in **Figure 7.4.9**, indicating the near purity of the infusion.

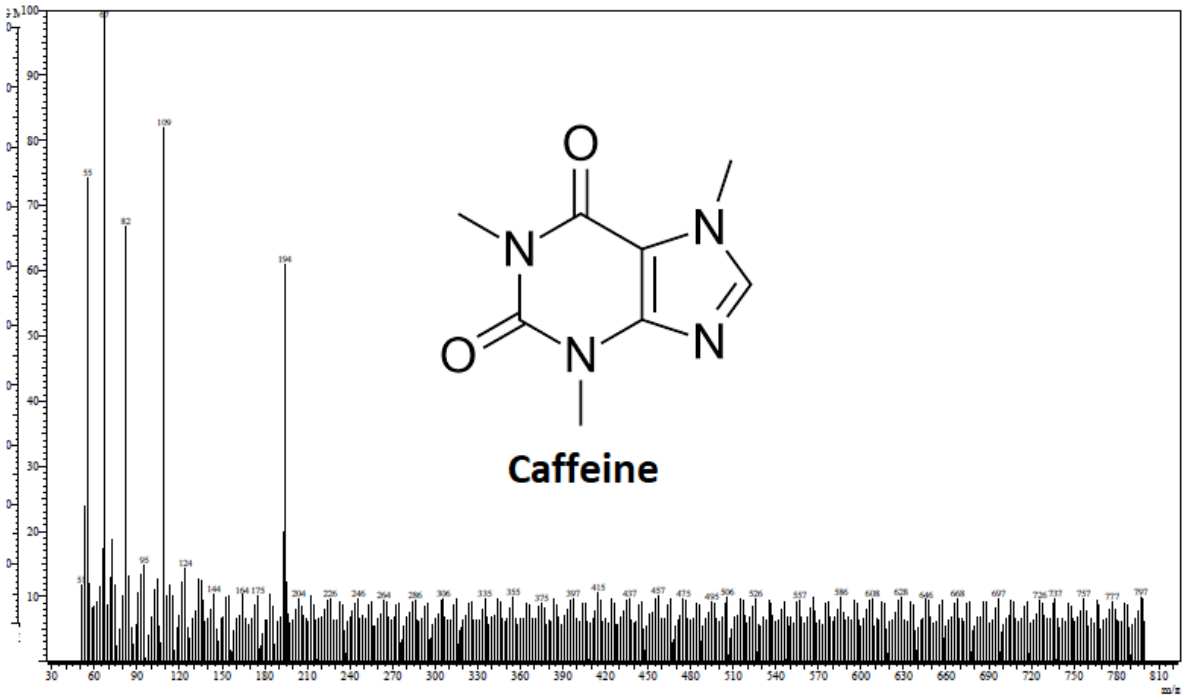


Figure 7.4.9: MS spectra of GC-MS identified compound in *C. nitida* hot infusion.

7.4.5 Discussion

Alterations in glucose homeostasis owing to suppressed glucose transportation across the BBB as well as the susceptibility of the brain's PUFAs to oxidative damage have been recognized as the main players in the pathogenesis and progression of neurodegeneration in T2D (Gejl et al. 2017; Hwang et al. 2017; Kim et al. 2015; Uttara et al. 2009). These alterations and oxidative damage are often characterized by morphological changes, neuronal loss, and metal accumulation (Folarin et al. 2017; Kim et al. 2015; Wrighten et al. 2009). The neuroprotective effect of some medicinal plants and their constituents have been reported, with antioxidative stress being a major mechanism (Bhattacharya et al. 2000; Kumar 2006; Uddin et al. 2013). In the present study, the neuroprotective effect of caffeine-rich infusion from *C. nitida* on neuropathology of the brain in T2D.

The brain has been reported for its low endogenous antioxidant system, which when coupled with its high PUFAs content, the excitotoxic and auto-oxidizable activities of neurotransmitters increases its susceptibility to oxidative stress (Patel 2016). In the present study, the depleted GSH level, SOD and catalase activities in the untreated diabetic rats ($p<0.05$) depicts an occurrence of

oxidative stress which can be attributed to hyperglycemia-induced generation of ROS. This is in consent with previous studies on the depleted level and activities of these antioxidants in brains of diabetic rats (Mastrocola et al. 2005; Moreira et al. 2003). The generation of ROS, such as $O_2^{\cdot -}$ and ·OH are toxic to neuronal cells and their production from persistent glucose oxidation have been reported (Maritim et al. 2003b). Glucose in its enediol form, undergoes oxidation in a transition-metal dependent reaction to an unstable enediol radical anion. Owing to its instability, the anion is converted reactive ketoaldehydes and $O_2^{\cdot -}$ (Maritim et al. 2003b). SOD dismutates the generated $O_2^{\cdot -}$ to H_2O_2, which if not acted on by catalase will be further broken down to give ·OH. Both H_2O_2 and ·OH can switch on the lipid peroxidation process, with PUFAs being the most susceptible owing to their double bonds. An initiation of lipid peroxidation due to hyperglycemia is portrayed in the present study by the elevated MDA level in the untreated diabetic rats (**Figure 7.4.1D**). H_2O_2 is also involved in the proinflammatory cascade as it can be converted to hypochlorite (HOCl) in the presences of myeloperoxidase and hydrochloric acid (HCl) (Patel 2016). Thus, the reversed levels of GSH and MDA, as well as SOD, catalase and myeloperoxidase activities in rats treated with *C. nitida* (**Figure 7.4.1 – 7.4.2**) depicts an antioxidative and anti-proinflammatory protective effect of the infusion against hyperglycemia-induced oxidative brain.

Increased ATPase activity leading to impaired facilitative transportation of glucose across the BBB and altered levels of ATP have been reported in neurodegenerative diseases (Falkowska et al. 2015; Magistretti and Allaman 2015; Mochel et al. 2012; Zhang et al. 2015). The increased ATPase activity in brains of untreated diabetic rats (**Figure 7.4.3**) may thus indicate an impaired glucose transportation on induction of T2D and depleted ATP level. The reduced ATPase activity in brains of rats treated with *C. nitida* portrays the ability of the infusion to inhibit hyperglycemia-induced ATPase alteration, which may also reflect an increased facilitative glucose uptake in the brain. This corroborates previous reports on the suppressive effects of C. nitida on ATPase activities in brain tissues of STZ-induced diabetic rats (Imam-Fulani et al. 2018).

The hydrolyzing effect of acetylcholinesterase on the neurotransmitter, acetylcholine has been linked with the pathogenesis and progression of neurodegenerative diseases (Kuhl et al. 1999; Mushtaq et al. 2014; Pavlov et al. 2009). Impaired cognition, and motor neuron dysfunction are amongst the neurodegenerative diseases linked to increased acetylcholinesterase activities (Hwang et al. 1999; Kuhad et al. 2008; Tabet 2006). The increased activity on induction of T2D (**Figure**

7.4.4) is in consent with reports on increased acetylcholinesterase activities in diabetic rat brains (Ghareeb and Hussen 2008; Kuhad et al. 2008), further indicating a hyperglycemia-induced brain injury. The reduced activities in the brains of rats treated with *C. nitida* portrays a neuroprotective potential of the infusion, which can be attributed to the caffeine constituent (**Figure 7.4.9**). This corroborates previous reports on the inhibitory effect of *C. nitida* and caffeine on acetylcholinesterase activities (Oboh et al. 2018; Salahdeen et al. 2014).

The role of cerebellum in the facilitation of movement, motor and muscular activities is well documented (Imosemi 2013; Popescu et al. 2009). Purkinje cells have been recognized as the major neurons present in the cerebellum (Fahrion et al. 2013; Lopez et al. 2009). Morphological changes in the brain have been implicated in most neurodegenerative diseases, and has been linked to oxidative stress (Ojo et al. 2014; Sidhu and Nehru 2004). The distorted layers of Purkinje and granular cells in cerebellar cortexes of the untreated diabetic rats (**Figure 7.4.5B**) depict morphological alterations. This is further obvious in the depleted Purkinje and glial cells as well as distorted networks of dendrites and axons (**Figure 7.4.6B**). These morphological changes insinuate a compromised neuronal integrity and can be attributed to hyperglycemia-induced oxidative stress (**Figure 7.4.1A**) as Purkinje cells have been reported for their vulnerability to oxidative stress (Chen et al. 2003; Kern and Jones 2006; Lopez et al. 2009). These alterations correlate with previous reports on structural changes in the cerebellar cortexes of diabetic rats (Hernández-Fonseca et al. 2009; Ozdemir et al. 2016). Restoration of the layers and improved networks of the dendrites and axon in cerebellar cortexes of *C. nitida*-treated diabetic rats (**Figures 7.4.5** and **7.4.6**) further portrays the neuroprotective effect of the infusion. This activity can be attributed to its caffeine constituent as the alleviative effect of caffeine on the loss of Purkinje neurons have been reported (Gonçalves et al. 2017).

The roles of elements, particularly metals in the normal functions of the brain has been reported. They often act as enzyme co-factors and are involved in most redox reaction (Uttara et al. 2009). Alterations in their distribution have been linked with neurodegeneration, particularly cerebellar toxicity (Folarin et al. 2017; Kamal and Kamal 2013). The altered elemental levels (**Figure 7.4.7**), particularly elevated levels of oxygen, aluminum and sodium may portray in the cerebellar cortexes of the untreated diabetic rats indicates toxicity as their accumulations have been reported in neurodegeneration (Kamal and Kamal 2013; Wojda et al. 2008). The oxygen level may portray

the induced redox imbalance depicted in **Figure 7.4.1**, as its accumulation have been implicated in the generation of $O_2^{\cdot -}$ (Patel 2016). Restoration of these elements to levels indistinguishable from the normal control in the treatment groups, further indicates the neuroprotective effect of *C. nitida* in T2D-brain injury.

The increased expression of Nrf2 in the Purkinje and granular layers in the cerebellar cortexes of the untreated diabetic rats (**Figure 7.4.**8) correlates with reports of exacerbated expressions on the onset of T2D (Wrighten et al. 2009; Zucker et al. 2014). A suppressed antioxidant system have also been linked with Nrf2 expression (He et al. 2012; Miao et al. 2012), which also correlates with the induced oxidative stress in the untreated diabetic rats (**Figure 7.4.1**). These, however, contradicts some reports on the beneficial roles of increased Nrf2 expression in the recruitment of the endogenous antioxidants system (Dieter 2014; Ma 2013). The attenuated expression in the cerebellar cortex of the treatment groups, may therefore insinuates a suppressive effect of *C. nitida* on hyperglycemia-induced Nrf2 expression in diabetic cerebellum.

7.4.6 Conclusion

Based on these results, it can be said that the hot infusion of *C. nitida* confers a neuroprotective effect against hyperglycemia-induced oxidative brain damage in T2D. This it does by attenuating oxidative stress, proinflammation, Nrf2 expression and inhibiting the activities of acetylcholinesterase and ATPase, while concomitantly preserving the integrity of the cerebellar cortex.

Acknowledgments

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CHAPTER 8

THE ANTI-OXIDATIVE AND ANTI-DIABETIC ACTIVITIES OF *DACRYODES EDULIS* (BUSH PEAR) *IN VITRO, EX VIVO,* AND *IN VIVO*

8.1 Dacryodes edulis

Figure 8.1

Figure 8.1.1: *Dacryodes edulis*. Common names: Bush pear, African plum; Butterfruit, Bush butter tree, Bush plum (English); *Ube* (Nigeria); *Safou* (Cameroon); and *Atanga* (Gabon). Synonyms: *Canarium mansfeldianum* Engl., *Pachylobus albiflorus* Guill, *Canarium saphu* Engl., and *Canarium edule Hook* F., Photo: http://tropical.theferns.info/image.php?id=Dacryodes+edulis (accessed on November 8, 2018 without permission).

8.1.2 Background

Dacryodes edulis (G. Don) H.J. Lam is an evergreen fruit tree indigenous to Africa where the fruits are usually utilized as foods, and other parts for medicinal purposes. It has been reported to

originate from central Africa and the Gulf of Guinea but found across the western coast of Africa ranging from Southern Nigeria to Equatorial Guinea (Rodrigues et al. 2018). It often grows to heights of 18-40 m (Burkill 1985; Verheij 2002). Its trunk is relatively short, with a pale gray rough bark. It consists of compound leaves which are glossy (Ajibesin 2011; Burkill 1985). The fruits vary in size and are ellipsoid drupe, with thin pink exocarps which ripens to give purple, blue-green, or brilliant black (Ajibesin 2011).

8.1.3 Ethnopharmacological uses

Different parts of the plant are used in the treatment of diabetes, malaria, hypertension, labor pain, retarded growth, skin diseases, tonsillitis, leprosy, oral and ear conditions, and epilepsy (Ajibesin 2011; Conrad and Uche 2013).

8.1.4 Biological activities

The antidiabetic properties of the fruits have been demonstrated (Oboh et al. 2015; Okolo et al. 2016). The antioxidant protective effects of the leaves and fruits have been reported (Agbor et al. 2007; Conrad and Uche 2013; Oboh et al. 2015). Its antimalarial and antimicrobial properties have also been reported (Miguel et al. ; Zofou et al. 2013; Zofou et al. 2011). The ability of oil from the fruits to elevate HDL-c have been demonstrated in rats (Leudeu et al. 2006). The aqueous and ethanol extracts of its leaves have been reported for their ability to normalize sickled erythrocytes, thereby insinuating its protective effect on sickle cell disease (Mpiana et al. 2007).

8.1.5 Phytochemistry

The phytochemical constituents of different parts of *D. edulis* include: xanthone, ascorbic acid 2,6-dihexadecanoate, isorhamnetin rhamnoside, ethylgallate, quercitrin, α-pinene, α-terpineol, myrcene and germacrene-D, α-cadinol, δ-cadinol and β-eudesmol, kaur-15-ene peonidin hexosides, isoquercitrin, petunidin, hyperin, cyanidin, sitosterol, urs-12-ene-3-ol acetate and sorhamnetin hexoside (Ajibesin 2011; Ella Missang et al. 2003; Erukainure et al. 2017b; Onocha et al. 1999).

8.1.6 Aim

The aims of this study are to investigate the antioxidative, antidiabetic, and cytotoxic activities of the leaves of *D. edulis* using *in vitro, ex vivo, in silico* and *in vivo* models.

8.2 *Dacryodes edulis* Enhances Antioxidant Activities, Suppresses DNA Fragmentation in Oxidative Pancreatic and Hepatic Injuries; and Inhibits Carbohydrate Digestive Enzymes linked to Type 2 Diabetes

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Preface: This article investigated the antioxidant and enzyme inhibitory activities of *D. edulis* extracts *in vitro* and *in silico*, as well as its modulatory effect on redox imbalance in oxidative pancreatic and hepatic injuries *ex vivo*. The phytochemical constituents were also investigated using GC-MS and FTIR. It has already been published in Biomedicine and Pharmacotherapy (**Erukainure OL,** Mopuri R, Oyebode OA, Koorbanally NA, Islam MA. (2017). *Dacryodes edulis* enhances antioxidant activities, suppresses DNA fragmentation in oxidative pancreatic and hepatic injuries; and inhibits carbohydrate digestive enzymes linked to type 2 diabetes. Biomed Pharmacoth. 96: 37–47).

8.1 Abstract

The leaves of *Dacryodes edulis* were investigated for their anti-oxidative vis-à-vis anti-diabetic potentials *in vitro*. Extracts from sequential extraction with solvents of increasing polarity (n-hexane, ethyl acetate, ethanol and aqueous) of the leaves were subjected to *in vitro* antioxidant assays using the 2,2'-diphenyl-1-picrylhydrazyl (DPPH) scavenging and Ferric reducing antioxidant power (FRAP) protocols respectively. Their inhibitory effects were investigated on α-glucosidase, pancreatic lipases, pancreatic ATPase and glucose-6-phospatase activities. Their antioxidant and anti-apoptotic effects on Fe^{2+} - induced oxidative injuries in pancreatic and hepatic

tissues were also investigated *ex vivo*. The most active extract (ethanol) was subjected to GC-MS and FTIR spectroscopic analysis to identify its bioactive chemical constituents. The extracts showed potent free radical scavenging activity and significantly ($p<0.05$) inhibited all studied enzymes, with the ethanol extract showing greater activities. Superoxide Dismutase (SOD) and Catalase activities were significantly ($p<0.05$) increased in both pancreatic and hepatic tissues with concomitant elevation of reduced glutathione (GSH) levels as well as reduced levels of malondialdehyde (MDA). The extracts significantly inhibited DNA fragmentation. These activities were dose – dependent. Amongst Compounds identified via GC-MS analysis of the ethanol extract, only Kaur-15-ene, Urs-12-ene-3-ol acetate and 2,3,23-trihydroxyolean-12-en-28-oic acid methyl ester showed strong binding affinities when docked with α-glucosidase (PDB ID:3TON). These results indicate the anti-oxidative, anti-diabetic and anti-obesogenic potentials of *D. edulis* leaves. Thus, giving credence to its antidiabetic folkloric claims

Keys: Antioxidants; *Dacryodes edulis*; Type 2 Diabetes; Medicinal plant

8.2.2 Introduction

The world has witnessed an increase in the prevalence of diabetes from 336 million individuals in 2011 to 415 million in 2015 depicting a 13.36% rise, with more than 45% undiagnosed (Atlas 2014). This is estimated to rise to 642 million in 2040, with Sub Saharan Africa accounting for 5.33% compared to 3.42% in 2015 (Atlas 2014). The urban areas have been shown to witness the highest increase, thus implicating rural–urban drift as well as changes in dietary patterns coupled with less physical activity and ageing population as the main factors in Africa (Bos and Agyemang 2013).

Type 2 diabetes (T2D) accounts for more than 90% of all diabetes, which is a major contributor to global mortality and morbidity (Atlas 2014). Unlike type 1 diabetes, it occurs when the body cannot effectively utilize the insulin produced. It generally arises from a combination of insulin resistance and pancreatic β-cell dysfunction, resulting to chronic hyperglycemia. In the progression of T2D, hyperglycemia results to elevated reactive oxygen species (ROS) production which causes oxidative stress due to the imbalance in endogenous antioxidant defense system (Tiwari et al. 2013a). Oxidative stress has been implicated in oxidative pancreatic and hepatic injuries, which further aggravates T2D (Saeed et al. 2012). This is characterized by decreased SOD

and catalase activities leading to accumulation of superoxide ($O_{2^{\cdot}}^{-}$), hydrogen peroxide (H_2O_2) and hydroxyl ($^{\cdot}OH$) which causes peroxidation of the cell membrane lipid and DNA insults (Oluwakemi and Olufeyisipe 2016).

The use of medicinal plants in the treatment and management of T2D is well documented (Ezuruike and Prieto 2014). Their efficacy has been attributed to the presence of phytochemicals with reported anti-oxidative and anti-diabetic activities (Erukainure et al. 2013; Saeed et al. 2012). Unlike synthetic drugs, the use plant originated natural medicines is believed to be associated with little or no side effects and are readily affordable. However, there are rising issues concerning toxicity of natural products (Ifeoma and Oluwakanyinsola 2013). Amongst such plants is *Dacryodes edulis*.

This paper aims to report the anti-oxidative potentials of the ethyl acetate, ethanol and aqueous extracts of *D. edulis* leaves on Fe^{2+} - induced oxidative injury and DNA fragmentation in pancreatic and hepatic tissues. As well as their antidiabetic effect by investigating their inhibitory activity on key carbohydrate and lipid digestive enzymes linked to T2D.

8.2.3 Materials and Methods

Kindly refer to chapter 2, subsections: 2.1.1, 2.1.3, 2.1.6, 2.3-2.4, 2.6, 2.8, 2.10.1, 2.10.4, 2.11, and 2.13 for details materials and methods.

8.4 Results

The total phenolic content of the ethanol extract of *D. edulis* was significantly ($p<0.05$) higher than the other extracts as depicted in **Figure 8.2.1**.

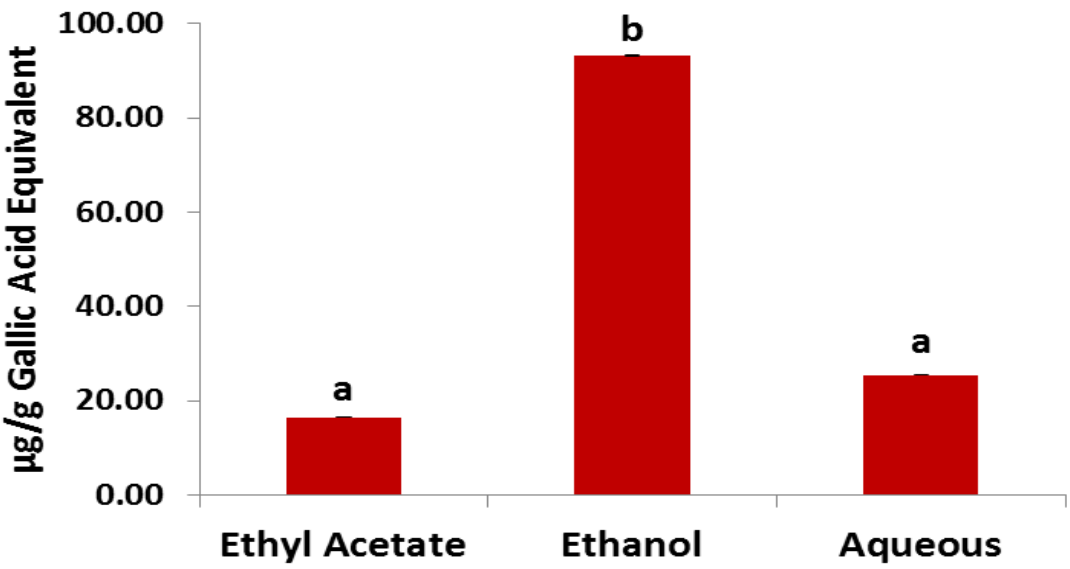

Figure 8.2.1: Total phenolic contents of the *D. edulis* extracts. Data are presented as mean ± SEM. [ab]Values with different letter above the bars for a given extract are significantly different from each other ($p < 0.05$, Tukey's HSD-multiple range post-hoc test, IBM SPSS for Windows).

All extracts showed significant ($p<0.05$) dose–dependent DPPH scavenging activity, with the ethanol extract showing the best activity as indicated by its low IC_{50} value as shown in **Table 8.2.1** and **Figure 8.2.2A.**

A dose–dependent and significant ($p<0.05$) pattern was observed for the FRAP activity of all the extracts as revealed in **Figure 8.2.2B**. The lowest IC_{50} value of the ethyl acetate extract, portrays the best activity (**Table 8.2.1**).

Table 8.2.1: IC_{50} values of biological activities of *D. edulis* leaf extracts

Activity	Ethyl Acetate (µg/mL)	Ethanol (µg/mL)	Aqueous (µg/mL)	Ascorbic Acid (µg/mL)	Gallic Acid (µg/mL)	Acarbose (µg/mL)	Orlistat (µg/mL)
DPPH	54.90	1.83	44.57	8.32	-	-	-
FRAP	43.03	381.66	235.04	29.98			
GSH (Pancreas)	20.89	27.54	45.7	-	>1000	-	-
GSH (Liver)	0.11	0.42	0.003	-	0.009	-	-
SOD (Pancreas)	>1000	>1000	>1000	-	575.43	-	-
SOD (Liver)	28.84	>1000	64.66	-	85.11	-	-
Catalase (Pancreas)	8.63	5.09	23.67	-	>1000	-	-
Catalase (Liver)	691.83	>1000	>1000	-	537.03	-	-
Lipid Peroxidation (Pancreas)	23.71	922.99	0.22	-	2.38	-	-
Lipid Peroxidation (Liver)	429..31	>1000	>1000	-	>1000	-	-
Alpha glucosidase	15.05	11.03	8.29	-	-	1.79	-
Lipase	1.51	1.56	1.41	-	-	-	1.24
ATPase	19.06	34.84	42.77	-	30.31	-	-
Glucose 6 Phosphate	0.66	3.59	0.05	-	1.48	-	-
DNA Fragmentation (Pancreas)	6.04	2.96	4.21	-	4.40	-	-

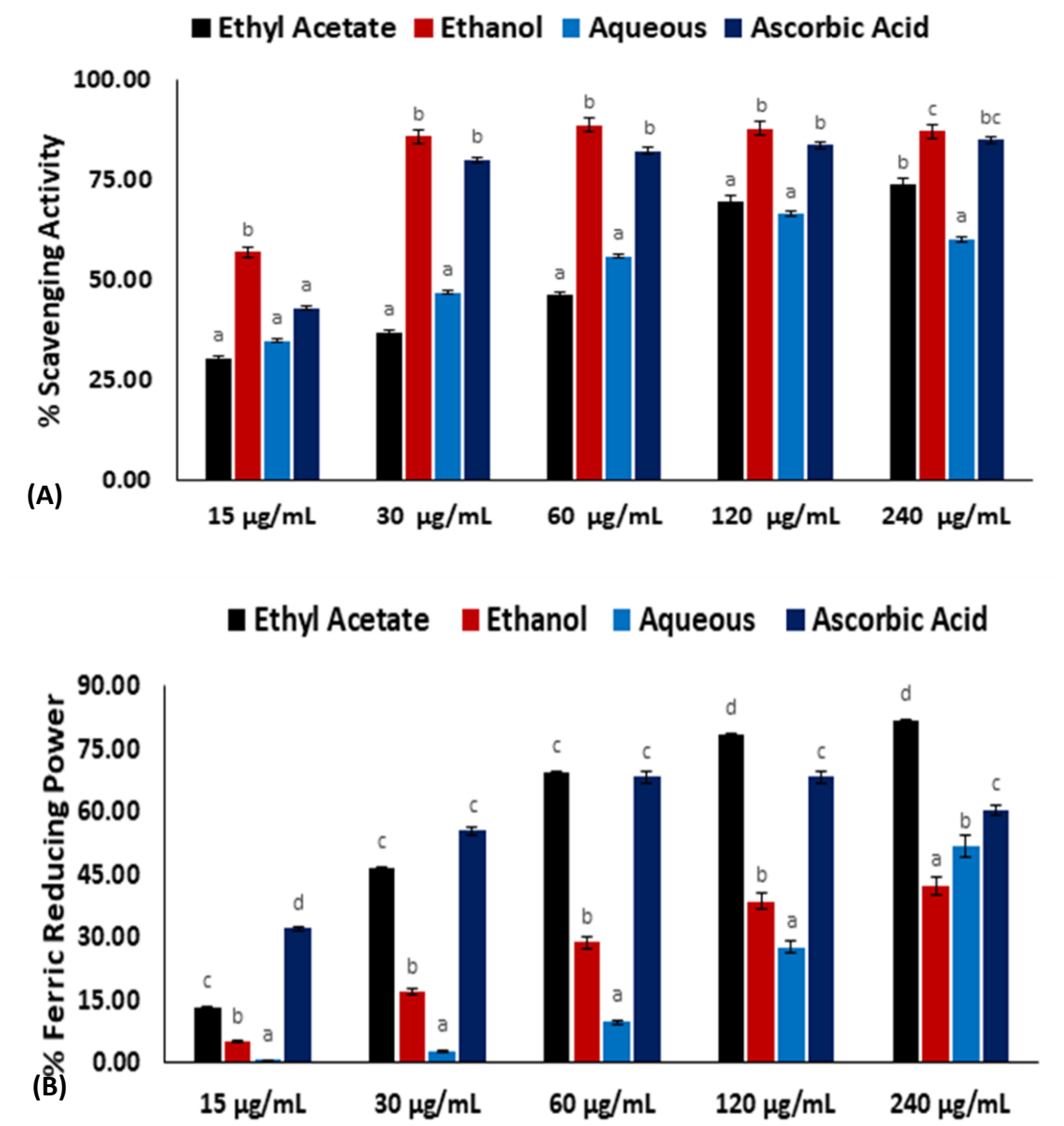

Figure 8.2.2: (A) DPPH scavenging and (B) Ferric reducing antioxidant power (FRAP) activities of *D. edulis* leaf extracts. Data are presented as mean ± SEM. [abc]Values with different letter above the bas for a given extract are significantly different from each other ($p < 0.05$, Tukey's HSD-multiple range post-hoc test, IBM SPSS for Windows).

Induction of oxidative injury with $FeSO_4$ significantly ($p<0.05$) decreased GSH level in pancreatic tissue as depicted in **Figure 8.2.3A**. The effect was even more pronounced in hepatic tissue (**Figure 8.2.3B**). Treatment with the extracts led to significant increase in both tissues depicting their therapeutic potentials, with the ethanol extract showing the best activity in both tissues. The increase in GSH level with increasing concentration indicates a dose–dependent effect.

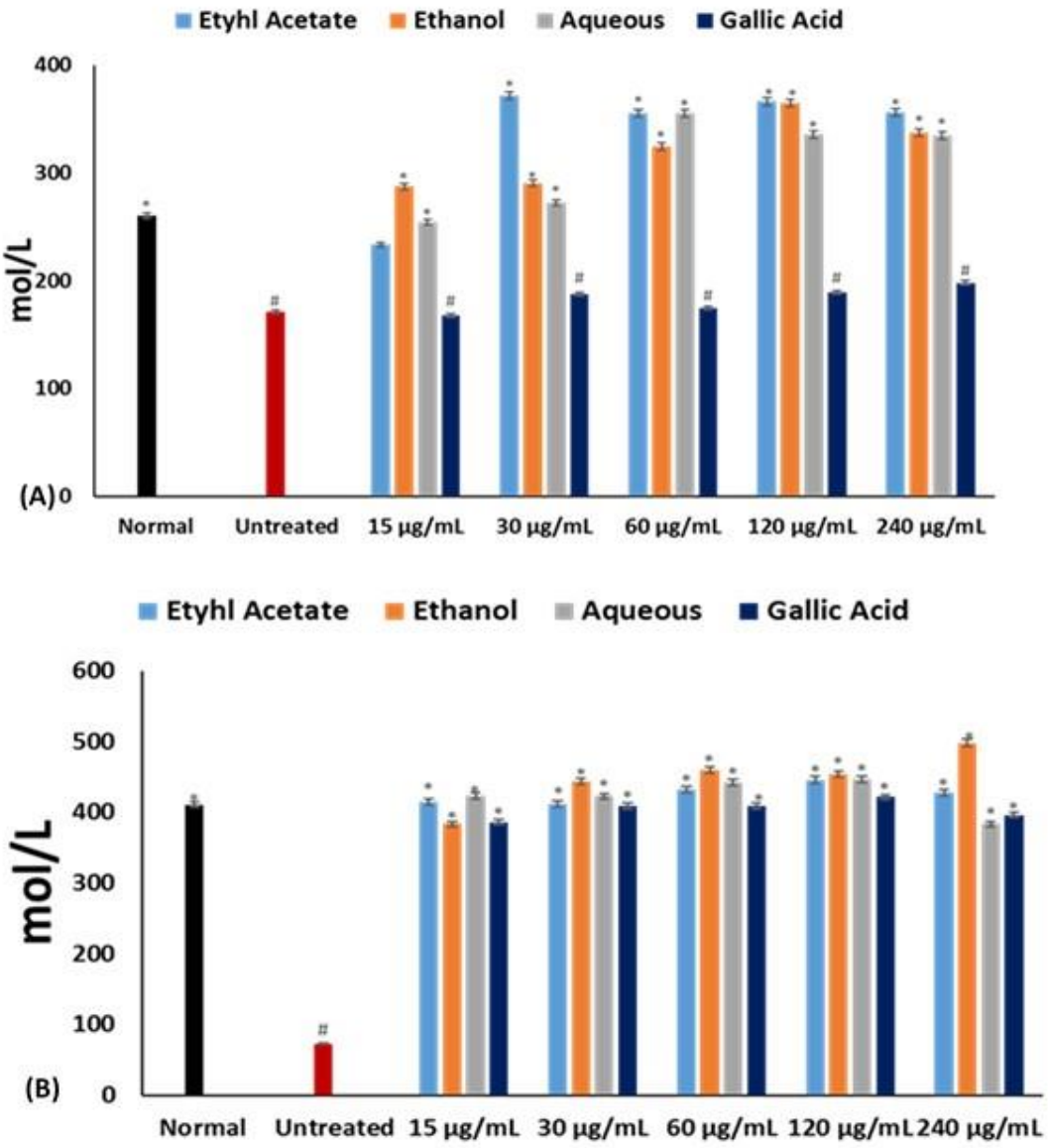

Figure 8.2.3: Effect of *D. edulis* leaf extracts on GSH level in oxidative (A) pancreatic and (B) hepatic injuries. Data are presented as mean ± SEM. *Significantly different from untreated sample and #Significantly different from normal sample ($p < 0.05$, Tukey's HSD-multiple range post-hoc test, IBM SPSS for Windows).

The SOD activities were observed to be significantly ($p<0.05$) reduced in both tissues on induction of oxidative injury, with that of the pancreatic tissue more pronounced as shown in **Figures 8.2.4A** and **8.2.4B**. A dose–dependent effect in the restoration of the activities to near normal was observed on treatment with the extracts in hepatic tissue, with the ethanol extract showing the best

activity in pancreatic tissues and ethyl acetate in hepatic tissues as depicted by their respective low IC_{50} values (**Table 8.2.1**).

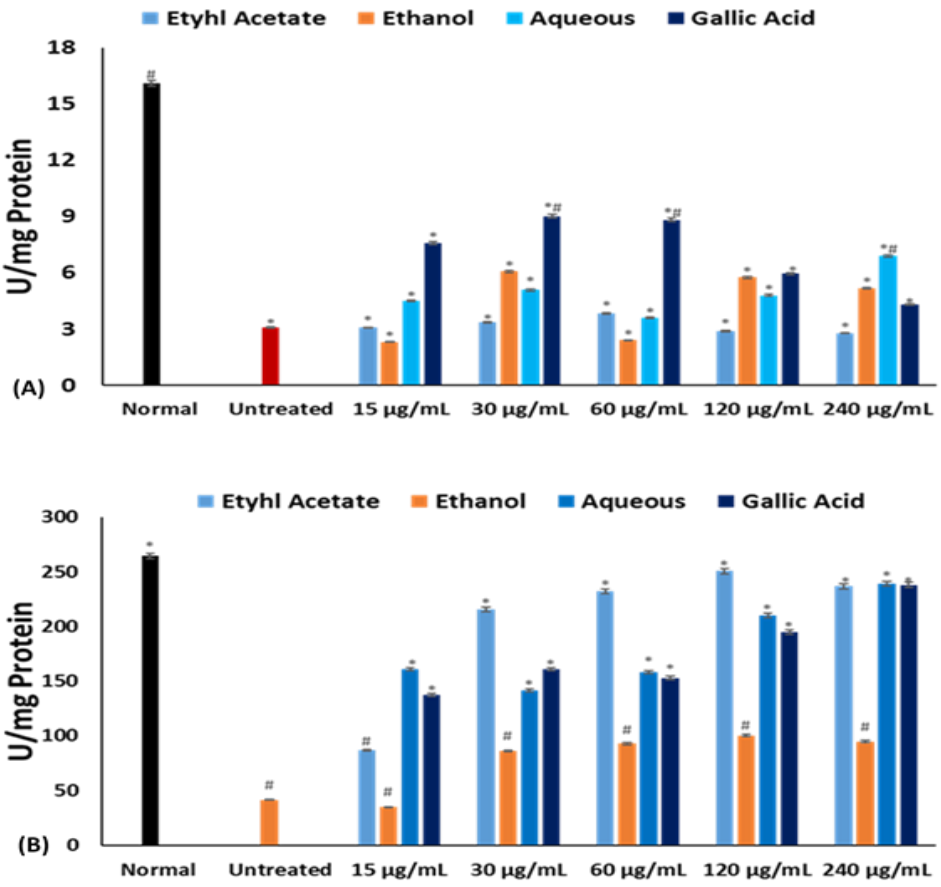

Figure 8.2.4: Effect of *D. edulis* leaf extracts on SOD activity in oxidative (A) pancreatic and (B) hepatic injuries. Data are presented as mean ± SD. #Significantly different from normal sample and *Significantly different from untreated sample ($p < 0.05$, Tukey's HSD-multiple range post-hoc test, IBM SPSS for Windows).

Reduced catalase activities were observed in both tissues on induction of oxidative injury with $FeSO_4$ as shown in **Figures 8.2.5A** and **8.2.5B**. Treatment with the extracts led to a dose–dependent reversion of the activities to near normal in hepatic tissue, with ethyl acetate showing the best activity in pancreatic tissue (**Figure 8.2.5A**) and aqueous in hepatic tissues (**Figure 8.2.5B**) as portrayed by their low IC_{50} values (**Table 8.2.1**).

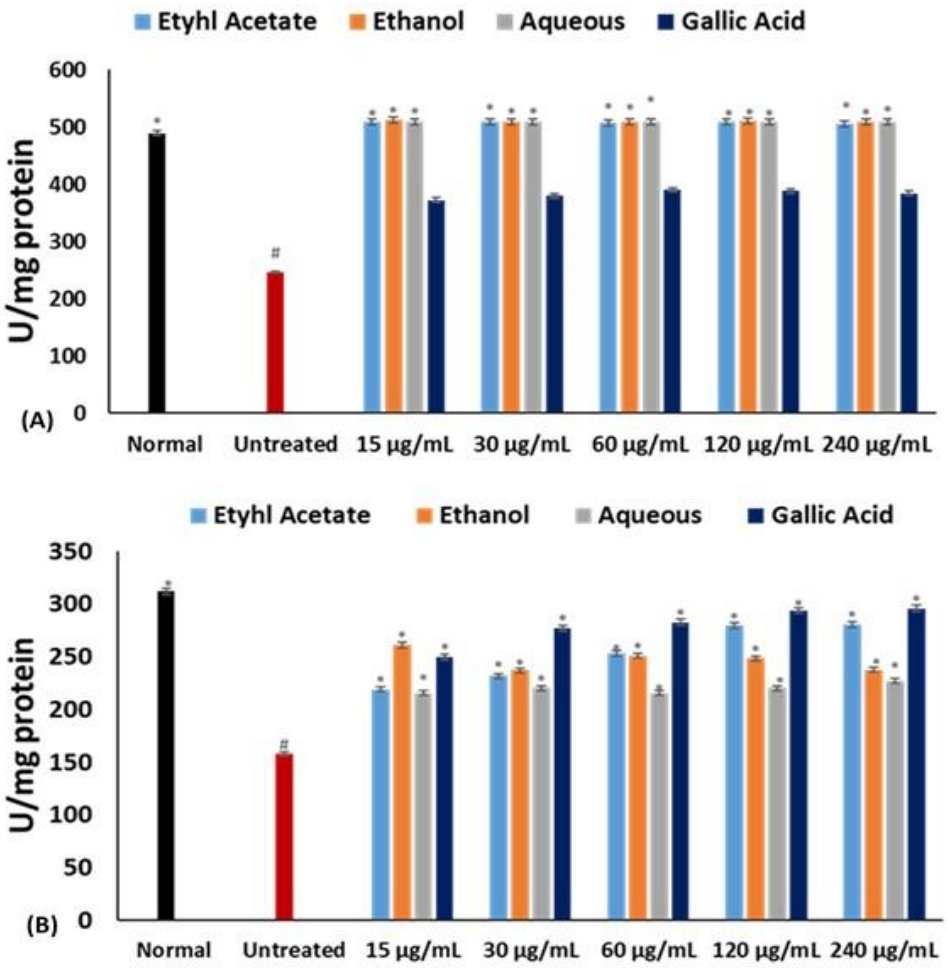

Figure 8.2.5: Effect of *D. edulis* leaf extracts on catalase activity in oxidative (A) pancreatic and (B) hepatic injuries. Data are presented as mean ± SD. *Significantly different from untreated sample and #Significantly different from normal sample ($p < 0.05$, Tukey's HSD-multiple range post-hoc test, IBM SPSS for Windows).

Induction of oxidative injury with $FeSO_4$ caused a significant ($p<0.05$) increase in MDA levels in both tissues as shown in **Figure 8.2.6A** and **8.2.6B**. This was significantly ($p<0.05$) reduced on treatment with the extracts in a dose–dependent pattern in hepatic tissue, with the ethyl acetate showing the best activity in pancreatic tissues (**Figure 8.2.6A**) and ethanol in hepatic tissues (**Figure 8.2.6B**) as revealed by their low IC_{50} values (**Table 8.2.1**).

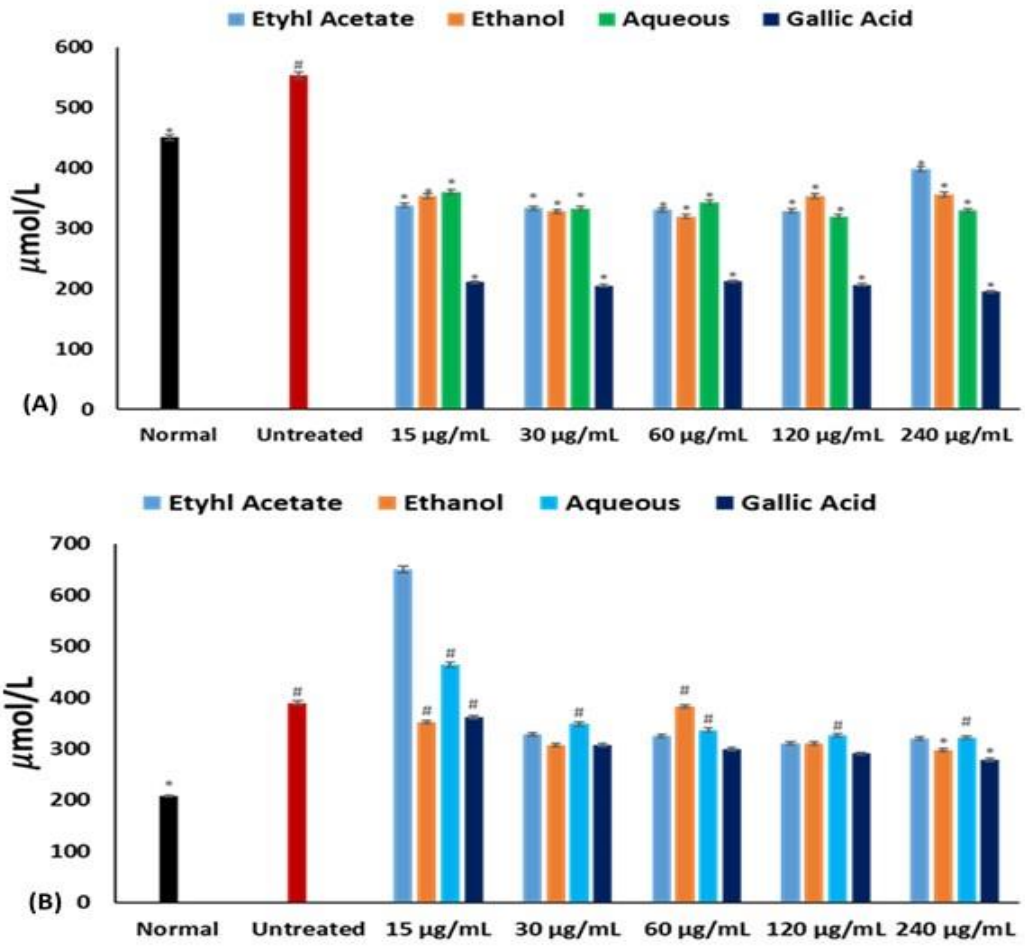

Figure 8.2.6: Effect of *D. edulis* leaf extracts on lipid peroxidation in oxidative (A) pancreatic and (B) hepatic injuries. Data are presented as mean ± SD. *Significantly different from untreated sample and #Significantly different from normal sample ($p < 0.05$, Tukey's HSD-multiple range post-hoc test, IBM SPSS for Windows).

α – glucosidase was significantly ($p<0.05$) inhibited on incubation with the extracts as depicted in **Figure 8.2.7A**. The aqueous extract showed the best inhibitory activity respectively and compared favorably to Acarbose as portrayed by their low IC_{50} values (**Table 8.2.1**).

The extracts showed significant ($p<0.05$) inhibitory activity on incubation with pancreatic lipase as shown in **Figure 8.2.7B**. The aqueous extract showed the best inhibitory activity and even more potent than orlistat as revealed by its low IC_{50} value (**Table 8.2.1**).

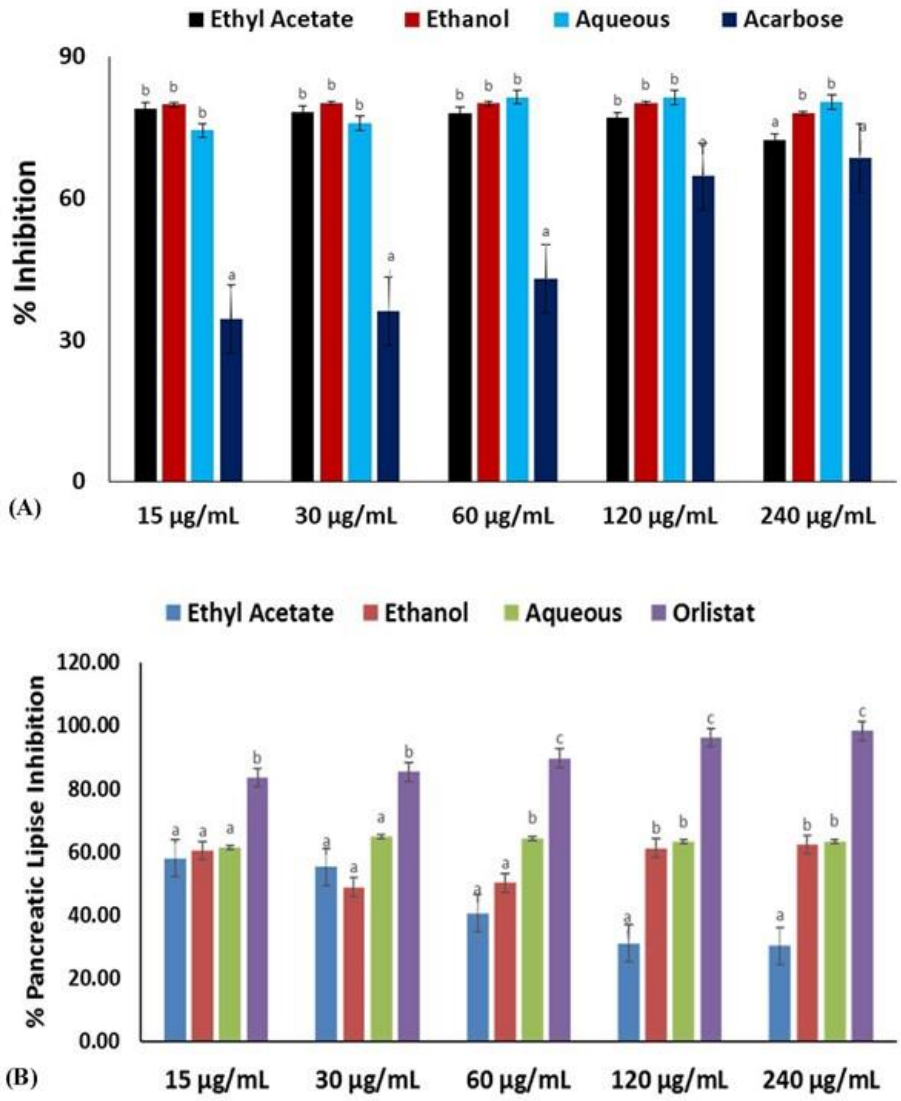

Figure 8.2.7: *(*A) α-glucosidase and (B) pancreatic lipase inhibitory activities of *D. edulis* leaf extracts. Data are presented as mean ± SEM. [ab]Values with different letter above the bars for a given concentration are significantly different from each other ($p < 0.05$, Tukey's HSD-multiple range post-hoc test, IBM SPSS for Windows).

Induction of oxidative injury led to increased ATPase activity in pancreatic tissues as depicted in **Figure 8.2.8**. A dose – dependent effect on reversion of the activity to near normal was observed on treatment with the extract, with the ethyl acetate showing the best activity as revealed by its low IC_{50} value (**Table 8.2.1**).

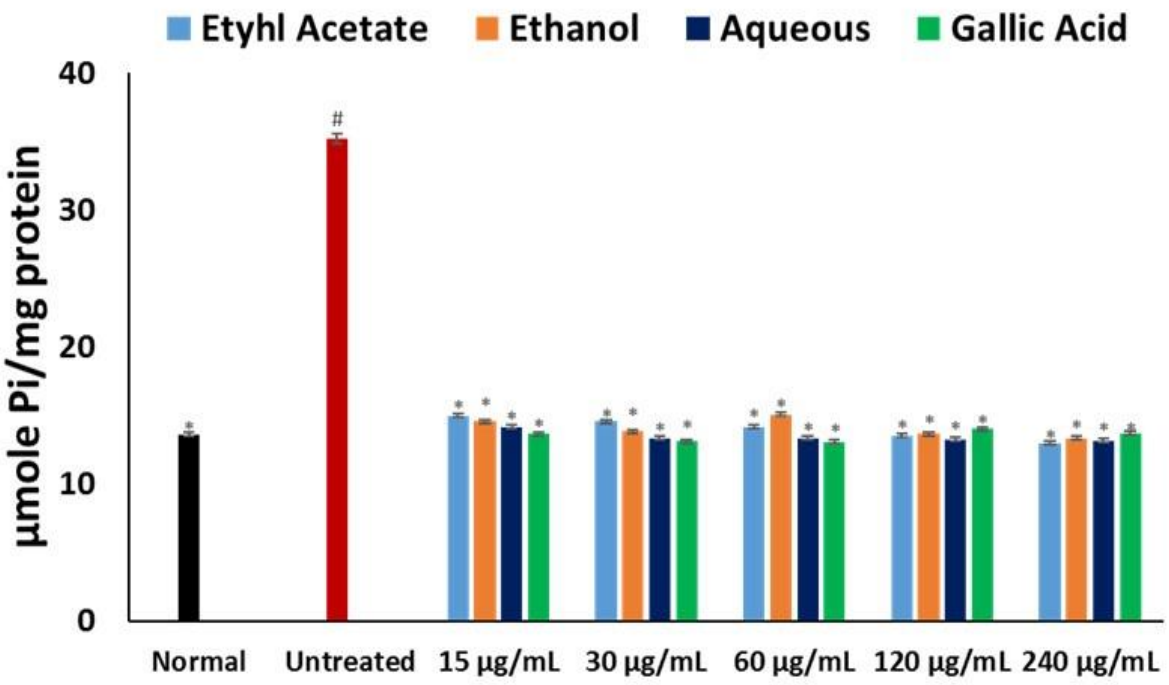

Figure 8.2.8: Effect of *D. edulis* leaf extracts on ATPase activity in oxidative pancreatic injury. Data are presented as mean ± SEM. *Significantly different from untreated sample and #Significantly different from normal sample ($p < 0.05$, Tukey's HSD-multiple range post-hoc test, IBM SPSS for Windows).

Induction of oxidative injury in hepatic tissues led to significant increase in glucose-6-phosphatase activity as shown in **Figure 8.2.9**. All extracts showed a significant ($p<0.05$) inhibition of the activity to near normal, with the aqueous showing the best activity as revealed by its low IC_{50} value (**Table 8.2.1**).

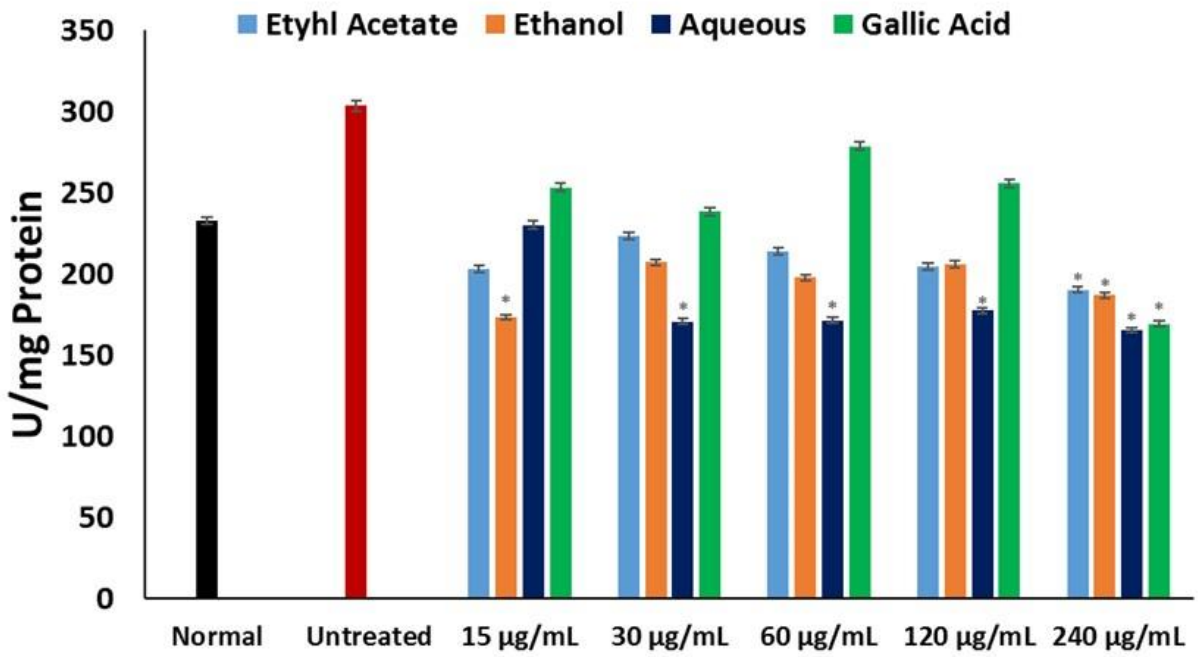

Figure 8.2.9: Effect of *D. edulis* leaf extracts on Glucose-6-Phosphatase activity in oxidative hepatic injury. Data are presented as mean ± SEM. *Significantly different from untreated sample and #Significantly different from normal sample ($p < 0.05$, Tukey's HSD-multiple range post-hoc test, IBM SPSS for Windows).

Increased DNA fragmentation was observed on induction of oxidative injury in hepatic tissue as depicted in **Figure 8.2.10**. This was significantly ($p<0.05$) suppressed on treatment with the extracts, with the ethanol showing the best activity as revealed by its low IC_{50} value (**Table 8.2.1**).

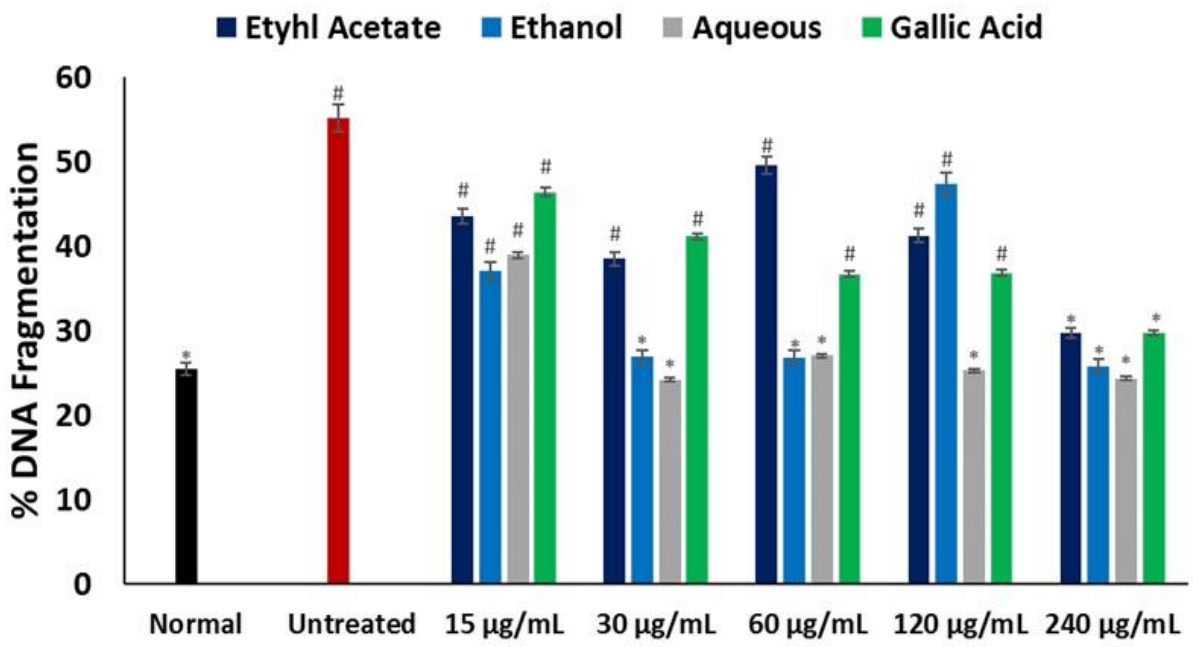

Figure 8.2.10: Effect of *D. edulis* leaf extracts on DNA fragmentation in oxidative hepatic injury. Data are presented as mean ± SD. *Significantly different from untreated sample and #Significantly different from normal sample ($p < 0.05$, Tukey's HSD-multiple range post-hoc test, IBM SPSS for Windows).

GC–MS analysis of the ethanol extract revealed the presence of Phytol acetate, Xanthone, Ascorbic acid 2,6-dihexadecanoate, Kaur-15-ene, *Trans* -Phytol, Octadecanoic acid, Ethyl 15-methylheptadecanoate, 9-(4-methoxyphenyl) xanthene, 2,3,23-trihydroxyolean-12-en-28-oic acid methyl ester, Sitosterol and Urs-12-ene-3-ol acetate as shown in **Figure 8.2.11** and **Table 8.2.2**.

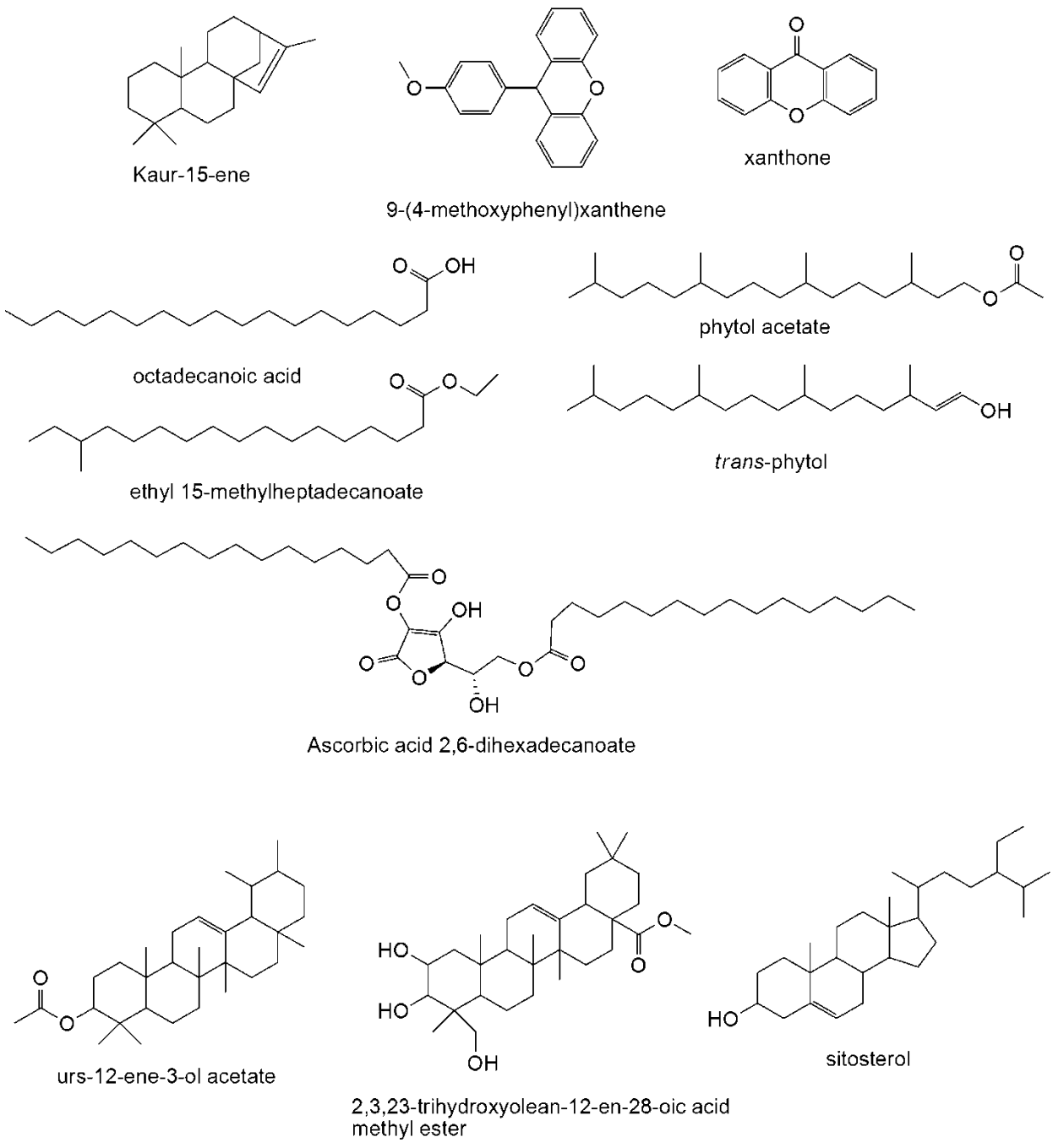

Figure 8.2.11: Structures of identified compounds in the ethanol extract of *D. edulis* leaf

Table 8.2.2: Identified compounds of the ethanol extract of *D. edulis* leaf by GC-MS

Compounds	RT (min)	Molecular mass	Relative abundance (%)
Phytol acetate	16.42	338	3.67
Xanthone	17.01	196	0.51
Ascorbic acid 2,6-dihexadecanoate	17.66	652	4.10
Kaur-15-ene	18.37	272	0.28
Trans-Phytol	19.10	296	2.03
Octadecanoic acid	19.55	284	2.58
Ethyl 15-methylheptadecanoate	19.83	298	0.90
9-(4-methoxyphenyl)xanthene	22.39	288	3.25
Urs-12-ene-3-ol acetate	24.45	468	0.63
2,3,23-trihydroxyolean-12-en-28-oic acid methyl ester	25.83	502	0.69
Sitosterol	29.77	414	1.55

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

FTIR spectroscopic analysis revealed the presence of aromatic C–H bending, N–H bend, C–C stretch (in–ring), N–O symmetric stretch, C–N stretch, C–O stretch, C=O stretch, $–CO_2H$, O–H stretch, N–H stretch chemical bonds as depicted in **Figure 8.2.12** and **Table 8.2.3**. These chemical bonds indicate the presence of alkyl halides; alcohols; esters; ethers; α,β–unsaturated aldehydes; ketones carboxylic acids; 1°, 2° amines; and amides functional groups.

Table 8.2.3: Quantitative analysis of FTIR spectra of ethanol extract of *D. edulis* leaf

Frequency (cm^{-1})	Assignment	Functional Group
720.31	Aromatics	Aromatic C-H Bending
767.39	Aromatics	Aromatic C-H Bending
819.30	Aromatics	Aromatic C-H Bending
1051.31	Aliphatic Amines	C–N stretch; C–O stretch
1159.36	Alkyl Halides	C–H wag ($–CH_2X$)
1202.12	Aliphatic Amines	C–N stretch
1248.86	Aromatic Amines, Alcohols, Carboxylic Acids, Esters, Ethers	N–O symmetric stretch; C–N stretch; C–O stretch
1281.59	Nitro Amines	N–O symmetric stretch
1363.16	Alkanes	C–H rock
1517.12	1° Amines; Aromatics	N–H bend; C–C stretch (in–ring)
1604.60	1° Amines; Aromatics	N–H bend; C–C stretch (in–ring)
1699.04	α,β–unsaturated aldehydes, ketones	C=O stretch
2851.69	Carboxylic Acids	$–CO_2H$
2920.57	Carboxylic Acids	$–CO_2H$
3257.71	Phenols; 1°, 2° amines, amides	O–H stretch; N–H stretch

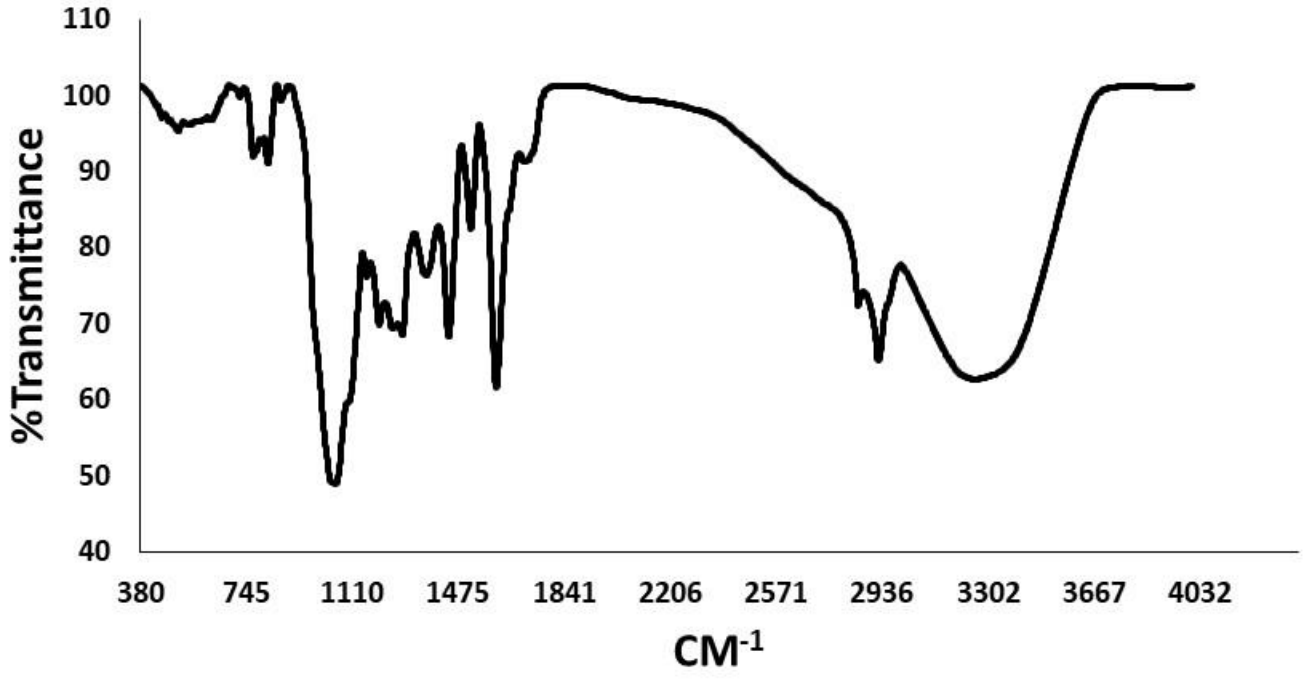

Figure 8.2.12: Fourier transform infrared (FT-IR) spectroscopy of the ethanol extract of *D. edulis* leaf

Except for octadecanoic acid, prediction of oral toxicity of the identified compounds on ProTox (tox.charite.de) web-based server showed they fell between toxicity classes 4 – 6 indicating their safety on consumption as shown in **Table 8.2.4**. Octadecanoic acid had the least toxicity class of 3, implying toxic if swallowed. While phytol acetate and ascorbic acid 2,6-dihexadecanoate had the highest toxicity class of 6, implying their safety if swallowed.

Table 8.2.4: Oral Toxicity Prediction and Binding Affinity of Identified compounds of the ethanol extract of *D. edulis* leaf by GC-MS

Compounds	**Predicted LD_{50} (mg/kg)**	**Toxicity Class**	**Prediction Accuracy (%)**	**Binding Affinity (Kcal/mol)**
Phytol acetate	8000	6	72.9	16.70
Xanthone	623	4	68.07	32.22
Ascorbic acid 2,6-dihexadecanoate	25000	6	69.26	67.96
Kaur-15-ene	5000	5	69.26	-25.90
Trans-Phytol	5000	5	100	19.46
Octadecanoic acid	130	3	100	38.11
Ethyl 15-methylheptadecanoate	4800	5	100	21.01
9-(4-methoxyphenyl)xanthene	370	4	68.07	37.82
Urs-12-ene-3-ol acetate	4800	5	70.97	-142.73
Sitosterol	890	4	72.90	18.27
2,3,23-trihydroxyolean-12-en-28-oic acid methyl ester	-	-	-	-80.49

The selected conformations of the identified compounds were docked into the active site of alpha glucosidase using GOLD software. In the binding pocket, common H-bonding interactions were formed between all docked ligands and LYS356, LYS347, ALA355 and ASP969. The specific H-bonding interaction with GLU354 was observed for some of the compounds. Among the 11 compounds docked, only Kaur-15-ene (-25.00 Kcal/mol), Urs-12-ene-3-ol acetate (-142.73 Kcal/mol) and 2,3,23-trihydroxyolean-12-en-28-oic acid methyl ester (-80.49 Kcal/mol) showed

strong binding affinities, with Urs-12-ene-3-ol acetate showing the best activity (**Figure 8.2.13A – 8.2.13C** and **Table 8.2.4**). The others showed weak binding affinity as indicated by their binding energy (**Table 8.2.2**). In the binding pocket, common H-bonding interactions were formed between all docked ligands and LYS356, LYS347, ALA355 and ASP969. The specific H-bonding interaction with ALA355 was only found in molecule 6.

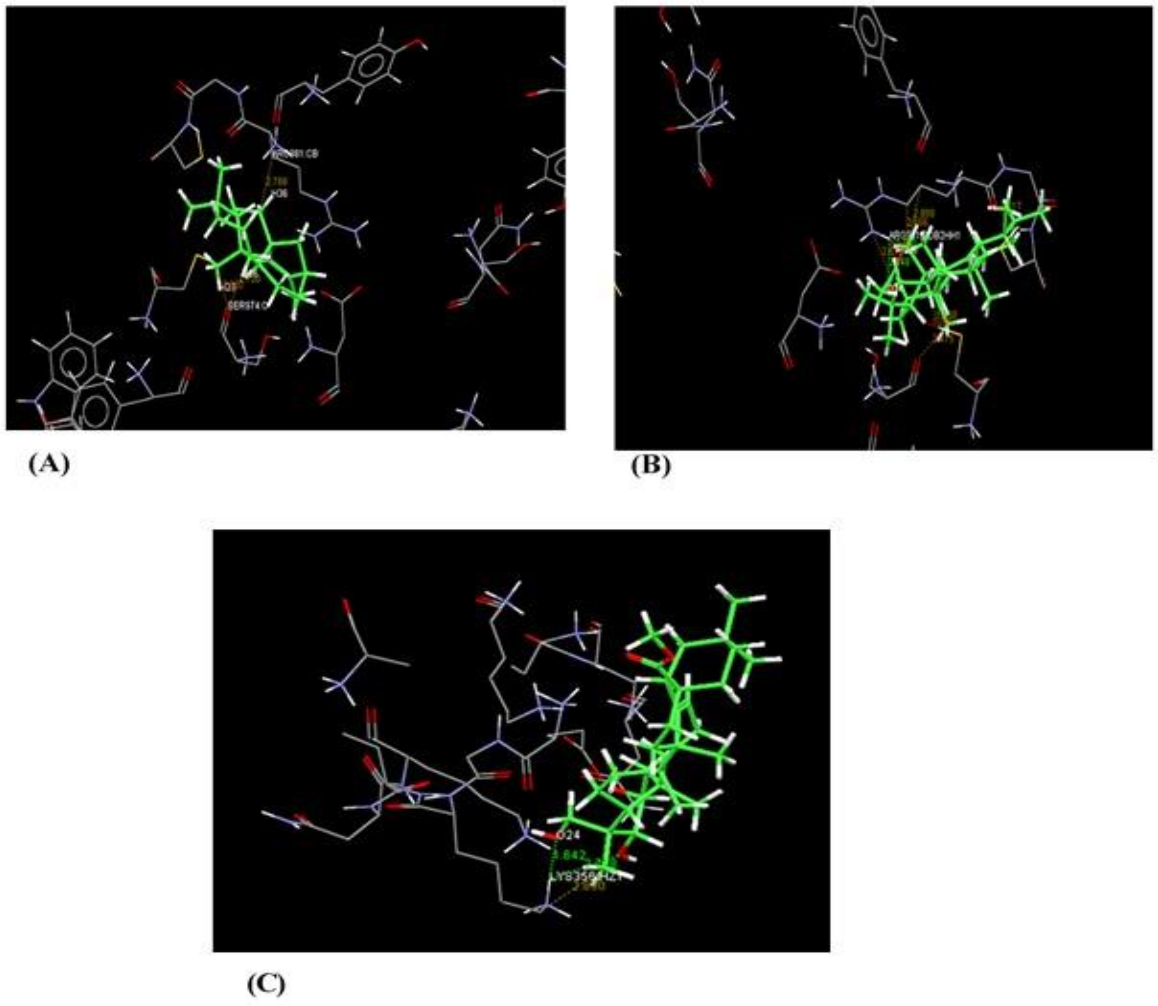

Figure 8.2.13: Docked conformation of (A) kaur-15-ene; (B) urs-12-ene-3-ol acetate; and (C) 2,3,23-trihydroxyolean-12-en-28-oic acid methyl ester in the binding pocket of α-glucosidase (PDB ID:3TON)

8.2.5 Discussion

The role of iron in the etiology and pathogenesis of T2D and its complications has been reported.(Andrews 1999) Alteration of optimal heath owing to iron deficiency and iron overload makes it the proverbial double edged sword. Its ability to exist in two ionic states: ferrous (Fe^{2+}) and ferric (Fe^{3+}) makes it a major co-factor for several enzymes involved in redox reactions (Andrews 1999; Rajpathak et al. 2009). This also makes it a potent pro-oxidant. Thus, high concentration levels may elevate T2D and its complication via increased redox imbalance (Rajpathak et al. 2009). In this study, oxidative injury was induced *ex vivo* in pancreatic and hepatic tissues with $FeSO_4$ and treated with extracts of *D. edulis* leaves.

Several *in vitro* studies have linked high total phenolic content with increased free radical scavenging activities (Anahita et al. 2015; Hossain and Shah 2015; Saeed et al. 2012). This corresponds to the observed high total phenolic content and DPPH scavenging activity of the ethanol extract **(Figure 8.2.2A and 8.2.2B).**

GSH has been documented as the first line of defense in the body's endogenous antioxidant system, with the highest concentration found in the liver.(Mohamed et al. 2016; Tiwari et al. 2013a) It is regarded as a marker of oxidative stress at cellular level, thus implying the reduced levels observed in the untreated tissues (**Figures 8.2.3A** and **8.2.3B**) indicates an occurrence of oxidative stress. Several studies have implicated a reduced GSH level in β-cell dysfunction and in the pathogenesis of T2D complications. (Tiwari et al. 2013) The observed reduced GSH levels in the tissues can be attributed to an overwhelming effect of ROS produced due to the oxidation of Fe^{2+} to Fe^{3+}. The increased levels on treatment with the extracts therefore portrays an anti-oxidative protective potential against Fe^{2+} - induced oxidative injuries, which corresponds to the observed FRAP activities (**Figure 8.2.2B**).

Generation of superoxide ($O_2^{\cdot -}$) and hydroxyl (˙OH) radicals have been recognized as major mechanism of iron toxicity and if not scavenged, can lead to oxidative damage (Davari et al. 2013). The protective role of SOD against oxidative cellular damage is well documented. It catalyzes the dismutation of $O_2^{\cdot -}$ to H_2O_2, which is further converted into oxygen and water by catalase. SOD level in pancreatic β–cells is 50% that of hepatic tissues, while catalase have been reported to be extremely low making 1% that of hepatic tissues (Pi et al. 2010; Tiedge et al. 1997). Thus, making

the pancreatic β–cells more susceptible to oxidative damage compared to other organ tissues. The significantly reduced SOD and catalase activities (**Figure 8.2.4A – 8.2.5B**) in the untreated tissues further portrays an occurrence of oxidative injury on incubation with $FeSO_4$. Increased activities in the treated tissues, also indicates an anti-oxidative potential.

The role of iron on the induction of lipid peroxidation is well documented (Andrews 1999; Rajpathak et al. 2009). H_2O_2 not neutralized by catalase reacts with Fe^{3+} via Fenton reaction to produce $^{\bullet}OH$ (Aslan et al. 2000). This attacks the membrane lipid, triggering a peroxidative reaction which produces highly reactive aldehydes, including MDA, acrolein, 4-hydroxynonenal (HNE), 4-oxononenal (ONE), and isolevuglandins (IsoLGs) (Guo et al. 2012a). Increased MDA levels have been reported in T2D and its complications (Saddala et al. 2013). Incubation of pancreatic and hepatic tissues with $FeSO_4$ led to significant increase in MDA level, signifying an occurrence of lipid peroxidation (**Figure 8.2.6A** and **8.2.5B**). This also indicates a decline in the endogenous antioxidant system. The decreased MDA level on treatment with the extracts corresponds to the observed increased GSH level, SOD and catalase activities (**Figure 8.2.3A – 8.2.5B**). It also portrays an anti-peroxidative effect.

Inhibition of intestinal α – glucosidase has been demonstrated to delay and prolong carbohydrate digestion, thus reducing the rate of glucose absorption with concomitant attenuation of glucose-induced insulin secretion (Bischoff ; Bischoff 1995). The dose – dependent inhibition by the extracts (**Figure 8.2.7A**) portrays their ability to delay carbohydrate digestion, thereby reducing the rate of glucose absorption. This therefore indicates an anti-hyperglycemic potential. The strong binding affinities coupled with the molecular docking of Kaur-15-ene, Urs-12-ene-3-ol acetate and 2,3,23-trihydroxyolean-12-en-28-oic acid methyl ester with α – glucosidase (**Figure 8.2.13A – 8.2.13C** and **Table 8.2.3**), indicates a strong inhibitory effect on the enzyme. This corroborates with the observed inhibitory effect of the ethanol extract on α – glucosidase (**Figure 8.2.7A**) and can be thus be attributed to a synergetic effect of these compounds.

Pancreatic lipase plays a major function in dietary fat absorption by hydrolyzing triglycerides into monoglycerides and free fatty acids (Lunagariya et al. 2014). Its role in the progression of obesity is well documented. Thus, making it a major inhibitory target for the treatment and management of obesity. The dose – dependent inhibition by the extracts indicates an anti-obesogenic potential

(**Figure 8.2.7B**). This correlates with the observed α – glucosidase activity (**Figure 8.2.7A**), thus further portraying the potential of the plant in the management of T2D and its complication.

Decrease in pancreatic ATPase activity particularly Na^+K^+-ATPase leading to β – cell membrane depolarization and Ca^{2+} influx has been suggested to be a major mechanism of insulin secretion (Costa et al. 2010; Owada et al. 1999). Na^+, K^+-ATPase activity has been suggested as a possible mechanism by which glucose initiate insulin secretion via promotion of membrane depolarization (Owada et al. 1999). Thus, the increased ATPase activity in the oxidative injured pancreatic tissues may portray an impaired membrane depolarization reflecting a decrease in insulin secretion. The reduced activity on treatment with the extract may therefore indicate increased membrane polarization, implying an increase in insulin secretion (**Figure 8.2.8**).

The role of glucose-6-phosphatase in the homeostatic regulation of blood glucose levels is well documented (Ghosh et al. 2004; Van Schaftingen and Gerin 2002). Its increased activity leading to hyperglycemia in T2D have been reported (Ghosh et al. 2004). Thus, the increased activity in the untreated oxidative injured hepatic tissue (**Figure 8.2.9**) may indicate an increased gluconeogenesis and glycogenolysis. The significant ($p<0.05$) decrease on treatment with the extracts further portrays an anti-hyperglycemic potential, with increased glycogenesis.

Increased apoptosis characterized by DNA fragmentation and cellular shrinkage has been reported in pancreatic β – cells and hepatocytes in T2D (Donath et al. 1999). This can be attributed to increasing hyperglycemia and hyperlipidemia, with oxidative stress playing an influential role (Allameh et al. 2010). The role of Fe^{2+} in oxidative injury via the Fenton pathway, therefore implicates iron in the triggering of apoptosis. The increased DNA fragmentation on incubation of hepatic tissues with Fe^{2+} portrays an apoptotic activity (**Figure 8.2.10**). This corresponds to previous report on the induction of apoptotic cell death in isolated rat hepatocytes by iron overload via oxidative stress (Allameh et al. 2010). The decreased DNA fragmentation on treatment with the extracts indicates an anti-apoptotic activity in oxidative injured hepatic tissue. This correlates with the observed anti-oxidative activities of the extracts (**Figure 8.2.3A – 8.2.6B**).

Owing to its consistent high activities, the ethanol extract of *D. edulis* leaves was subjected to GC–MS analysis to have an idea of its likely chemical constituents (**Figure 8.2.11** and **Table 8.3.2**). The antioxidant properties of the identified phytochemical constituents have been documented

particularly phytol and its derivatives (Santos et al. 2013), 2,3,23-trihydroxyolean-12-en-28-oic acid methyl ester (Abdullah et al. 2016), and Urs-12-ene-3-ol acetate (El-Kashef et al. 2015). The observed anti-oxidative activities of the extract can therefore be attributed to the synergetic effect of the identified phytochemicals. Furthermore, the identified functional groups (**Figure 8.2.12** and **Table 8.2.3**) particularly the carboxylic acids and phenols corresponds to the identified compounds (**Figure 8.2.11**) and total phenol content (**Figure 8.2.1**). N–H bend, N–O symmetric C–C stretch and C=O stretches indicate an electron – deficient moiety, making it susceptible to oxidative reaction (Harrold 2013). Thus, a potent antioxidant and may be responsible for the antioxidative effect of the extracts. The presence of C–H rock of alkane indicate metabolic selectivity of the extract as alkanes has a large impact on the metabolism that a drug undergoes or not (Demeyer et al. 1994). The oxygen atom present in phenols, ether and esters makes them good hydrogen acceptors, which also portrays a potent antioxidative activity (Harrold 2013) and may be responsible for the reducing power of the extract (**Figure 8.2.2B**). The high concentration of amides and amines is of great significance. They are protonated by hydrochloric acid as the pass through the gut making them water soluble and prevents their absorption into the bloodstream from the intestinal tract (Manallack et al. 2013).

The risk of toxicity of the use of medicinal plant is of major concern, particularly issues concerning standardization, characterization and preparation (Ezuruike and Prieto 2014). Studies have revealed that administration of plant extracts triggers different biological activities in the body, some of which may be toxic effects (Ezuruike and Prieto 2014; Yüce et al. 2006). The observed toxicity classes of the identified compounds of the ethanol extract indicates the safety of the extract on oral consumption (**Table 8.3.4**). However, further studies on their cytotoxic effect on normal cell lines are recommended.

8.2.6 Conclusion

These results suggest the anti – oxidative and anti-hyperglycemic potential of *D. edulis* leaves, with the ethanol extract showing the overall best activity. These can be attributed to the synergetic effect of the identified phytochemical compounds. These findings give credence to the folkloric claims of the use of *D. edulis* leaves as major ingredient in the treatment and management of T2D and its complications. However, *in vivo* studies are required to further validate its anti-hyperglycemic activity as well as the underlying molecular mechanisms.

8. 3 Antidiabetic Properties of the Butanol Fraction of *Dacryodes edulis* L. Ethanol Extract: Modulatory Effects on Redox Imbalance and Nrf2 Expression; and Inhibitory Effects on Glycolytic and Cholinergic Enzymes Activities in Type 2 Diabetic Rats

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Preface: This article investigated the antidiabetic effect of the butanol fraction of *D. edulis* ethanol extract in type 2 diabetic rats. Its antioxidative, anti-proinflammatory, antilipemic and insulin stimulatory effects were investigated. Its inhibitory effect on glycolytic, cholinergic and carbohydrate digesting enzymes were investigated. Its ability to inhibit Nrf2 expression was also investigated *in vivo*. Additionally, its stimulatory effect on glucose uptake and cytotoxicity in 3T3 adipocytes cells was investigated.

8.3.1 Abstract

The antidiabetic effect of the butanol fraction of *Dacryodes edulis* L. ethanol extract (BFDE) was investigated in fructose-streptozotocin induced type 2 diabetic rats. Diabetic rats were grouped into 4. A group served as a negative control and was administered water only, another was administered metformin (200 mg/kg bodyweight), while the other 2 were administered BDFE at 150 and 300 mg/kg bodyweight respectively. Two other groups of normal rats were administered water and BFDE (300 mg/kg bodyweight) respectively, with the formal serving as normal control. The rats were sacrificed after 6 weeks of intervention. Treatment with the fraction to significant

($p<0.05$) reduction in blood glucose level, with concomitant increase in serum insulin secretion. It also caused significant ($p<0.05$) elevation of reduced glutathione level, superoxide dismutase, catalase, α-amylase, and ATPase activities, while concomitantly depleting myeloperoxidase activity, NO and MDA levels of the serum and pancreas. The pancreatic β-cell function and morphology were significantly improved in BFDE-treated rats, with restoration of the pancreatic capillary networks. Treatment with BFDE significantly ($p<0.05$) inhibited glycogen phosphorylase, fructose 1,6 biphosphatase, glucose 6 phosphatase, and acetylcholinesterase activities, with while suppressing Nrf2 expression. HPLC analysis of the fraction revealed the presence of gallic acid, vanillic acid, vanillin, and (-)-epicatechin. These results portray the antidiabetic and antioxidative properties of BFDE, which may be attributed to the synergistic effect of the identified phenolics.

Keywords: Antioxidant; *Dacryodes edulis*; Nrf2; Phenolics; Type 2 diabetes

8.3.2 Introduction

The use medicinal plants in the treatment of various diseases and ailments such as diabetes mellitus (DM), cancers, and malaria dates to time immemorial, with their folkloric uses integrated in the cultural systems and practices of their indigenous flora. These plants also contribute to the indigenous health system of most countries in the present day, with their increasing utilizations attributed to the paradigm shift from synthetic drugs to plant-based natural products (Erukainure et al. 2018). The efficacy of folkloric claims of these plants has been validated by several studies and attributed the medicinal properties to their phytochemical constituents, with phenolics being amongst the most studied phytochemicals (Decker 1995). Amongst such plants is *Dacryodes edulis* L., belonging to the *Burseraceae* family.

Diabetes mellitus is amongst the many diseases folklorically treated with *D. edulis*. It is characterized by high blood glucose level (hyperglycemia) arising from disorders in carbohydrate, protein, and fatty acid metabolisms. These metabolic disorders have been attributed to inability of the pancreatic β-cells to secrete insulin as seen in type 1 diabetes (T1D) and/or inability of the cells to utilize the insulin secreted by the pancreatic β-cells as seen in type 2 diabetes (T2D) (Erukainure et al. 2018). T2D accounts for over 90% of all diabetes, making it the most prevalent (I.D.F. 2016; I.D.F. 2018). It is characterized by insulin resistance and β-cell dysfunction, leading

to persistent hyperglycemia and dyslipidemia (Bardini et al. 2012; Kahn 2003). Free radicals arising from persistent hyperglycemia will lead to oxidative stress, when its production surpasses the body's endogenous antioxidant system (Maritim et al. 2003; Tiwari et al. 2013b). Oxidative stress has been linked with the pathogenesis, pathophysiology and progression of micro and macro – complications associated with T2D such retinopathy, nephropathy, and cardiopathy (Evans et al. 2002; Giacco and Brownlee 2010). This has been evidenced by several pre-clinical and clinal studies using antioxidants in the treatment and management of T2D and its complications (Sabu and Kuttan 2002; Tiong et al. 2013). Oxidative stress has also been reported to aggravate pancreatic β-cell dysfunction and insulin secretion by triggering apoptosis of the pancreatic β-cell involving the caspase cascade (Liadis et al. 2005; Montane et al. 2014). Increased expression of the nuclear factor erythroid 2–related factor 2 (Nrf2) has also been implicated in the suppression of antioxidants and the early onset of T2D (He et al. 2012; Miao et al. 2012).

Thus, the study aims at investigating the antidiabetic properties of the butanol fraction of *D. edulis* leaves ethanol extract in T2D rats. The inhibitory effects of the fraction on major glycolytic, cholinergic and carbohydrate hydrolyzing enzymes were also investigated. Additionally, its ability to modulate pancreatic oxidative injury and morphology as well as suppress the expressions of (Nrf2) and caspase-3 were investigated.

8.3.3 Materials and Methods

Kindly refer to chapter 2, subsections: 2.1.1, 2.1.3, 2.1.5, 2.1.6, 2.4.1, 2.9, 2.10.1, 2.10.3, 2.11, 2.12 – 2.13 for details materials and methods.

8.3.4 Results

Incubation of *D. edulis* fractions with α-glucosidase led to significant ($p<0.05$) inhibition by all the fractions to levels indistinguishable from the standard drug, acarbose as shown in **Figure 8.3.1**. The inhibitions were dose dependent, with the BuOH fraction showing the best activity as indicated by its low IC_{50} value of 0.13 µg/mL (**Table 8.3.1**).

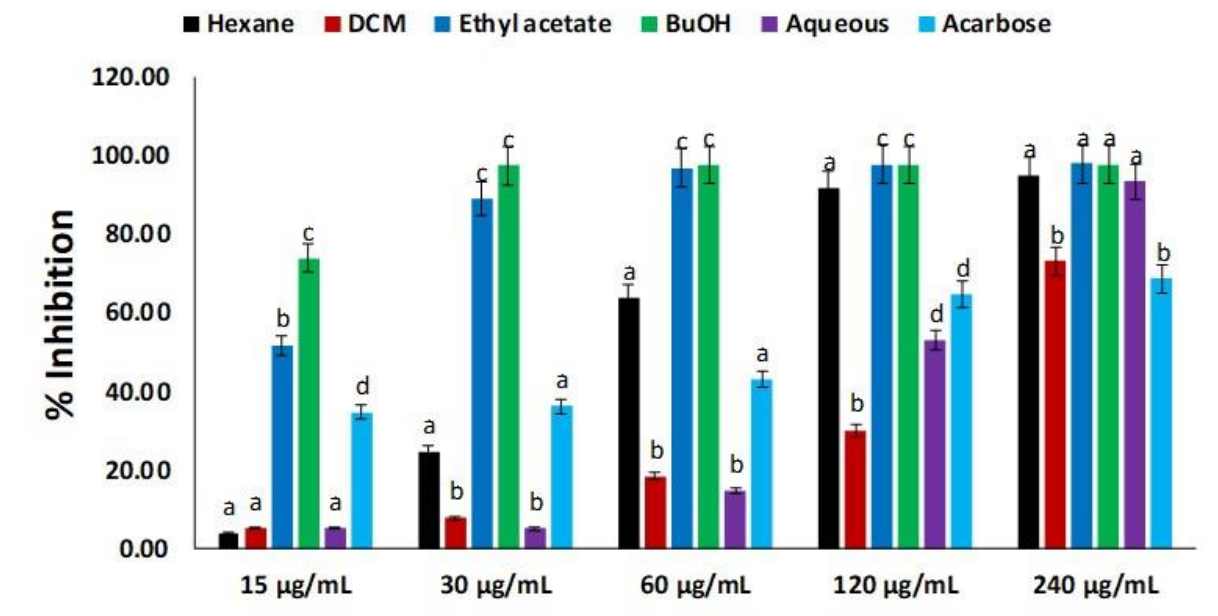


Figure 8.3.1: α-glucosidase inhibitory activities of fractions of *D. edulis* ethanol extract. Values = mean ± SD; n = 3. [abcd]Values with different letter above the bars for a given concentration are significantly different ($p < 0.05$) from each other.

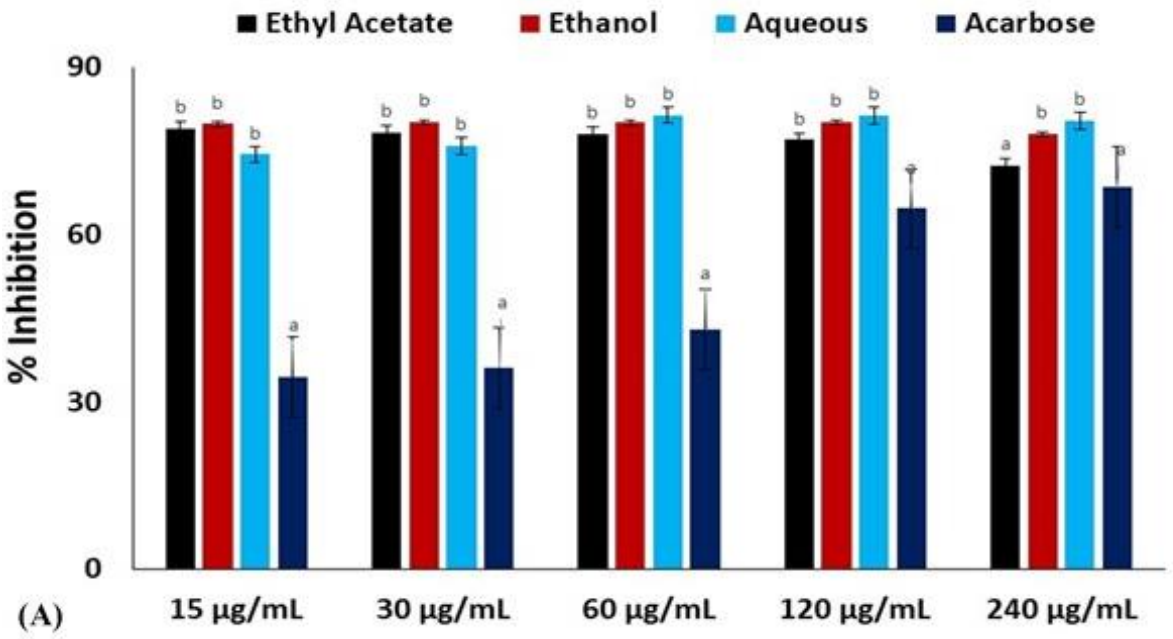
Table 8.3.1: IC_{50} values of α-glucosidase inhibitory activities of fractions of D. edulis ethanol extract

Fractions	**IC_{50} values**
Hexane	50.97
DCM	164.92
Ethyl acetate	4.85
BuOH	0.13
Aqueous	97.70
Acarbose	62.42

Values = mean; n = 3

Figure 8.3.2A shows the effect of a 1.5 h treatment of extract on glucose uptake/utilization in 3T3-L1 adipocytes, while **Figure 8.3.2B** shows they effect of the extract treatment on the adipocytes viability during the 1.5 h treatment period. Data showed that the fraction increased glucose uptake in adipocytes by 10, 8 and 52% at 30, 60 and 120 µg/mL, respectively, while 1 µM metformin

increased glucose uptake by 96% (**Figure 8.3.2A**). Only the 120 µg/mL extract and insulin significantly increased ($p < 0.05$) glucose uptake, although the effect of insulin was significantly higher ($p < 0.05$) than that of the extract (**Figure 8.3.2A**). The computed GU_{50} (concentration of extract in µg/mL causing 50% glucose uptake increase) of extract was 123.7 µg/mL. Interestingly, **Figure 8.3.2B** showed that the viability of the adipocytes after the treatment period was 87, 136 and 166% at 30, 60 and 120 µg/mL extract concentration, respectively, which suggests that the extract did not markedly reduce the cells' viability after treatment. However, the proliferative tendency of the fraction on the adipocytes, particularly at 120 µg/mL, may influence its glucose uptake activity.



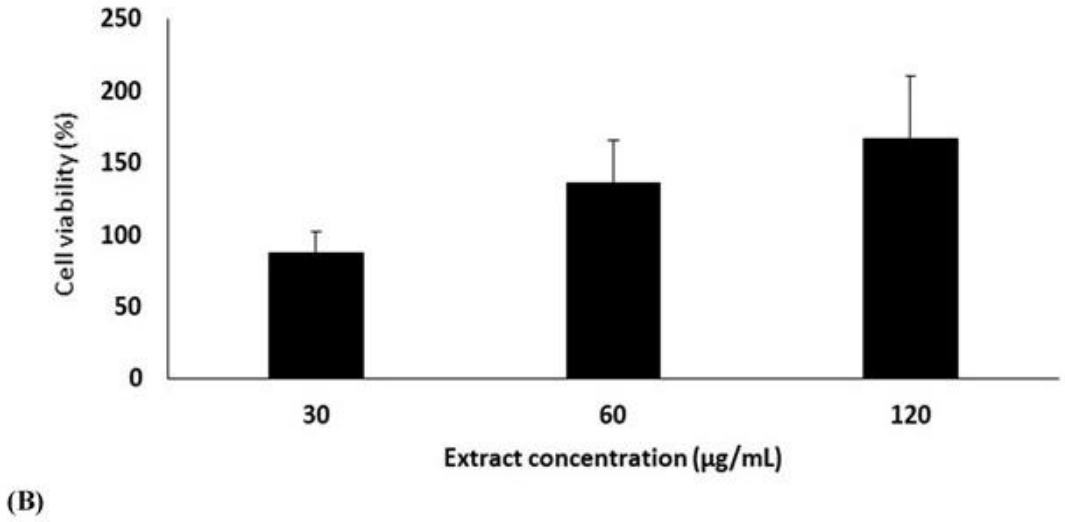
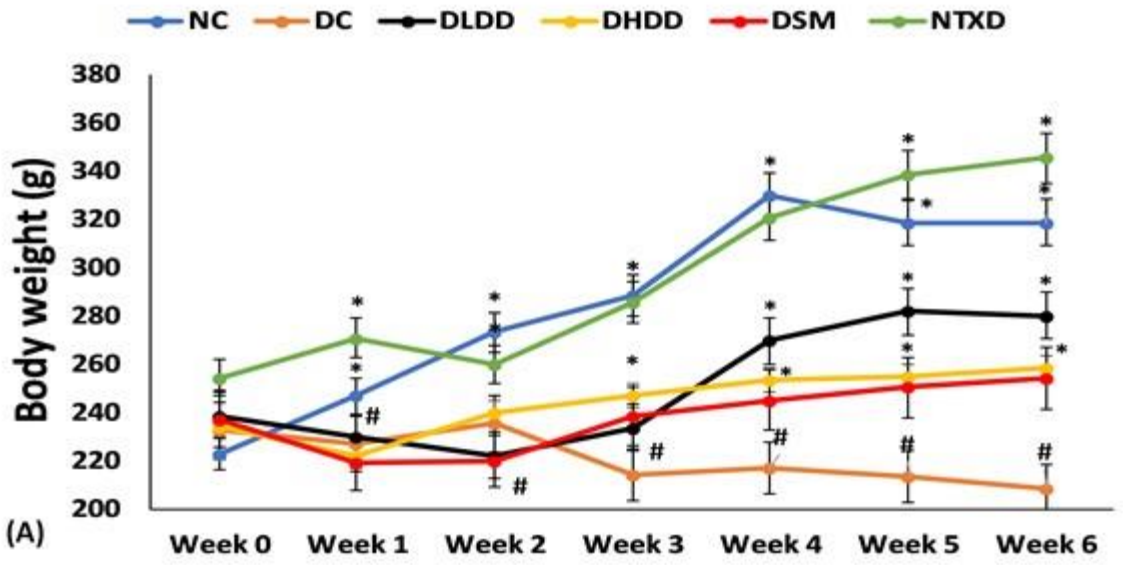
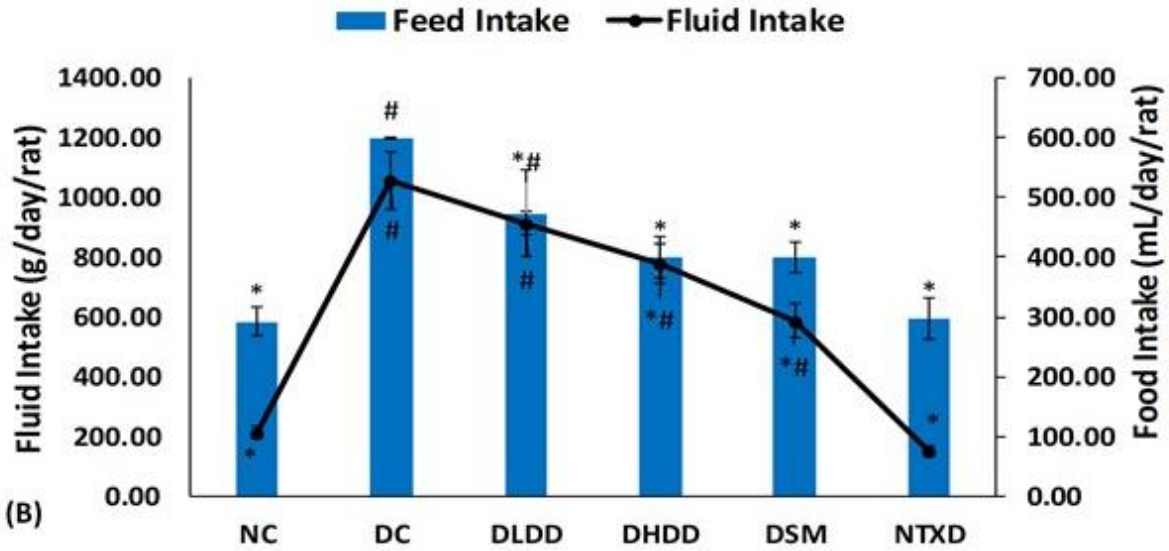


Figure 8.3.2: Effect of BFDE on (A) Glucose uptake and (B) cell viability on 3T3-L1 adipocytes. Data = mean ± SD; n = 3. [abc]Values are significantly different from each other when comparing control different treatment in 3T3-L1 adipocytes ($p < 0.05$; Tukey's HSD multiple range post-hoc test, IBM, SPSS, version 23)

Induction of T2D led to significant ($p<0.05$) loss in body weight, with concomitant increase in food and fluid intake as depicted in **Figures 8.3.3A and 8.3.3B**. This also led to reduced pancreatic weight and relative weight (**Figure 8.3.3C**). Treatment with BFDE significantly ($p<0.05$) reversed these changes, with rats treated with high dose of BFDE showing reversibility higher than the standard antidiabetic drug, metformin.


NC
DC
DLDD
DHDD
DSM
NTXD
Body weight (g)
380
360
340
320
300
280
260
240
220
200
(A)
Week 0
Week 1
Week 2
Week 3
Week 4
Week 5
Week 6


Feed Intake
Fluid Intake
Fluid Intake (g/day/rat)
1400.00
1200.00
1000.00
800.00
600.00
400.00
200.00
0.00
Food Intake (mL/day/rat)
700.00
600.00
500.00
400.00
300.00
200.00
100.00
0.00
(B)
NC
DC
DLDD
DHDD
DSM
NTXD

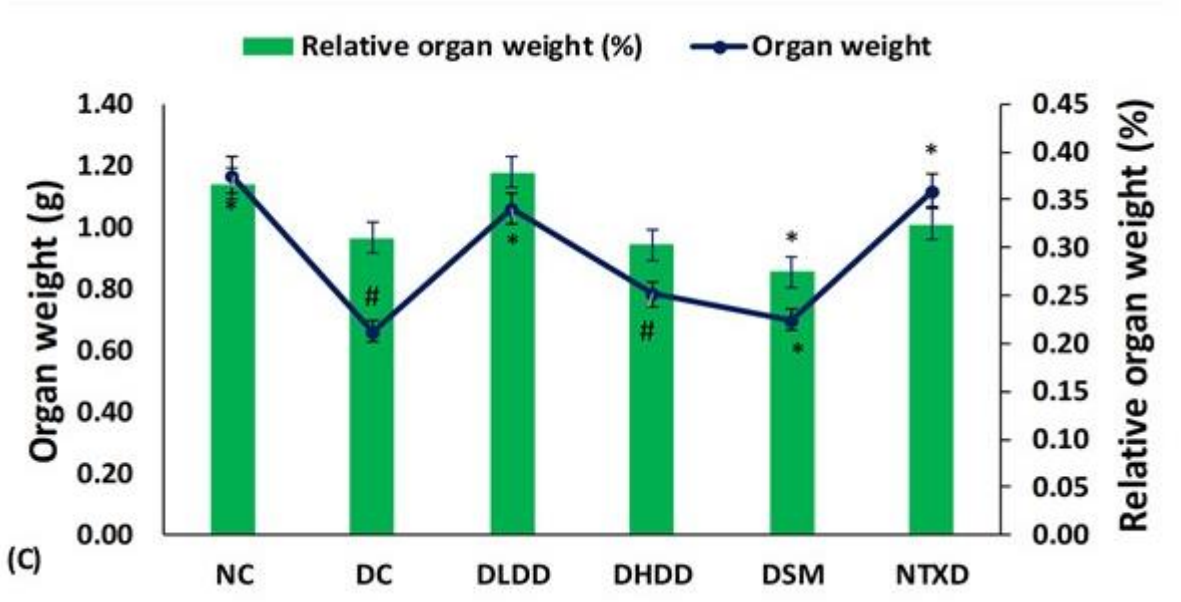


Figure 8.3.3: (A) Body weight; (B) feed and fluid intake; and (C) weight and relative weight of pancreas of experimental groups. Values = mean ± SD; n = 5 (NC and NTXD) and 7 (DC, DLDD, DHDD, and DSM). *Statistically ($p<0.05$) significant to DC, #statistically significant ($p<0.05$) to NC. **NC** = normal rats, **DC** = diabetic control, **DLDD** = diabetic rats + *D. edulis* (150 g/kg bw), **DHDD** = diabetic rats + *D. edulis* (300 g/kg bw), **DSM** = diabetic rats + metformin (200 g/kg bw), and **NTXD** = Normal rats + *D. edulis* (300 g/kg bw).

As shown in **Figure 8.3.4A**, induction of T2D led to increased blood glucose level with concomitant reduction in serum insulin level (**Figure 8.3.4C**) with significantly ($p<0.05$) increased HOMA- IR and reduced HOMA-β function (**Figure 8.3.4D**). These were significantly ($p<0.05$) reversed in all treatment groups, with BFDE treated rats showing 33.75 and 44.067% reduction in blood glucose levels for low and high doses respectively (**Figure 8.3.4A**). OGTT analysis revealed an increased level of blood glucose, which peaked at 30 min for all groups except metformin treated (DSM) (**Figure 8.3.4B**). The glucose level reduced with increasing time during the test.

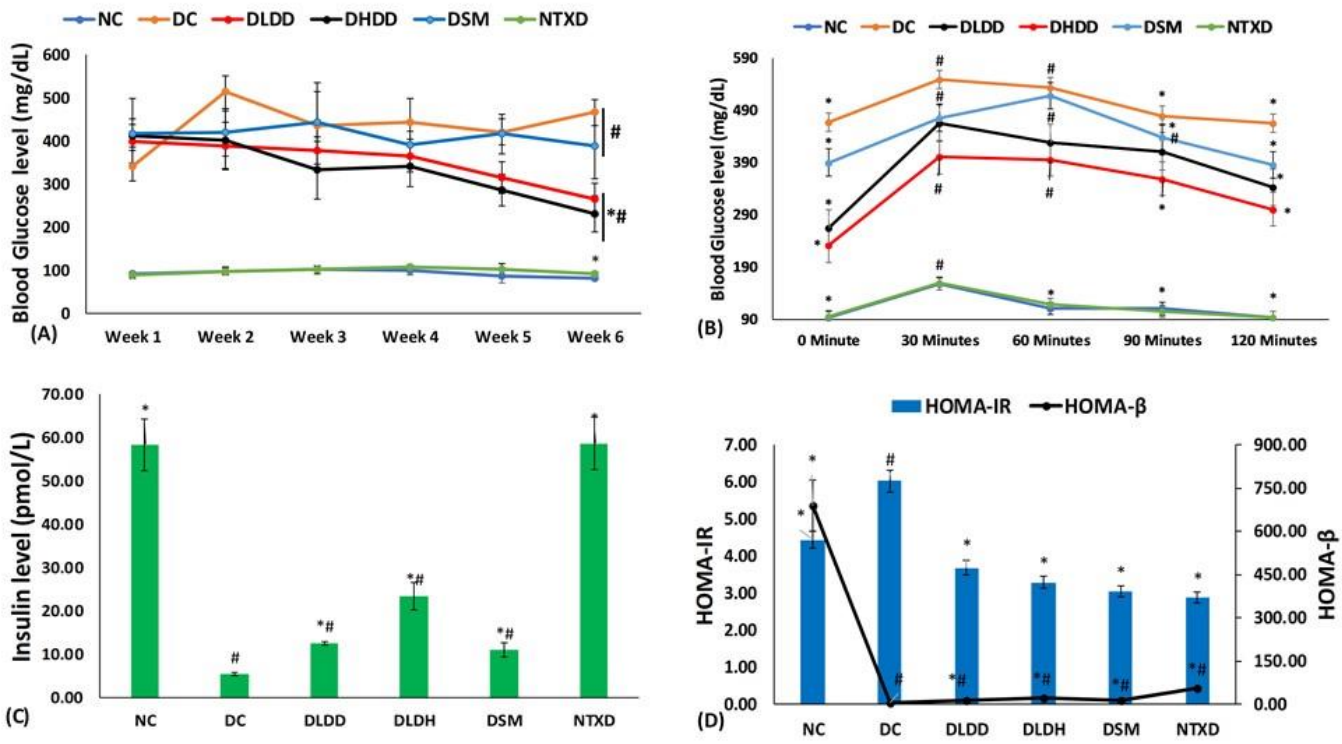


Figure 8.3.4: (A) Blood glucose level; (B) oral glucose tolerance; (C) serum insulin; and (D) HOMA-IR and β-cell function of experimental groups. Values = mean ± SD; n = 5 (NC and NTXD) and 7 (DC, DLDD, DHDD, and DSM). *Statistically ($p<0.05$) significant to DC, #statistically significant ($p<0.05$) to NC **NC** = normal rats, **DC** = diabetic control, **DLDD** = diabetic rats + *D. edulis* (150 g/kg bw), **DHDD** = diabetic rats + *D. edulis* (300 g/kg bw), **DSM** = diabetic rats + metformin (200 g/kg bw), and **NTXD** = Normal rats + *D. edulis* (300 g/kg bw).

Induction of T2D did not cause any significant increase in TC, but treatment with BFDE led to a significant ($p<0.05$) decrease as depicted in **Figure 8.3.5**. However, induction of T2D significantly ($p<0.05$) increased TG and LDL-c levels, while concomitantly suppressing HDL-c level. The TG level of all treatment groups were significantly reduced to levels indistinguishable from the normal control. Treatment with BFDE had little or no effect on LDL-c level, while treatment with low dose of BFDE led to significant ($p<0.05$) increased level of HDL-c.

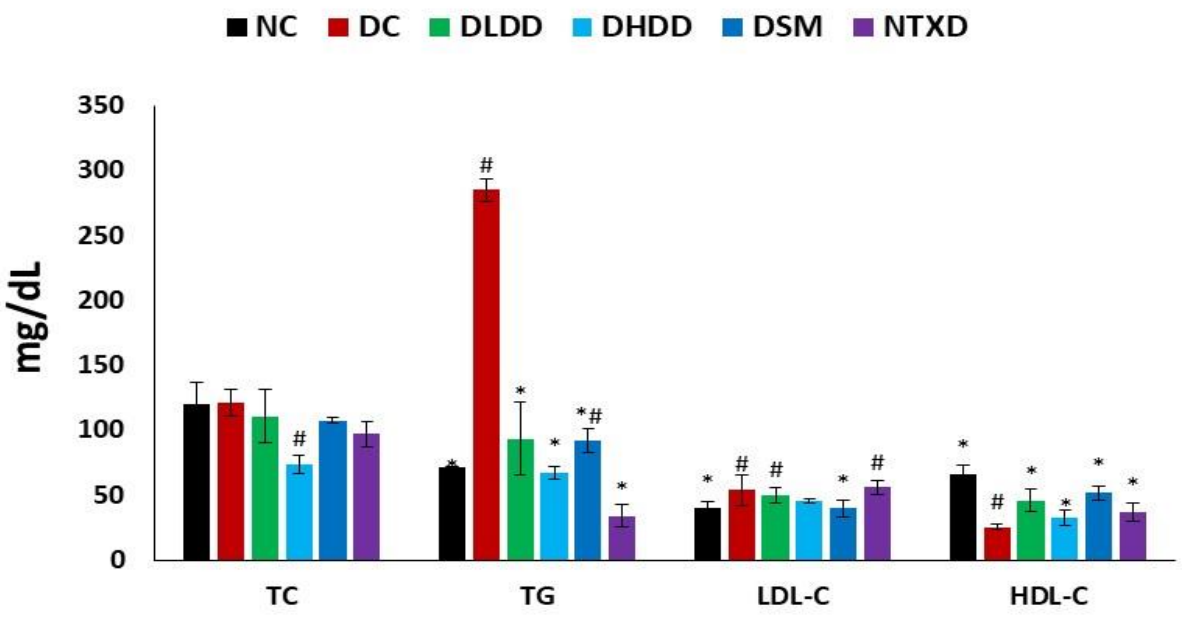


Figure 8.3.5: Serum lipid profile of experimental groups. Values = mean ± SD; n = 5 (NC and NTXD) and 7 (DC, DLDD, DHDD, and DSM). *Statistically ($p<0.05$) significant to DC, #statistically significant ($p<0.05$) to NC. **NC** = normal rats, **DC** = diabetic control, **DLDD** = diabetic rats + *D. edulis* (150 g/kg bw), **DHDD** = diabetic rats + *D. edulis* (300 g/kg bw), **DSM** = diabetic rats + metformin (200 g/kg bw), and **NTXD** = Normal rats + *D. edulis* (300 g/kg bw).

There were significant ($p<0.05$) increases in serum and pancreatic α-amylase activities respectively on induction of T2D as shown in **Table 8.3.2**. These were significantly ($p<0.05$) reduced in all treatment groups, with rats treated with low dose of BFDE showing a better inhibitory activity for serum α-amylase, while the high dosed exhibited a higher pancreatic α-amylase activity.

As shown in **Table 8.3.2**, there was a significant ($p<0.05$) decrease in serum ATPase activity on induction of T2D. This was significantly ($p<0.05$) increased in all treatment groups, with the BFDE high dose – treated rats exhibiting the highest activity.

Table 8.3.2: Enzyme activities of experimental groups.

Enzyme Activities	NC	DC	DLDD	DHDD	DSM	NTXD
Serum α-amylase	25.38±3.50*	72.36±4.25#	31.21±3.86*	46.41±5.36#	41.57±9.53*	34.11±2.41*
Pancreatic α-amylase	46.90±4.25*	89.72±4.21#	24.32±1.86*	8.42±1.39*	28.02±3.37*	27.29±2.45*
Serum ATPase	279.18±5.60*	201.24±17.89#	251.38±5.97*	255.70±7.20*	241.92±12.42*	235.30±6.15*
Pancreatic ATPase	144.35±21.59*	234.49±7.781#	133.27±15.14*	119.62±25.23*	103.95±14.28*	95.48±13.90*#
Acetylcholinesterase	29.56±3.24*	44.20±3.24#	24.68±1.69*	26.98±1.22*	22.96±3.76*	35.97±3.16*
Glycogen Phosphorylase	174.22±9.98*	209.89±17.50#	158.36±23.04*	133.54±3.67*	169.35±24.65*	143.32±14.14*
Fructose 1,6 Biphosphatase	177.19±7.93*	247.46±9.90#	147.32±6.80*	135.66±7.07*	191.78±19.49*	156.31±10.57*
Glucose 6 Phosphatase	153.68±3.06*	223.14±12.03#	138.54±9.64*	139.98±5.43*	176.92±23.96*	160.16±13.00*

Values = mean ± SD; n = 5 (NC and NTXD) and 7 (DC, DLDD, DHDD, and DSM). *Statistically ($p<0.05$) significant to DC, #statistically significant ($p<0.05$) to NC. **NC** = normal rats, **DC** = diabetic control, **DLDD** = diabetic rats + *D. edulis* (150 g/kg bw), **DHDD** = diabetic rats + *D. edulis* (300 g/kg bw), **DSM** = diabetic rats + metformin (200 g/kg bw), and **NTXD** = Normal rats + *D. edulis* (300 g/kg bw).

Induction of T2D significantly ($p<0.05$) increased pancreatic ATPase activity as shown in **Table 8.3.2**. This was significantly ($p<0.05$) reversed in all treatment groups to levels indistinguishable from the normal controls. Rats treated with high dose of BFDE exhibited a better inhibitory effect compared to the low dose treated.

Pancreatic acetylcholinesterase activity was significantly ($p<0.05$) increased on induction of T2D as shown in **Table 8.3.2**. Treatment with both doses of BFDE led to a significant ($p<0.05$) reduction to levels indistinguishable from the normal control. This was similar for the other treatment groups.

BFDE significantly ($p<0.05$) inhibited T2D – induced glycolytic enzyme activities as revealed by the decreased activities of glycogen phosphorylase, fructose 1,6 biphosphatase, and glucose 6 phosphatase to levels indistinguishable from the normal controls (**Table 8.3.2**).

Induction of T2D led to significant ($p<0.05$) decrease in GSH level, SOD, and catalase activities with concomitant increase in MDA and NO levels, and myeloperoxidase activities as depicted in **Table 8.3.2**. These were significantly ($p<0.05$) reversed in all treatment groups, with rats treated with BFDE exhibiting levels indistinguishable from the normal controls.

As shown in **Table 8.3.3**, induction of T2D led to significant ($p<0.05$) increase in ALP, CK-MB, fructosamine and uric acid levels. These was significantly reduced in all treatment groups, with rats treated with high dose of BFDE exhibiting reduced levels indistinguishable from the normal control. T2D had little or no significant effect on ALT and urea levels as compared to the normal control.

Table 8.3.3: Antioxidant status of (A) serum and (B) pancreas of experimental groups

(A)

	GSH (mol/L)	SOD (U/mg protein)	Catalase (U/mg protein)	MDA (mol/L)	NO (µml)	Myeloperoxidase (U/mg protein)
NC	64.28±11.90*	86.21±8.13*	636.37±29.48*	63.09±4.57*	0.075±0.006*	100.27±9.68*
DC	36.65±2.47#	40.23±3.32#	490.64±42.75#	71.19±4.52#	0.102±0.007#	121.08±18.63#
DLDD	49.55±5.17*	123.08±21.34*	635.05±6.04*	52.54±2.33*	0.044±0.003*	52.14±4.77*
DHDD	71.48±3.81*	128.83±15.83*	601.13±18.35*	51.98±2.79*	0.034±0.005*	33.85±4.26*
DSM	53.31±2.27*	68.97±8.78*	650.50±4.82*	50.09±9.53*	0.053±0.008*	47.44±1.53*
NTXD	41.48±2.39*	132.18±36.57*	657.31±12.12*	45.20±5.39*	0.065±0.001#	80.74±6.48*

(B)

	GSH (mol/L)	SOD (U/mg protein)	Catalase (U/mg protein)	MDA (mol/L)	NO (µml)	Myeloperoxidase (U/mg protein)
NC	104.87±3.09*	659.96±57.9*	783.32±28.21*	40.96±5.79*	0.24±0.02*	20.31±5.75*
DC	54.06±5.01#	150.86±10.6#	711.48±34.46#	71.75±3.60#	0.70±0.02#	35.35±3.61#
DLDD	101.48±9.92*	639.37±30.47*	789.19±12.16*	50.28±5.99*	0.16±0.04*	12.39±2.65*
DHDD	116.97±2.61*	664.51±15.24*	785.64±19.61*	65.25±13.78*	0.10±0.02*	13.67±2.99*
DSM	51.80±6.38#	583.33±31.8*	754.29±40.8*	55.08±5.99*	0.34±0.03*	17.85±1.81*
NTXD	45.35±2.13#	462.64±27.8*	782.47±4.97*	47.03±4.46*	0.25±0.01*	18.62±0.49*

Values = mean ± SD; n = 5 (NC and NTXD) and 7 (DC, DLDD, DHDD, and DSM). *Statistically ($p<0.05$) significant to DC, #statistically significant ($p<0.05$) to NC. **NC** = normal rats, **DC** = diabetic control, **DLDD** = diabetic rats + *D. edulis* (150 g/kg bw), **DHDD** = diabetic rats + *D. edulis* (300 g/kg bw), **DSM** = diabetic rats + metformin (200 g/kg bw), and **NTXD** = Normal rats + *D. edulis* (300 g/kg bw).

Table 8.3.4: Toxicity biomarkers of experimental groups

Enzyme Activities	NC	DC	DLDD	DHDD	DSM	NTXD
ALP U/L	101.00±5.55*	1235.00±29.88$^{\#}$	425.67±63.81*	364±10.80*	582.33±10.67*	123.33±28.99*
ALT U/L	91.00±4.24	87.50±0.71	88.67±0.58	89±8.54	88.00±3.20	89.00±1.00
CK-MB U/L	625.00±15.80*	959.00±25.10$^{\#}$	1104.30±159.19$^{\#}$	727±46.95*	739.40±12.89$^{\#}$	1544.25±61.97*#
Fructosamine mg/dL	656.00±12.6*	743.00±15.56$^{\#}$	698.00±43.62*	605±17.68*	658.50±12.02*	558.00±30.45*
Urea mg/dL	58.00±0.71	46.00±5.67	34.00±5.66#	28.50±4.95*$^{\#}$	31.50±6.81$^{\#}$	63.33±0.58*
Uric acid mg/dL	2.46±0.24	3.48±0.70	2.55±0.68	2.07±0.87	1.60±0.23*$^{\#}$	2.75±0.38

Values = mean ± SD; n = 5 (NC and NTXD) and 7 (DC, DLDD, DHDD, and DSM). *Statistically ($p<0.05$) significant to DC, #statistically significant ($p<0.05$) to NC. **NC** = normal rats, **DC** = diabetic control, **DLDD** = diabetic rats + *D. edulis* (150 g/kg bw), **DHDD** = diabetic rats + *D. edulis* (300 g/kg bw), **DSM** = diabetic rats + metformin (200 g/kg bw), and **NTXD** = Normal rats + *D. edulis* (300 g/kg bw).

Induction of T2D led to alteration of the pancreatic morphology as depicted by the inflamed/necrotic pancreatic β-cells (red colored) as well as the decreased number of acinar and normal β-cells as shown in **Figure 8.3.6B**. Treatment with BDFE led to restoration of the morphology as evident by the increased number of the β- and acinar cells as well as decreased number of inflamed cells (**Figure 8.3.6C** and **Figure 8.3.6D**) when compared to the intact morphology of the normal rats (**Figure 8.3.6A**). This was same for all the treatment groups.

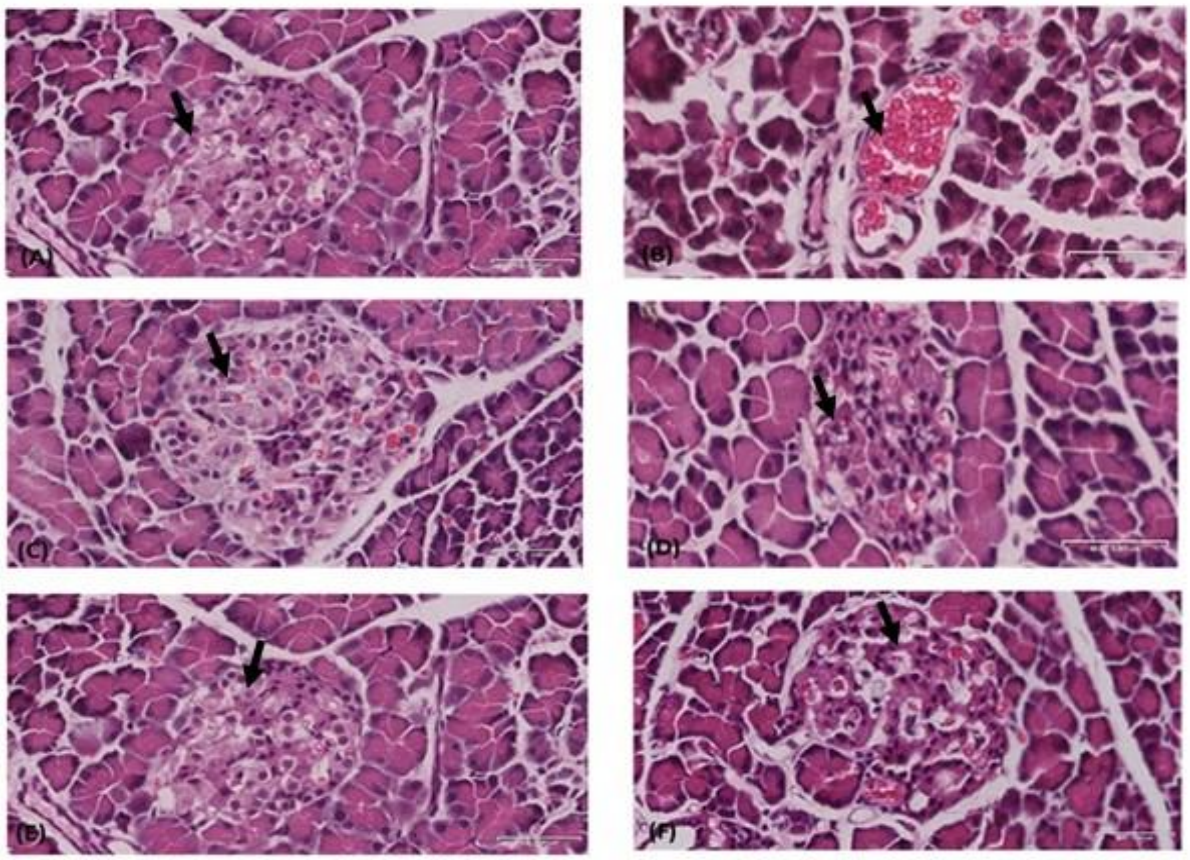


Figure 8.3.6: Morphological changes in pancreatic tissues of experimental groups. Magnification: 10x. (A) = NC, (B) = DC, (C) = DLDD, (D) = DHDD, (E) = DSM, and (F) = NTXD. **NC** = normal rats, **DC** = diabetic control, **DLDD** = diabetic rats + *D. edulis* (150 g/kg bw), **DHDD** = diabetic rats + *D. edulis* (300 g/kg bw), **DSM** = diabetic rats + metformin (200 g/kg bw), and **NTXD** = Normal rats + *D. edulis* (300 g/kg bw). Arrow: β-cells

Figure 8.3.7A reveals intact clusters of acini, enriched with vast networks of capillaries in pancreatic tissues of normal rats. Induction of T2D led to alteration of the capillary networks,

leading to the conspicuousness of the acini clusters and enlargement of the cells as shown in **Figure 8.3.7B**. The capillary networks were restored in all the treatment groups, with the BFDE low dose exhibiting much networks (**Figure 8.3.7C – 8.3.7E**). The acini cells were also reduced on treatment with BFDE. Administration of BFDE to normal rats led enlarged and conspicuous pancreatic acini cells, with altered capillary networks (**Figure 8.3.7F**).

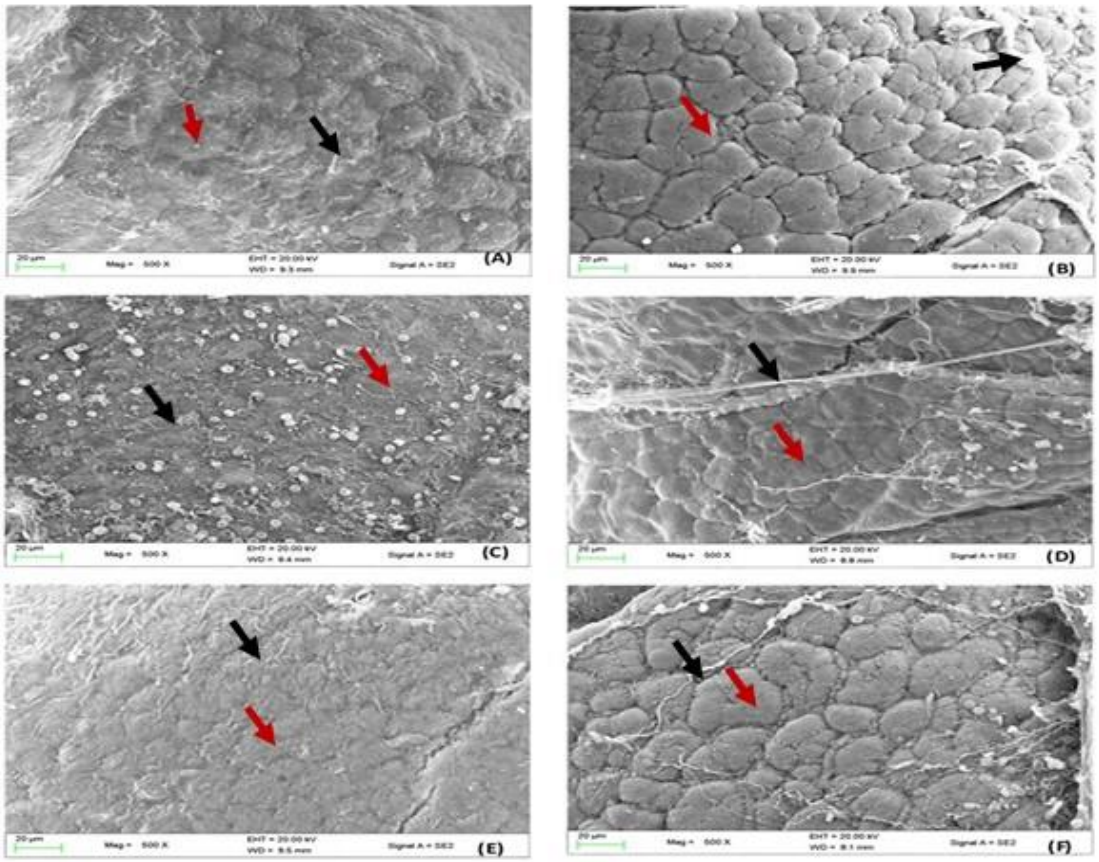


Figure 8.3.7: Electron microscopy of pancreatic tissues of experimental groups. Magnification = 1000x. (A) = NC, (B) = DC, (C) = DLDC, (D) = DHDC, (E) = DSM, and (F) = NTXC. **NC** = normal rats, **DC** = diabetic control, **DLDD** = diabetic rats + *D. edulis* (150 g/kg bw), **DHDD** = diabetic rats + *D. edulis* (300 g/kg bw), **DSM** = diabetic rats + metformin (200 g/kg bw), and **NTXD** = Normal rats + *D. edulis* (300 g/kg bw). Arrows: Black = blood vessel; Red = acini

As shown in **Figure 8.3.8**, induction of T2D had little or no effect on pancreatic caspase-3 expression. The expressions were however increased (not significant) in BFDE treated diabetic groups (**Figure 8.3.8C** and **8.3.8D**).

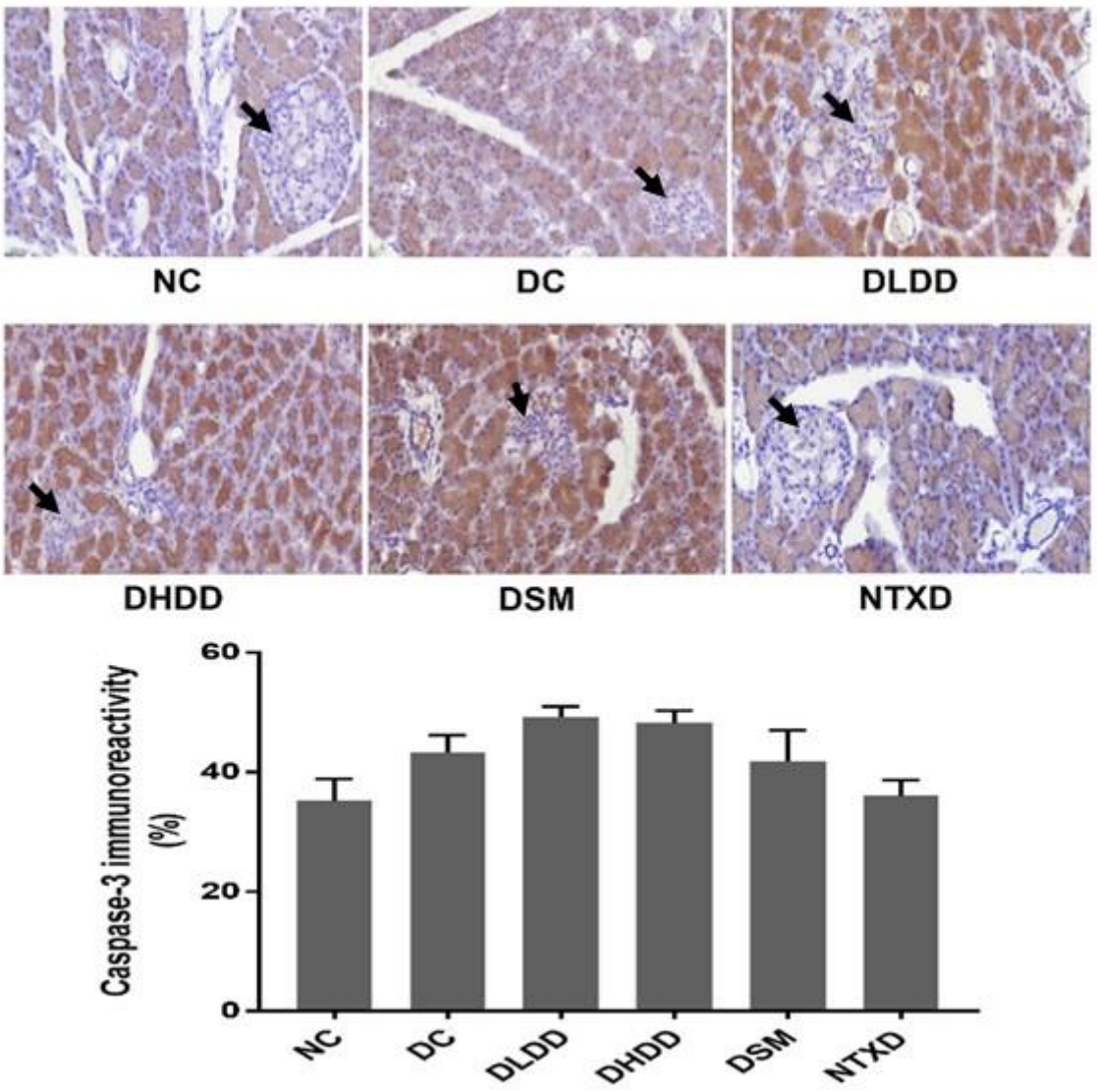


Figure 8.3.8: Immunohistochemistry photomicrographs and immunoreactivity of caspase3 expression in pancreatic tissues of experimental groups. Magnification = 200x. NC = normal rats, DC = diabetic control, DLDD = diabetic rats + *D. edulis* (150 g/kg bw), DHDD = diabetic rats + *D. edulis* (300 g/kg bw), DSM = diabetic rats + metformin (200 g/kg bw), and NTXD = Normal rats + *D. edulis* (300 g/kg bw). Arrow: β – cells

Induction of T2D led to significant expression of Nrf2 in the pancreas as shown in **Figure 8.3.9**. Treatment with BFDE led to significant ($p<0.05$) inhibition of the expressions.

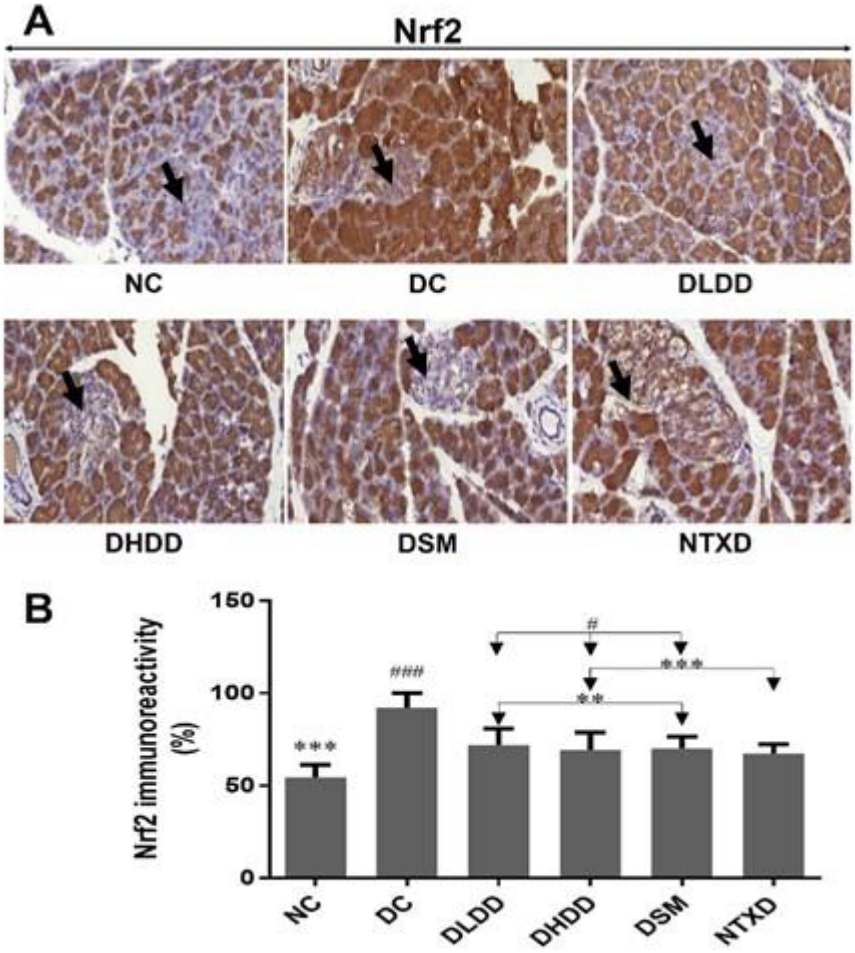


Figure 8.3.9: (A) Immunohistochemistry photomicrographs and (B) immunoreactivity of Nrf2 expression in pancreatic tissues of experimental groups. Values = mean ± SD; n = 5 (NC and NTXD) and 7 (DC, DLDD, DHDD, and DSM). *Statistically ($p<0.05$) significant to DC, #statistically significant ($p<0.05$) to NC. Magnification = 200x. **NC** = normal rats, **DC** = diabetic control, **DLDD** = diabetic rats + *D. edulis* (150 g/kg bw), **DHDD** = diabetic rats + *D. edulis* (300 g/kg bw), **DSM** = diabetic rats + metformin (200 g/kg bw), and **NTXD** = Normal rats + *D. edulis* (300 g/kg bw). Arrow: β – cells

HPLC analysis of BFDE revealed the presence of gallic acid, vannilic acid, (-)-epicatechin, and vanillin as depicted in **Figure 8.3.10**. (-)-epicatechin had the highest peak, indicating the highest concentration.

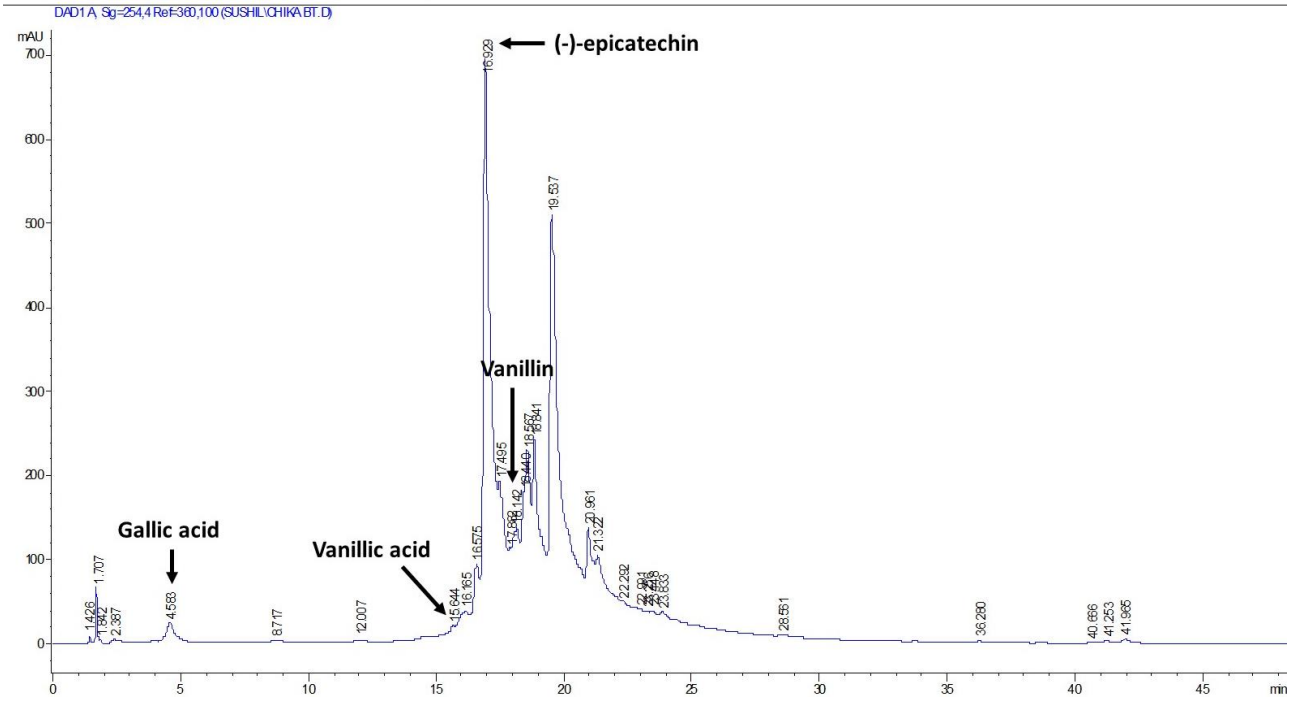


Figure 8.3.10: HPLC chromatogram of butanol fraction of *D. edulis* ethanol extract

8.3.5 Discussion

The increasing epidemy of DM also connotes an increasing cost of its treatment, which has been reported to be an economic burden for most developing countries with little and/or poor health infrastructures. The IDF projection of 156% increase in the number people living with diabetes by 2045 in Africa (I.D.F. 2018), raises a lot of concerns on how to tackle the rising epidemic in sub-Saharan Africa. Several studies have suggested the use of alternative medicine in the treatment and management of DM, with particular interest in medicinal plants owing to their availability and affordability (Ekor 2014; Mohammed et al. 2014). In this study, the antidiabetic properties of the butanol fraction of *D. edulis* ethanol extract were investigated in T2D rats.

The increased food and fluid intake as well as weight loss in untreated diabetic group (**Figures 8.3.2A** and **8.3.2B**), indicates an occurrence of polyphagia and polydipsia. Polyphagia and polydipsia as well as weight loss have been reported as key symptoms of DM (Ibrahim et al. 2016; Okon et al. 2012). Thus, the ability of BFDE to suppress these symptoms, may portray a beneficial effect in the treatment and management of T2D.

Hyperglycemia arising from pancreatic β-cell dysfunction, insulin insufficiency and resistance has been recognized as the major characteristics of T2D (Abdul-Ghani et al. 2006; Cerf 2013; Donath and Halban 2004). This is in consent with the elevated blood glucose level (**Figure 8.3.4A**), depleted serum insulin level (**Figure 8.3.4C**), decreased HOMA-β value (**Figure 8.3.4D**), and elevated HOMA-IR value (**Figure 8.3.4D**) of the untreated diabetic rats. The depleted serum level indicates an insulin insufficiency, while the decreased HOMA-β and elevated HOMA-IR values indicate β-cell dysfunction and insulin resistance respectively which corroborates with the elevated blood glucose level. This also correlates with the compromised pancreatic β-cell morphology (**Figure 8.3.6B**) and reduced weight (**Figure 8.3.4C**). Thereby indicating an occurrence of T2D. The ability of BFDE to reduce the blood glucose level, elevate serum insulin level, improve β-cell dysfunction, suppress insulin resistance, improving the pancreatic morphology and weight, demonstrates its antidiabetic potentials and affirmation of its folkloric claims. The improved glucose tolerance in diabetic rats treated with BFDE (**Figure 8.3.4B**), corroborates the antidiabetic potentials of the fraction. This corresponds with previous reports on the ability of antidiabetic medicinal plants to stimulate insulin secretion and improve the pancreatic morphology, while regenerating β-cells (Erukainure et al. 2015; Ibrahim et al. 2016; Islam and Choi 2007). This was also demonstrated in the ability of the fraction to stimulate glucose uptake in 3T3 adipocytes cells (**Figure 8.3.2A**), with high a proliferative tendency (**Figure 8.3.2B**).

The increased activities of the studied glycolytic enzymes which covers for glycogen phosphorylase, fructose-1,6-biphosphatase and glucose-6-phosphatase in the untreated diabetic rats (**Table 8.3.2**) indicates increased glycogenolysis, gluconeogenesis and glycolysis. This corroborates previous reports on the elevation of these pathways in T2D (Clore et al. 2000; Guo et al. 2012b). Amplification of glycogenolysis and gluconeogenesis in T2D has been implicated in the continuous production of glucose from glycogen and non-carbohydrate, leading to increased blood glucose level. Increased glycolysis in T2D has been linked with the production of metabolites for the hexosamine, polyol, protein kinase C, and AGE pathways (Luo et al. 2016; Rolo and Palmeira 2006). Activation of these pathways corroborates the elevated blood glucose level (**Figure 8.3.4A**) as well as the diminished serum insulin level (**Figure 8.3.4C**) in the untreated diabetic rats. The latter has been reported for its glycogenic activity (Cersosimo et al. 2018; Gardner et al. 1993), with insulin resistance playing an influential role (Saltiel and Kahn

2001). The ability of BFDE to inhibit the activities of these enzymes (**Table 8.3.2**) may insinuate parts of its antidiabetic mechanism.

Inhibition of major dietary carbohydrate metabolizing enzymes particularly α-glucosidase and α-amylase has been recognized as a major antihyperglycemic mechanism which is employed by some commercial antidiabetic drugs such as acarbose and miglitol (Chelladurai and Chinnachamy 2018; Rahimzadeh et al. 2014). These enzymes contribute to increased postprandial blood glucose level by breaking down dietary carbohydrate to glucose. Thus, the increased α-amylase activities in the pancreas and serum of the untreated diabetic rats (**Table 8.3.2**) suggests an elevation of postprandial blood glucose level which may also be a contributor to the induced hyperglycemia (Fig. 4A). This is in consent with previous studies on amplified α-amylase activities in T2D (Aughsteen et al. 2005; Ewadh et al. 2014; Yadav et al. 2013). The distorted acinar cells (**Figure 8.3.6B**) and its clusters (**Figure 8.3.7B**) may also contribute to the increased α-amylase activities, as these cells functions in the secretion of digestive enzymes in the pancreas (Muniraj et al. 2015; Williams 2010). The reduced α-amylase activities in diabetic rats treated with BFDE therefore demonstrates the antihyperglycemic activity of the fraction. This is further evidenced by their reduced blood glucose level (**Figure 8.3.4A**) and improved acinar cell morphology (**Figures 8.3.6C** and **8.3.6D**) and clusters (**Figure 8.3.7C** and **8.3.7D**). This correlates with its high inhibitory potency on α-glucosidase activity *in vitro* (**Figure 8.3.1** and **Table 8.3.1**). Previous studies have also reported the ability of *D. edulis* leaf extracts to inhibit carbohydrate digestive enzymes *in vitro* (Erukainure et al. 2017b).

The increased pancreatic ATPase activity in the untreated diabetic rats (**Table 8.3.2**) may insinuate decreased insulin secretion as studies have linked a decreased activity coupled with depolarized β – cell membrane and Ca^{2+} influx to glucose-stimulated insulin secretion (Owada et al. 1999). This may also contribute to the reduced serum insulin level (**Figure 8.3.4C**). The decreased serum ATPase activity also corroborates previous studies which reported decreased activities in T2D (Kiziltunç et al. 1997; Zadhoush et al. 2015). The reversed activities in diabetic rats treated with BFDE, thus corroborates the increased serum level (**Figure 8.3.4C**) and further reflects the antidiabetic mechanism of the fraction. This correlates with previous studies on the ability of the *D. edulis* leaf extracts to inhibit pancreatic ATPase activity (Erukainure et al. 2017b).

Disturbances in lipid metabolism has been associated with the early stage of T2D (Erukainure et al. 2013). Often describes as dyslipidemia, it is characterized by elevated levels of TC, TG, LDL-c, and depleted HDL-c level. Insulin resistance has been implicated in these metabolic changes which renders the lipoproteins pathogenic in T2D (Ormazabal et al. 2018). Thus, the elevated TG, LDL-c and depleted HDL-c levels in the untreated diabetic rats (**Figure 8.3.5**) depicts diabetic dyslipidemia. This can be attributed to hyperglycemia (**Figure 8.3.4A**) and insulin resistance (**Figure 8.3.4D**) on induction of T2D. The reduced TG, LDL-c and elevated HDL-c levels in diabetic rats treated with BFDE suggests an antilipemic activity of the fraction. This also correlates with the reduced blood glucose levels (**Figure 8.3.4A**) and elevated serum insulin levels (**Figure 8.3.4C**) in BFDE-treated diabetic rats.

Oxidative stress and proinflammation have been implicated in hyperglycemia-induced destruction of the pancreatic β-cells, leading to alterations in insulin sufficiency and β-cell function (Evans et al. 2002; Evans et al. 2003). This has been linked to the extremely low levels of the endogenous antioxidant system in the pancreas, which makes it highly prone to oxidative attacks (Acharya and Ghaskadbi 2010; Donath 2014). The depleted GSH level, SOD and catalase activities in the untreated diabetic rats (**Table 8.3.3**) indicates oxidative stress. These antioxidants protect against the injurious activities of free radicals particularly superoxide anion ($O_2^{\bullet-}$). $O_2^{\bullet-}$ undergoes dismutation to hydrogen peroxide (H_2O_2) in a reaction catalyzed by SOD. H_2O_2 is then broken down by catalase to water (H_2O) and O_2 (oxygen). NO can react with $O_2^{\bullet-}$ to form the lethal nitric radical, peroxynitrite ($ONOO^-$). If not converted by catalase, H_2O_2 can be broken down to hydroxyl radical (•OH) which has been recognized as a trigger for lipid peroxidation. Excess H_2O_2 can also react with hydrochloric acid (HCl) to give hypochlorous acid (HOCl), in a reaction catalyzed by myeloperoxidase. Thus, the elevated level of MDA, NO, and myeloperoxidase activity in the untreated diabetic rats (**Table 8.3.2**) indicates lipid peroxidation, and proinflammation. This corroborates with the inflamed β-cells (**Figure 8.3.6B**). The reversed levels and activities of these biomarkers in diabetic rats treated with BFDE, therefore insinuates a protective effect of the fraction against hyperglycemia-induced oxidative stress and proinflammation. The latter is corroborated by the reduced acetylcholinesterase activities in BFDE-treated diabetic rats (**Table 8.3.2**), as increased pancreatic acetylcholinesterase activities have been correlated with inflammation of the tissue (Zhang et al. 2012).

Alteration in pancreatic perfusion have been reported in T2D, and has been implicated in β-cell dysfunction (Honka et al. 2014). Perfusion of the pancreas by multiple arteries from the abdominal aorta have been linked with maintenance the pancreatic islets as well as normal physiology of the endocrine (Honka et al. 2014; Jansson et al. 2016). Thus, the altered capillary network on the pancreatic suffices of the untreated diabetic rats (**Figure 8.3.7B**) suggests an altered perfusion. The increased networks in that of diabetic rats treated with BFDE insinuates an increased perfusion, which may also contribute to the improved β-cell function (**Figure 8.3.4D**).

The detrimental effect associated with apoptosis of the pancreatic β-cells has been reported in T2D, with hyperglycemia-induced oxidative stress and inflammation playing key roles (Acharya and Ghaskadbi 2010; Kaneto et al. 1996; Rojas et al. 2018; Tomita 2016). Caspase cascade has been implicated in the progression of the extrinsic apoptotic pathway, with caspase-3 very effective in apoptosis of the β-cells (Liadis et al. 2005; Tomita 2016). In this study, induction of T2D had little or no effect on the expression of caspase 3 (**Figure 8.3.8**), thus suggesting that the induction mechanism may not involve caspase-3-dependent β-cell apoptosis.

The increased pancreatic Nrf2 expression in the untreated diabetic rats (**Figure 8.3.9**) corroborates previous studies which correlated exacerbated expressions with onset T2D (He et al. 2012; Miao et al. 2012). This also correlates with reports on its activation of oxidative stress (Wrighten et al. 2009; Zucker et al. 2014). Thus, insinuating its exacerbated expression may contribute to the pancreatic oxidative stress (**Table 8.3.3**). The decreased expressions in diabetic rats treated with BFDE (**Figures 8.3.9C** and **8.3.9D**), further insinuates the antidiabetic properties of the fraction and may also be a possible antidiabetic mechanism.

The elevated level of fructosamine in the untreated diabetic rats (**Table 8.3.4**) indicates an activation of the glycation cascade, as fructosamine is a product of an early stage glycated protein which undergoes oxidative cleavage to produce advanced glycation end (AGE) products (Ibrahim et al. 2016; Sen et al. 2011). The reduced levels in diabetic rats treated with BFDE, insinuates the ability of the fraction to arrest the glycation cascade.

The elevated levels of ALP, CK-MB and uric acids in the untreated diabetic rats (**Table 8.3.4**), insinuates liver, heart and kidney toxicities (Erukainure et al. 2018; Mortazavi et al. 2016). The elevation of these toxicity biomarkers has been demonstrated in diabetic rats (Čaušević et al. 2010;

Erukainure et al. 2017a; Fazel et al. 2005). Inflammation of the liver, heart and kidney has been implicated in their leakage to the bloodstream (Giordano et al. 2015; Kim et al. 2008; Peppes et al. 2008). The reduced levels of these biomarkers in both diabetic and normal rats treated with BFDE as well as non-cytotoxic effect of BFDE on 3T3-adipocytes (**Figure 8.3.2B**), indicate the safety of the fraction on both diabetic and healthy tissues. This correlates with previous reports on the predicted safety of the *D. edulis* extracts if consumed orally (Erukainure et al. 2017b).

The antidiabetic properties of BFDE can be attributed to its chemical constituents, particularly the identified phenolics consisting of gallic acid, vanillic acid, vanillin, and (-)-epicatechin (**Figure 8.3.10**). As common with phenolics, the identified compounds have been reported as potent antioxidants (Makni et al. 2011; Rein et al. 2000; Rice-Evans et al. 1997) and may be responsible for the antioxidative activity of the fraction in diabetic rats (**Table 8.3.3**). Their antidiabetic properties which encompasses improved insulin sensitivity (Josic et al. 2010), suppressed insulin resistance (Cremonini et al. 2016; Latha and Daisy 2011), and glucose uptake (Prasad et al. 2010) have been reported. This may also contribute to the ability of the fraction to elevate serum insulin level (**Figure 8.3.4C**) and suppress insulin resistance (**Figure 8.3.4D**). The identified compounds may work synergistically to bring about the antidiabetic effect of the fraction.

8.3.6 Conclusion

These results insinuate the therapeutic effects of BFDE against T2D and its complications, as evidenced by the ability of the fraction to suppress hyperglycemia via increased insulin secretion, improved pancreatic β-cell function, modulation of glycolytic and carbohydrate hydrolyzing enzymes, attenuation of hyperlipidemia, pancreatic oxidative stress, and Nrf2 expression. These activities may be attributed to the synergetic effect of the identified phenolic constituents.

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CHAPTER 9

GENERAL DISCUSSIONS AND CONCLUSIONS

9.1 General Discussions

The increasing number of people living with diabetes mellitus (DM) makes it one of the fastest growing global epidemy. It is amongst the most common global chronic diseases, with a global prevalence of 425 million reported in 2017 (Cho et al. 2018; I.D.F. 2018) depicting a 2.4% increase in the 2015 estimate of 415 million (I.D.F. 2016; Ogurtsova et al. 2017). The International Diabetes Federation (IDF) projected a 48% rise in 2045 of the current figures to 629 million, with Africa experiencing a 156% increase (Cho et al. 2018; I.D.F. 2018). The projected rise in the number of diabetics in Africa can portrays a huge burden for the continent, owing to the cost of treating and managing of DM coupled to her poor health infrastructures. This has led to a paradigm shift from commercial antidiabetic drugs to the use of natural products. These natural products are majorly plant-based which are readily available, accessible and affordable. Often referred to as medicinal plants, these plants have been utilized in the treatment and management of various ailments and diseases such as cancer, malaria, and DM from time immemorial. Africa has been reported for the richness and endemicity of her indigenous flora, encompassing millions of medicinal plants employed in the treatment of DM and other diseases (Mohammed et al. 2014; Scott et al. 2004). However, there are still scientific dearth in the efficacies of some of these plants as well as their mechanism of actions. Although the use of medicinal plants is regarded safe, there are however concerns on their toxicity vis-à-vis standardization, characterization, and preparation (Shan et al. 2007). There have been concerns of herbal – herbal, herbal – food, and/or herbal – drug interactions owing to the cocktails of phytochemicals that are present in these plants (Ezuruike and Prieto 2014; Yüce et al. 2006). Hence, this study was carried out to investigate the antidiabetic, antioxidant and toxicological properties of Raffia palm (*Raffia hookeri*) wine, *Vernonia amygdalina, Clerodendrum volubile, Dacryodes edulis, Cola nitida,* and *Phaseolus lunatus* using *in vitro*, *ex vivo*, *in silico* and *in vivo* models. The metabolic pathways and metabolites involved were also investigated *ex vivo* and *in vivo*.

The first plant to be studied is the Raffia palm (*Raphia hookeri*) wine which was sub divided into 3 batches. The two of the batches were subjected to open air fermentation for 24 and 48 h

respectively, the other batch left unfermented. They were all concentrated at -50°C to yield concentrates which all showed significant ($p<0.05$) dose-dependent antioxidant activities *in vitro*. Their inhibitory effect on α-glucosidase, α-amylase and intestinal glucose absorption were also dose-dependent but increased with increasing time of fermentation. They further portrayed their antidiabetic potentials by stimulating glucose uptake in isolated paos muscles and yeast cells, while inhibiting intestinal glucose absorption and glucose diffusion *ex vivo*. The antioxidant activities of the palm wine samples were also demonstrated by their ability to protect against oxidative pancreatic injury *ex vivo* by elevating SOD and catalase activities, while depleting levels of NO, MDA, and myeloperoxidase activities. Their protective effect against oxidative injury also involved inhibition of citric acid cycle metabolites and pathways involved in SOD generation, while activating vitamins, lipid, steroids, inositol and its phosphates, and sulfate/sulfite metabolic pathways. The unfermented palm wine portrayed an antidiabetic activity by significantly ($p<0.05$) depleting blood glucose levels of T2D rats, with concomitant increased serum insulin concentration, while concomitantly improving pancreatic β-cell function and numbers. This activity can be attributed to its ability to inhibit the activities of glycogen phosphorylase, fructose-1,6-biphosphatase, glucose-6-phosphatase, ATPase and α-amylase. It also led to down regulation of Nrf2 expression in the pancreatic tissue and cerebellar cortex of T2D rats, while enhancing the antioxidant activities of the serum, pancreas, brain and testes. It improved the morphology of the pancreas, cerebellar cortex and testes, while restoring the neuronal integrity and perfusion of the cerebellar cortex and pancreatic tissues respectively. It also led to replenishment of cholesterol and squalene levels, while activating pathways for plasmalogen synthesis, mitochondrial beta-oxidation of long chain saturated fatty acids in the testicular tissues. These activities can be attributed to the synergetic effect of the identified phytochemicals comprising of non-calories sugars, phenolics, unsaturated fatty acids, and alkaloids. Considering the its availability and its high consumption owing to its sweetness, raffia palm wine can be explored in the development of nutraceuticals or food adjunct for the treatment and management of T2D and its complications.

The second plant investigated was *Phaseolus lunatus*. It was subjected to aqueous extraction which was concentrated at -50°C to yield the aqueous extract. The extract showed significant free radical scavenging and ferric reducing activities *in vitro*, which insinuates an antioxidant potential. Its antioxidant activities were further affirmed by the ability of the extract to elevate the GSH level, SOD and catalase activities, with concomitant depletion of MDA levels in oxidative hepatic injury

ex vivo. The protective effect of the extract against oxidative hepatic injury was also shown to involve inhibition of hepatic ATPase activities and arrest of pancreatic DNA fragmentation. Its antidiabetic activities were demonstrated by its inhibitory effects on α-glucosidase and α-amylase activities as increased activities of these enzymes have been implicated in the postprandial rise in blood glucose level (Chukwuma and Islam 2015). Some commercial antidiabetic drugs such as acarbose bring about their action via inhibition of these enzymes. The antidiabetic potential was further demonstrated by the ability of the extract to inhibit glucose 6 phosphatase activity and intestinal glucose absorption, while stimulating muscle glucose *uptake ex vivo*. The identified phytochemical constituents of *P. lunatus* were predicted *in silico* to be safe as they fell between the toxicity classes of 5 and 6. These antioxidant and antidiabetic activities of *P. lunatus* can be attributed to the synergetic effect of the identified phytochemical and amino acids constituents. As a legume and the predicted toxicity class, *P. lunatus* can be explored in the development of functional foods or included in diets for the treatment and management of T2D and its complications.

Vernonia amygdalina is the third plant that was studied for its antioxidant and antidiabetic properties. The hot infusion, ethyl acetate, ethanol and aqueous extracts displayed potent antioxidant activities by significantly scavenging free radicals and reducing ferric oxidation. The potency was further revealed by their ability to protect against oxidative injuries in the liver and brain as they elevated the GSH level, SOD and catalase activities, and depleted MDA level *ex vivo*. Their antidiabetic activities were demonstrated by their ability to inhibit the activities of α-glucosidase and α-amylase, which was further ascertain by their ability to stimulate muscle glucose uptake. The hot infusion also arrested DNA fragmentation in oxidative hepatic injury, indicating an antiapoptotic potential (loannou and Chen 1996). The neuroprotective effect of the hot infusion against glucose-induced neurotoxicity was demonstrated by its ability to stimulate brain glucose uptake. This uptake also involved increasing antioxidant and anti-inflammatory activities, and inhibition of ATPase activities, while suppressing the oxidative activation of the pyruvate metabolism pathway. Histochemical analysis of the *V. amygdalina* leaves revealed the distribution of the phytochemicals, acidic lipid, mucilage and pectin, lipids, polyphenols and alkaloids in the mid rib, glandular trichome and epidermis. This corresponded with the identified phytochemicals of the hot infusion, ethyl acetate and ethanol extracts. The synergetic effects of these phytochemicals may be responsible for the antioxidant and antidiabetic properties of *V.*

amygdalina. This study further affirms the antidiabetic properties of this leafy vegetable. It can also be utilized in the development of novel drugs or nutraceuticals for treating neurodegeneration in T2D.

The flowers and leaves of *Clerodendrum volubile* were investigated for their antioxidant, antidiabetic, and cytotoxic activities *in vitro* and *ex vivo*. The ethyl acetate, ethanol, aqueous extracts of the flowers displayed potent antioxidant activities by significantly ($p<0.05$) scavenging free radicals and reducing ferric oxidation. This was further evidenced by the ability of the extracts to elevate GSH level, SOD and catalase activities, as well as reduce MDA levels in oxidative hepatic and pancreatic injuries respectively, *ex vivo*. The ameliorative effect of the extracts on these organs was shown to involve inhibition of ATPase activity and arrest of DNA fragmentation. Their antidiabetic potential was revealed by their ability to inhibit the activities of α-glucosidase and α-amylase. Similarly, the dichloromethane (DCM) fraction of *C. volubile* leaves ethanol extract displayed potent antioxidant and antidiabetic activities *in vitro*. However, it was cytotoxic in human embryonic kidney (HEK-293) cells. Its cytotoxic mechanism involved exacerbation of oxidative stress and proinflammation as evidenced by the depleted GSH level, SOD and catalase activities, and elevated myeloperoxidase activity, NO and MDA levels. The fraction also led to morphological changes of the cells portraying an occurrence of apoptosis and necrosis. *In silico* toxicological analysis of the identified phytochemicals constituents of the fraction predicted them as potent inhibitors of CYP1A2, CYP2C9, and CYP2C9. The predicted low maximum tolerated human dose by most of the compounds also portrays a toxic potential of the fraction. This study gives credence to the folkloric use of this plant in the tresatment and management of DM, and can be utilized in the development of novel antidiabetic drugs. However, the toxic effect of the leaf's DCM fraction should be taken into consideration in the development of such drugs.

The hot infusion of *Cola nitida* was investigated for its antioxidant and antidiabetic properties. The infusion displayed significant ($p<0.05$) antioxidant potentials as portrayed by their ability to scavenge free radicals and reduce ferric oxidation *in vitro.* This was further corroborated by their ability to elevate GSH level, SOD and catalase activities, while concomitantly depleting MDA levels in oxidative hepatic injury *ex vivo*. Its anti-hepatoxicity mechanisms involves modulation of the hepatic functional chemistry to levels indistinguishable from the normal tissues, with concomitant activation of catecholamine biosynthesis, phenylalanine and tyrosine metabolism,

tyrosine metabolism, and urea cycle pathways. The infusion also displayed an antiapoptotic potential by its arrest of DNA fragmentation in oxidative hepatic injury. Its antidiabetic potentials were displayed by its inhibition of α-glucosidase and α-amylase *in vitro.* This was further ascertained by its ability to reduce blood glucose level in T2D rats, with concomitant increase in serum insulin levels while improving pancreatic β-cell function and glucose tolerance. It also caused improvement in the pancreatic and cerebellar cortex morphologies, while restoring pancreatic perfusion and neuronal integrity. Its antidiabetic and neuroprotective effects were also corroborated by its ability to inhibit the activities of glycogen phosphorylase, fructose 1,6 biphosphatase, glucose-6-phosphatase, ATPase α-amylase, and acetylcholinesterase. Its antioxidant activities were further ascertained by its ability to modulate redox imbalance in the serum, brain and pancreatic tissues as evidenced by the increased GSH level, SOD and catalase activities, and reduced MDA level. The depleted NO level and myeloperoxidase activity insinuates an anti-proinflammatory potential of the infusion. Suppressed expressions of Nrf2 in the pancreas and brains by the infusion, portrays a molecular mechanism that may contribute to its antidiabetic and antioxidant effect in T2D. This was further affirmed by the ability of its identified main phytoconstituents (caffeine and theobromine) to molecularly interact with Nrf2 *in silico*. *In silico* toxicity analysis of caffeine revealed it to be hepatotoxic and a substrate of P-glycoprotein. The Adenosine Receptor A2a was identified as its toxicity target. Its toxicity class of 3 insinuates a toxic potential. These results further validate the efficacy of *C. nitida* in the treatment and management of T2D and neurodegenerative complications. However, the toxicity of its main constituent, caffeine should be taken into consideration.

The ethyl acetate, ethanol and aqueous extracts of *Dacryodes edulis* were investigated for their antioxidant and antidiabetic activities. The extracts displayed potent antioxidant activities as revealed by their significant ($p<0.05$) free radical scavenging and reducing power activities *in vitro*. This is further corroborated by their ability to modulate redox imbalance in oxidative pancreatic and hepatic injuries *ex vivo*. The antidiabetic potentials of the extract were demonstrated by their ability to α-glucosidase, ATPase and glucose 6 phosphatase activities *in vitro* and *ex vivo*. This was also reflected by the ability of the identified compounds to molecularly interact with α-glucosidase. They also displayed an antiapoptotic potential by arresting DNA fragmentation in oxidative hepatic injury. Based on these results, the ethanol extract was further fractionated via liquid-liquid fractionation using gradient-based solvents to yield the hexane, DCM, ethyl acetate,

butanol (BuOH) and aqueous fractions. The antidiabetic potentials of these fractions were determined via their ability to inhibit α-glucosidase activity *in vitro,* with the BuOH fraction giving the most potent activity. To further affirm its antidiabetic potentials, the BuOH fraction was significantly stimulated glucose uptake in 3T3-L1 adipocytes, with little or no cytotoxic effect. The antidiabetic properties of the fraction were further confirmed by its ability to deplete blood glucose level, with concomitant increase in serum insulin concentration while improving β-cell function and suppressing insulin resistance. The fraction also led to improve the pancreatic morphology, with concomitant restoration of the pancreatic perfusion. The inhibition of glycogen phosphorylase, fructose 1,6 biphosphatase, glucose 6 phosphatase, ATPase α-amylase, and acetylcholinesterase activities by the extracts may also contribute to the observed antihyperglycemic activity. The ability of the fraction to attenuate dyslipidemia portrays its antilipemic effect as portrayed by the depleted levels of total cholesterol, LDL-cholesterol and triglyceride, while elevating HDL-cholesterol level. Its modulatory effect on redox imbalances in the serum and pancreatic tissues, portrays an antioxidant protective effect in T2D as revealed by the elevated GSH level, SOD and catalase activities, as well as depleted levels of MDA. The anti-proinflammatory potential of the fraction is portrayed by its ability to deplete NO level and myeloperoxidase activities in the serum and pancreatic tissues. The inhibition of pancreatic Nrf2 by the fraction may also reflect an antidiabetic (He et al. 2012; Miao et al. 2012) and antioxidant potential (Wrighten et al. 2009; Zucker et al. 2014). These activities can be attributed to the synergetic activities of the identified compounds, which are majorly phenolics. These results affirm the efficacy of this leaves in the treatment and management of T2D. It can be exploited in the development of novel affordable antidiabetic drugs.

9.2 General Conclusions

These results insinuate the antioxidant and antidiabetic properties of raffia palm wine, *V. amygdalina, C. volubile, D. edulis, C. nitida,* and *P. lunatus* as revealed by their antioxidant activities; ability to inhibit key carbohydrate digestive and glycogenic enzymes; reduction of blood glucose levels; and stimulation of insulin secretions. These activities were often better than those the standard commercial drugs. Of the studied plants, Raffia palm wine had the best antidiabetic activity, with little or no toxicity. This was followed by *D. edulis* and *C. nitida*. The DCM fraction of *C. volubile* leaves was the most toxic, while the high caffeine content of *C. nitida* infusion poses

a toxicity threat and a herbal-drug interaction. Raffia palm wine and *C. nitida* can further be utilized in the development of nutraceuticals and/or functional food for the management of neurodegeneration in T2D as they restored and maintained the neuronal integrity of the brain. Raffia palm wine can also be utilized in the treatment and management of T2D-induced male infertility. Metabolomic analysis of tissues treated with some of the studied plants, suggests suppression of the oxidative-activated glycolytic and citric acid cycle pathways. These activities can be attributed to the synergetic activities of the identified phytoconstituents. However, results from cytotoxic activities and *in silico* toxicology analysis suggest caution in their usage in order to reduce the occurrence of herbal – herbal, herbal – food, and herbal – drug interactions.

9.3 Recommendations

Clinical studies on the antidiabetic activities of these plants are recommended to further ascertain their efficacies in humans. Considering the facts that these plants are readily available and most of them (raffia palm wine, *P. lunatus, V.* amygdalina, *C. nitida* and *C. volubile*) consumed as food, they can be utilized in the design and development of functional foods and/or nutraceutical and employed in dietary therapies for treating and managing T2D and its complications.

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APPENDIX 1

ANIMAL ETHICS APPROVAL

BIOMEDICAL RESOURCE UNIT

UNIVERSITY OF KWAZULU-NATAL

April 4, 2017

Dear Prof Islam
Chair: Animal Research Ethics Committee
c/o School of Life Sciences

RE: ATTENDANCE OF LAS COURSE

This letter certifies that Mr Ochuko L. Erukainure have attended the Laboratory Animal Course that was hosted by the Biomedical Resource Unit.

The course was held on the 16 – 17 March 2017 and entailed the following:

Introduction to laboratory animal sciences. Bioethics and Animal experimentation. Animal Research Methodology. Experimental design, environmental enrichment and occupational safety.

The course was completed satisfactorily and may be allowed to initiate his research after the relevant practical procedures was done to a level of competency that was signed off by the veterinarian in charge.

Kind Regards

Dr SD Singh BVSc. (Mumbai) MS (Illinois) LAS (Utrecht) CVE (Pretoria)
HOD: Biomedical Resource Unit
Veterinarian

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

02 August 2017

Mr Ochuko L Erukainure (216073060)
School of Life Sciences
Westville Campus

Dear Mr Erukainure,

Protocol reference number: AREC/020/017D
Project title: Studies on the anit-hyperglycemic and toxicological effects of some anti-diabetic African Medicinal Plants *in vitro*, *ex vivo* and *in vivo*

Full Approval – Research Application

With regards to your revised application received on 23 June 2017. The documents submitted have been accepted by the Animal Research Ethics Committee and **FULL APPROVAL** for the protocol has been granted.

Please note: Any Veterinary and Para-Veterinary procedures must be conducted by a SAVC registered VET or SAVC authorized person.

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

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

The ethical clearance certificate is only valid for a period of one year from the date of issue. Renewal for the study must be applied for before 02 August 2018.

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

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

Yours faithfully

.....
Dr Sam Singh
Deputy Chair: Animal Research Ethics Committee

/ms

Cc Dean & Head of School: Dr A Olaniran
Cc Registrar: Mr Simon Mokoena
Cc NSPCA
Cc BRU – Dr L Bester

Animal Research Ethics Committee (AREC)
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APPENDIX 2

PUBLICATIONS FROM THIS THESIS

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FULL ARTICLE

WILEY Journal of Food Biochemistry

Histochemistry, phenolic content, antioxidant, and anti-diabetic activities of *Vernonia amygdalina* leaf extract

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University of KwaZulu-Natal; National Research Foundation, South Africa

Abstract

Vernonia amygdalina leaves were investigated for their histochemical properties and antidiabetic activities. Histochemical analysis of the leaf revealed distributions of acidic lipid, mucilage, and pectin, lipids, polyphenols, and alkaloids at the mid rib, glandular trichome, and epidermis. HPLC analysis of the leaves hot water infusion revealed the presence of quercetin and (-)-epi-catechin. The infusion had significant ($p < 0.05$) 2,2′-diphenyl-1-picrylhydrazyl scavenging activity and ferric reducing antioxidant power. Ex vivo antioxidative analysis revealed the ability of the infusion to increase glutathione level, superoxide dismutase, and catalase activities, while concomitantly depleting malondialdehyde level and DNA fragmentation in Fe^{2+}-induced hepatic injury. The infusion showed significant ($p < 0.05$) inhibitory activity against α-glucosidase and pancreatic lipase. It also inhibited intestinal glucose absorption and enhanced muscle glucose uptake, respectively. The ability of the infusion to abate oxidative stress, DNA fragmentation and stimulate muscle glucose uptake may suggest the antioxidative, anti-apoptotic, and insulin-sensitizing activity of *V. amygdalina*.

Practical applications

Vernonia amygdalina (bitter leaf) is among the common leafy vegetables in West Africa reported for its various medicinal and nutritional properties. It is utilized as a food ingredient as well as supplement for the treatment and management of type 2 diabetes (T2D). Its ability to inhibit intestinal glucose absorption, enhance muscle glucose uptake, and protect against hepatic oxidative stress gives more credence to its reported antidiabetic properties. Being a common leafy vegetable, *V. amygdalina* can be a cheap source of nutraceutical for the treatment and management of T2D and its complications.

KEYWORDS
antioxidative, enzyme inhibition, histochemistry, HPLC, *Vernonia amygdalina*

1 | INTRODUCTION

The use of medicinal plants in the treatment and management of several ailments and diseases dates to time immemorial, making these plants and their uses an incumbent part of most cultures. About 80% of the third world population are estimated to still rely on medicinal plants as their main healthcare source (Ekor, 2014), which indicates the relevance of these plants in modern days. Several studies have attributed the medicinal properties of these plants to their phytochemical constituents (Bruneton, 1993; Van Wyk, Oudtshoorn, &

CHAPTER

6

Clerodendrum volubile: Phenolics and Applications to Health

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1 INTRODUCTION

The health benefits of phenolics are well documented [1,2]. Phenolics are secondary metabolites in plants responsible for most of their health-promoting properties [3]. They are utilized in plants as defense mechanisms against pathogens and protection against ultraviolent rays [4]. They also contribute to the color, acidity, taste, fragrance, and oxidative permanence of plants [5].

Chemically, phenolics possess an aromatic ring with one or more hydroxyl groups, including functional derivatives such as methyl ethers, esters, and glycosides [6]. They are classified into phenolic acids, hydroxycinnamic acids, flavonoids, and lignans, which all arise from the amino acids, phenylalanine or tyrosine. The amino acids are deaminated to cinnamic acids, before entering the phenylpropanoid pathway [7]. One or more hydroxyl groups are then introduced into the phenyl rings to form the common carbon skeleton, the C6—C3 phenylpropanoid unit (Pereira et al. [7]). This is well reflected in the C6 aromatic ring of hydroxybenzoic acids, C6—C3 structure of hydroxycinnamic acids, C6—C3—C6 structure of flavonoids, and C6—C4—C6 structure of lignans [8].

2 PHENOLICS AND HEALTH

Several studies have reported the inverse correlation between phenolics intake and chronic human diseases such as cardiovascular diseases, diabetes, cancer, obesity, and inflammation [5,9,10]. This is dependent on their absorption and metabolism by the gastrointestinal (GI) system into the circulatory system and transportation to the liver [11]. Their structures play a major influential role in this regard by determining conjugation with other phenolics, degree of glycosilation/acylation, molecular size, and solubility [11–13].

These medicinal properties have been attributed to the potent antioxidative effect of phenolics, owing to their ability to (i) scavenge free radicals, (ii) chelate divalent cations, and (iii) modulate endogenous antioxidant enzymes [5,11]. These abilities are dependent on the hydroxylation of the aromatic rings. Phenolics have also been reported to bring about their medicinal effect by modulating intracellular signaling cascades involved in cellular metabolism [11,13].

2.1 *Clerodendrum volubile*

Phenolics are widely distributed in plants and are responsible for most of their medicinal properties [3]. The phenolic contents of some of these plants have been analyzed and their protective activities reported. Among such plants is *Clerodendrum volubile*, which belongs to the genus *Clerodendrum* L. under the Labiatae family.

The genus *Clerodendrum* L. is very large and diverse with over 580 identified species, comprising small trees, shrubs, and herbs widely distributed in the tropics and subtropics [14]. Most of these species are employed as folk medicine in Asian and African continents [14].

Clerodendrum volubile is indigenously known as obenetete by the Urhobos and Itsekiris, as well as eweta, dagba, or marugbo by the Yorubas all in Southern Nigeria [15,16]. It is often regarded as a magic leaf owing to its use in folkloric medicine for the treatment and management of several diseases such as diabetes mellitus, cancers, arthritis, ulcers, and neurological disorders [17]. Despite these folkloric claims there are, however, limited

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FULL ARTICLE

WILEY Journal of Food Biochemistry

Phaseolus lunatus (lima beans) abates Fe^{2+}-induced hepatic redox imbalance; inhibits intestinal glucose absorption and major carbohydrate catabolic enzymes; and modulates muscle glucose uptake

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Funding information
The Research Office, University of KwaZulu-Natal (UKZN), Durban, and National Research Foundation (NRF), Pretoria, South Africa

Abstract

The antioxidative and antidiabetic effects of the aqueous extract of *Phaseolus lunatus* were investigated in vitro and ex vivo. The seeds were subjected to aqueous extraction, after defatting with n-hexane. The extract (15, 30, 60, 120, 240 µg/ml) showed significant ($p < 0.05$) free radical scavenging and enzyme inhibitory activities in vitro. It significantly ($p < 0.05$) elevated glutathione level with concomitant depletion of malondialdehyde level as well as increased superoxide dismutase and catalase activities in rat liver. Fe^{2+}-induced hepatic DNA fragmentation was also significantly ($p < 0.05$) suppressed. The extract significantly inhibited intestinal glucose absorption and increased muscle glucose uptake with and without insulin. The inhibitory activities and reversion of hepatic redox imbalance, as well as inhibition of intestinal glucose absorption and increased muscle glucose uptake by *P. lunatus* suggest an antihyperglycemic and antioxidative effect. These can be ascribed to the synergistic effect of the phytochemicals and amino acids identified in the extract.

Practical applications

Lima beans (*Phaseolus lunatus*) is among the many underutilized legumes with reported nutritional benefits. It is utilized locally in Nigeria in treating and managing diabetes and its complications, but without any scientific proof. The ability of the legume to inhibit digestive enzymes linked to postprandial blood glucose spike, as well as inhibition of intestinal glucose absorption and increased muscle glucose uptake substantiates its antidiabetic folkloric uses. The amino acid constituents also indicate its nutritional properties. These findings will promote its utilization not only as an antidiabetic functional food but also as a nutritional meal.

KEYWORDS
antihyperglycemic, antioxidative, enzymes inhibition, legumes, *Phaseolus lunatus*

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Clerodendrum volubile inhibits key enzymes linked to type 2 diabetes but induces cytotoxicity in human embryonic kidney (HEK293) cells via exacerbated oxidative stress and proinflammation

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ARTICLE INFO

Keywords:
Apoptosis
Cytotoxicity
Oxidative stress
Proinflammation
Type 2 diabetes

ABSTRACT

The toxicity and safety associated with the use of medicinal plants remains a major concern. In this study, the antidiabetic properties of the dichloromethane (DCM) fraction of *C. volubile* leaves were investigated *in vitro*. Its cytotoxic effect and mechanism of toxicity were also investigated in Human Embryonic Kidney (HEK293) cells. The fraction was subjected to *in vitro* antioxidant assays using the 2,2′-diphenyl-1-picrylhydrazyl (DPPH) scavenging and Ferric reducing antioxidant power (FRAP) protocols. Its enzyme-inhibitory properties were investigated on α-glucosidase and α-amylase activities. Gas Chromatography Mass Spectroscopy (GC–MS) and Fourier Transform Infrared (FTIR) spectroscopic analysis were used to identify its phytoconstituents. Cytotoxicity was determined *via* MTT assay. The treated cells were assayed for reduced glutathione (GSH), non-protein thiol, nitric oxide and malondialdehyde (MDA) levels, as well as Superoxide Dismutase (SOD), catalase, myeloperoxidase and ATPase activities. Cell apoptosis and/or morphological changes were determined using the acridine orange and ethidium bromide (AO/EB) dual staining method. The fraction showed significant ($p < 0.05$) antioxidant and enzyme-inhibitory activity. It showed significant ($p < 0.05$) cytotoxic effect against HEK293 cells with concomitant depletion of antioxidative and elevation of proinflammatory biomarkers. Morphological changes were examined in the cells with an apoptotic index of 0.84. 1,1-Dodecanediol, diacetate was identified as the most predominant compound, while aromatics and amines as the most functional groups present in the fraction. These results suggest the antidiabetic and cytotoxic effects of *C. volubile* leaves. The toxicity can be attributed to induced oxidative stress and proinflammation with concomitant depletion of ATP leading to apoptosis of the cells.

1. Introduction

Type 2 diabetes accounts for more than 90% of all diabetes types, making it the most prevalent and a major contributor to diabetes-related mortality and morbidity [1,2]. Unlike type 1 diabetes, it arises from inability of the body to utilize the insulin produced and/or insufficient production of insulin [2]. It is characterized by pancreatic β-cell dysfunction and insulin resistance, leading to hyperglycemia [3,4]. Chronic hyperglycemia gives rise to increased production of free radicals, which when surpasses the endogenous antioxidant system induces oxidative stress [5]. Oxidative stress has been implicated in the pathogenic micro- and macro-vascular complications associated with type 2 diabetes [6–8]. Mitigation of oxidative stress as well as inhibition of carbohydrate and lipid metabolizing enzymes particularly the α – glucosidase, α – amylase and pancreatic lipase have been demonstrated to be therapeutic in the management of type 2 diabetes and its complications [9,10].

The use of medicinal plants in the treatment and management of type 2 diabetes have been in practice from time immemorial. This has been attributed to their nutritional and phytochemical constituents with reported anti-oxidative and anti-diabetic activities [8,11]. Amongst such plants is *Clerodendrum volubile*, commonly known among other leafy vegetables in south-south Nigeria for its food and medicinal values. Due to its multiple medicinal uses, it is commonly referred to as magic leaf [12]. It is used in the treatment of diabetes, ulcer, arthritis, rheumatism, dropsy, cancer, amongst other diseases [12]. Its aqueous

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Caffeine – rich infusion from *Cola nitida* (kola nut) inhibits major carbohydrate catabolic enzymes; abates redox imbalance; and modulates oxidative dysregulated metabolic pathways and metabolites in Fe^{2+}-induced hepatic toxicity

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ARTICLE INFO

Keywords:
Antioxidative
Cola nitida
Kola nuts
Metabolomics
Type 2 diabetes

ABSTRACT

The antioxidative and antidiabetic effects and toxicity of caffeine-rich infusion of *Cola nitida* were investigated using *in vitro*, *ex vivo* and *in silico* models. *C. nitida* was infused in boiling water and allowed to cool before concentrating at ≤ 50 °C. HPLC analysis of the infusion revealed a caffeine content of 80.08%. The infusion showed potent *in vitro* antioxidant activity by significantly ($p \leq 0.05$) scavenging 2,2′-diphenyl-1-picrylhydrazyl (DPPH). It significantly ($p < 0.05$) inhibited α-glucosidase and α-amylase activities. Treatment of Fe^{2+} induced oxidative hepatic tissues with the infusion led to increase Superoxide Dismutase (SOD) and catalase activities, and glutathione (GSH) level as well as decreased malondialdehyde (MDA) level. FTIR spectroscopy of hepatic metabolite revealed restoration of oxidative–induced depleted functional groups by the infusion. LC–MS analysis of the metabolite also revealed restoration of most depleted metabolites with concomitant generation of 4-*O*-Methylgallic, (-)-Epicatechin sulfate, L-Arginine, L-tyrosine, Citric acid and Decanoic acid in infusion-treated tissues. Pathway analysis of the identified metabolites revealed the presence of 21 metabolic pathways involved in normal hepatic tissues, 12 in oxidative injured tissues and 17 in the treated tissues. Treatment with the infusion restored 4 metabolic pathways common to the normal tissue and further activated 4 additional pathways. Prediction of oral toxicity of caffeine showed it to belong to class 3, with a LD_{50} of 127 mg/kg. Its toxicity target was predicted as Adenosine Receptor A2a. It was also predicted to be an inhibitor of CYP1A2. These results suggest the antioxidative and antidiabetic properties of *C. nitida* infusion, with caffeine as the major constituent.

1. Introduction

Diabetes mellitus (DM) remains one of the fast growing and unabated scourge in the world. It is a metabolic disorder affecting carbohydrate, protein and lipid metabolism [1]. It is characterized by dearth of insulin secretion as seen in type 1 diabetes or inability to utilize insulin secreted in the case of type 2 diabetes (T2D). Between these two types, T2D has been recognized as a major contributor to global mortality and morbidity [2]. It accounts for 12% of global healthcare expenditure with five million deaths only in 2015 [2]. Insulin resistance and pancreatic β-cell dysfunction leading to hyperglycemia have been implicated in the progression of T2D [3]. Increased hyperglycemia elevates the production of reactive oxygen species (ROS) which triggers an imbalance in the endogenous antioxidant system resulting to oxidative stress [4]. Oxidative stress plays an influential role in the chronic pathogenic micro- and macro-vascular complications associated with T2D such as nephropathy, neuropathy, microangiopathy, retinopathy and even death [5,6].

The economic cost of diabetes remains a huge burden to most developing countries with low gross domestic product (GDP). The cost of synthesized drugs coupled to their side effects have led to a paradigm shift to medicinal plants [7]. Their folkloric use of medicinal plants dates back time immemorial and can be attributed to their phytochemical constituents [8]. Amongst such plants is the kola nut fruit, *Cola nitida*.

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Original article

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

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ARTICLE INFO

Keywords:
Antioxidants
Dacryodes edulis
Type 2 diabetes
Medicinal plant

ABSTRACT

The leaves of *Dacryodes edulis* were investigated for their anti-oxidative and anti-diabetic potentials *in vitro*. Extracts from sequential extraction with solvents of increasing polarity (*n*-hexane, ethyl acetate, ethanol and aqueous) of the leaves were subjected to *in vitro* antioxidant assays using the 2,2′-diphenyl-1-picrylhydrazyl (DPPH) scavenging and Ferric reducing antioxidant power (FRAP) protocols respectively. Their inhibitory effects were investigated on α-glucosidase, pancreatic lipases, pancreatic ATPase and glucose-6-phospatase activities. Their antioxidant and anti-apoptotic effects on Fe^{2+} − induced oxidative injuries in pancreatic and hepatic tissues were also investigated *ex vivo*. The ethanol extract was subjected to Gas chromatography mass spectroscopy (GC–MS) and Fourier transform infrared (FTIR) spectroscopic analysis to identify its bioactive chemical constituents. The extracts showed potent free radical scavenging activity and significantly ($p < 0.05$) inhibited all studied enzymes, with the ethanol extract showing greater activities. Superoxide Dismutase (SOD) and Catalase (CAT) activities were significantly ($p < 0.05$) increased in both pancreatic and hepatic tissues with concomitant elevation of reduced glutathione (GSH) levels as well as reduced levels of malondialdehyde (MDA). The extracts significantly inhibited DNA fragmentation. These activities were dose − dependent. Amongst compounds identified, only Kaur-15-ene, Urs-12-ene-3-ol acetate and 2,3,23-trihydroxyolean-12-en-28-oic acid methyl ester showed strong binding affinities when docked with α-glucosidase (PDB ID:3TON). These results indicate the anti-oxidative, anti-diabetic and anti-obesogenic potentials of *D. edulis* leaves, which gives credence to its antidiabetic folkloric claims.

1. Introduction

The world has witnessed an increase in the prevalence of diabetes from 336 million individuals in 2011 to 415 million in 2015 depicting a 13.36% rise, with more than 45% undiagnosed [1]. This is estimated to rise to 642 million in 2040, with Sub Saharan Africa accounting for 5.33% compared to 3.42% in 2015 [1]. The urban areas have been shown to witness the highest increase, thus implicating rural–urban drift as well as changes in dietary patterns coupled with less physical activity and ageing population as the main factors in Africa [2].

Type 2 diabetes (T2D) accounts for more than 90% of all diabetes, which is a major contributor to global mortality and morbidity [1]. Unlike type 1 diabetes, it occurs when the body cannot effectively utilize the insulin produced. It generally arises from a combination of insulin resistance and pancreatic β-cell dysfunction, results in chronic hyperglycemia. In the progression of T2D, hyperglycemia results to elevated reactive oxygen species (ROS) production which causes oxidative stress due to the imbalance in endogenous antioxidant defense system [3]. Oxidative stress has been implicated in oxidative pancreatic and hepatic injuries, which further aggravates T2D [4]. This is characterized by decreased superoxide dismutase (SOD) and catalase activities leading to accumulation of superoxide ($O_2\cdot^-$), hydrogen peroxide (H_2O_2) and hydroxyl ($\cdot OH$) which causes peroxidation of the cell membrane lipid and DNA insults [5].

The use of medicinal plants in the treatment and management of T2D is well documented [6]. Their efficacy has been attributed to the presence of phytochemicals with reported anti-oxidative and anti-diabetic activities [4,7]. Unlike synthetic drugs, the use of plant originated

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APPENDIX 3

ACCEPTED MANUSCRIPTS FROM THIS THESIS

MEBR: Your manuscript entitled Vernonia Amygdalina Del. Stimulated Glucose Uptake in Brain Tissues Enhances Antioxidative Activities; and Modulates Functional Chemistry and Dysregulated Metabolic Pathways

From: Gregory Konat (em@editorialmanager.com)

To: loreks@yahoo.co.uk

Date: Tuesday, 4 December 2018, 03:26 GMT+1

Ref.: Ms. No. MEBR-D-18-00320R1
Vernonia Amygdalina Del. Stimulated Glucose Uptake in Brain Tissues Enhances Antioxidative Activities; and Modulates Functional Chemistry and Dysregulated Metabolic Pathways
Metabolic Brain Disease

Dear Mr. Erukainure,

I am pleased to tell you that your work has now been accepted for publication in Metabolic Brain Disease.

Thank you for submitting your work to this journal.

With kind regards

Dr. Gregory Konat
Editor-in-Chief
Metabolic Brain Disease

Journal of Food Biochemistry

Decision Letter (JFBC-08-18-0641.R1)

From: rotimi.aluko@umanitoba.ca

To: loreks@yahoo.co.uk, daramzjay09@gmail.com, chykochi@yahoo.com, matsabisamg@ufs.ac.za, Koorbanally@ukzn.ac.za, islamd@ukzn.ac.za

CC: carmen.lammi@unimi.it

Subject: Journal of Food Biochemistry - Decision on Manuscript ID JFBC-08-18-0641.R1

Body: 25-Oct-2018

Dear Mr. Erukainure:

It is a pleasure to accept your manuscript entitled "Raffia Palm (Raphia hookeri) Wine inhibits Glucose Diffusion; Exacerbates Antioxidative Activities; and Modulates Dysregulated Pathways and Metabolites in Oxidative Pancreatic Injury" in its current form for publication in the Journal of Food Biochemistry. The comments of the reviewer(s) who reviewed your manuscript are included at the foot of this letter.

First Look NEW: Please note although the manuscript is accepted the files will now be checked to ensure that everything is ready for publication, and you may be contacted if final versions of files for publication are required.

"Your article cannot be published until the corresponding author has signed the appropriate license agreement. Within the next few days the corresponding author will receive an email from Wiley's Author Services system which will ask them to log in and will present them with the appropriate license for completion."

Thank you for your fine contribution. On behalf of the Editors of the Journal of Food Biochemistry, we look forward to your continued contributions to the Journal.

Sincerely,
Dr Rotimi Aluko
Editor in Chief, Journal of Food Biochemistry
rotimi.aluko@umanitoba.ca

Associate Editor
Comments to the Author:
(There are no comments.)

Reviewer(s)' Comments to Author:

Reviewer: 1

Comments to the Author

APPENDIX 4

OTHER PUBLICATIONS

Acta Pharm. **68** (2018) 425–439 Original research paper
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Acalypha wilkesiana 'Java white': Identification of some bioactive compounds by GC-MS and their effects on key enzymes linked to type 2 diabetes

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

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

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Diabetes has gradually become a major health concern in both developed and developing countries, while controlling hyperglycemia is one of the major challenges in the management of this disease (1). Apart from hyperglycemia, almost 50 % of diabetic patients suffer from at least one or two diabetic complications such as diabetic retinopathy, cardiomyopathy, nephropathy, neuropathy and lower limb amputations, which are more noticeable in elderly patients (2). Approximately 108 million people were diagnosed with diabetes in 1980, which has increased to 422 million in 2014, with a projected increase to 642 million by the year 2030 (2).

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Original research article

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

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Boerhaavia diffusa
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Glucose uptake
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ABSTRACT

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

1. Introduction

Diabetes mellitus (DM) is regarded as the most common endocrine disorder in humans. According to the World Health Organization [1], at least 1.5 million deaths in 2015 alone were directly caused by diabetes globally. Among the different occurrences of diabetes, type 2 diabetes (T2D) remains the most common, and major public health challenge of the 21st century [2]. It is described as a versatile and degenerative illness that is affiliated with several metabolic flaws and organ malfunction or damage [3]. The eminent pathogenic features of T2D include pancreatic beta cell impairment and insulinemia which ultimately results in defective insulin secretion and persistent hyperglycemia [4]. Insulin resistance is also associated with poor lipid metabolism, which aggravates metabolic syndrome and lipidemia in the progression of T2D [5].

In peripheral tissues like adipose and muscle tissues, insulin resistance is an indicator for inadequate cellular glucose uptake, which leads to hyperglycemia in T2D [6,7]. Hyperglycemia-induced oxidative stress has been implicated in the symptoms, progression and complications of T2D [8,9]. Persistent hyperglycemia is responsible for elevated levels of mitochondrial energy production, glucose oxidation, protein glycosylation and lipid peroxidation, which leads to an increased output of free radicals and reactive oxygen species (ROS) and eventually, oxidative stress [10]. The pancreas is one of the most vulnerable organs to oxidative stress, because of its low levels of free radical scavenging ability compared to other organs [11]. Free radicals

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Flowers of *Clerodendrum volubile* exacerbate immunomodulation by suppressing phagocytic oxidative burst and modulation of COX-2 activity

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ABSTRACT

The immunomodulatory potentials of the crude methanolic extract and fractions [n-hexane (Hex), n-dichloromethane (DCM), ethyl acetate (EtOAc) and n–butanol (BuOH)] of *Clerodendrum volubile* flowers were investigated on whole blood phagocytic oxidative burst using luminol-amplified chemiluminescence technique. They were also investigated for their free radicals scavenging activities. The DCM fraction showed significant ($p < 0.05$) anti-oxidative burst and free radical scavenging activities indicating high immunomodulatory and antioxidant potencies respectively. Cytotoxicity assay of the DCM fraction revealed a cytotoxic effect on CC-1 normal cell line. GCMS analysis revealed the presence of triacetin; 3,6-dimethyl-3-octanol; 2R – Acetoxymethyl-1,3,3–trimethtyl – 4t – (3-methyl-2-buten-1-yl) – 1c – cyclohexanol and Stigmastan – 3,5–diene in DCM fraction. These compounds were docked with the active sites of cyclooxygenase-2 (COX-2). Triacetin, 3,6-dimethyl-3-Octanol, and 2R-Acetoxymethyl-1,3,3-trimethtyl-4t-(3-methyl-2-buten-1-yl)-1c-cyclohexanol docked comfortably with COX-2 with good scoring function (-CDocker energy) indicating their inhibitory potency against COX-2. 3,6–dimethyl-3-Octanol, displayed the lowest predicted free energy of binding ($-21.4\ kcal\ mol^{-1}$) suggesting its stronger interaction with COX-2, this was followed by 2R – Acetoxymethyl-1, 3, 3-trimethtyl-4t-(3-methyl-2-buten-1-yl)-1c-cyclhexanol (BE = $-20.5\ kcal\ mol^{-1}$), and triacetin (BE = $-10.9\ kcal\ mol^{-1}$). Stigmastan – 3,5–diene failed to dock with COX-2. The observed suppressive effect of the DCM fraction of *C. volubile* flower methanolic extract on phagocytic oxidative burst indicates an immunomodulatory potential. This is further reflected in its free scavenging activities and synergetic modulation of COX-2 activities by its identified compounds *in silico*.

1. Introduction

Reactive oxygen species (ROS) has long been shown to be produced during mitochondrial electron transport chain and/or oxido-reductase and metal–catalyzed oxidative reactions in aerobic tissues [1]. Under normal physiological condition, these free radicals are scavenged by antioxidants. However, oxidative stress sets in when there is a depletion of antioxidants or when the production of free radicals overwhelms the body's antioxidant system causing an imbalance between the free radicals and the antioxidant system [2]. Oxidative stress has been implicated in the peroxidation of membrane lipids, oxidation of protein sulfhydryl groups and disruption of the DNA [1].

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Article

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Monodora myristica (African nutmeg) modulates redox homeostasis and alters functional chemistry in sickled erythrocytes

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Abstract

The antioxidative effect of *Monodora myristica* seed acetone extract and its effect on chemical functional groups were investigated in sickled erythrocytes as well as molecular modeling of the antisickling potentials of its secondary metabolites. The extract was subjected to gas chromatography–mass spectrometry to identify the compounds present, which were then docked into the allosteric-binding site of deoxy-hemoglobin. The extract was incubated with sickled erythrocytes at 37°C for 6, 12, and 24 h and were subjected to antioxidative analysis for reduced glutathione (GSH), superoxide dismutase (SOD), catalase, and lipid peroxidation (LPO). Chemical functional group of the treated cells was analyzed via Fourier transform infrared spectroscopy (FTIR). The predominant compounds identified were 17-octadecynoic acid; oleic acid, androstan-3-one, 17-hydroxy-2-methyl- (2.beta.,5.beta.,17.beta.)-; estran-3-one, 17-(acetyloxy)-2-methyl-, (2.alpha., 5.alpha., 17.beta.), and (+)-3-carene, 10-(acetylmethyl)-. They all fitted well within the active site of Hb with good binding affinity, as evidenced by the negative CDocker interaction energies of their complexes ranging between −54.4 and −26.7 kcal/mol. Treatment with the extract exacerbated SOD and catalase activities as well as GSH level, while LPO was suppressed. This antioxidative activity was time and/or dose dependent, with 6 and 12 h incubation showing the optimum activity. FTIR analysis of the treated cells showed the presence of hydrophobic functional groups. The synergetic molecular interaction of the major compounds of the extract with the α-dimer of Hb depicts an antisickling effect of *M. myristica* acetone extract. This is accompanied by exacerbation of endogenous antioxidant enzymes activity and modification of the functional chemistry of the cells.

Keywords

Antisickling, antioxidative stress, *Monodora myristica*, and sickle cell disease

Introduction

Sickle cell disease (SCD) remains one of the most prevalent inherited hemoglobinopathy among Africans, with sub-Saharan Africa being the most hit.[1] It occurs due to a single mutation in the β-globin chain, causing valine to be substituted with glutamic acid at the sixth amino acid position,[2] thereby leading to the production of abnormal hemoglobin (HbS). Several studies have linked the pathogenesis of SCD to polymerization of HbS, which causes the erythrocyte to possess a crescent- or sickle-shape structure.[2,3] This triggers the release of hemoglobin (Hb) and iron into the plasma. The SCD is often associated with painful vaso-occlusive episodes characterized by endothelial dysfunction

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frontiers in Pharmacology

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Suppressive Effects of *Clerodendrum volubile* P Beauv. [Labiatae] Methanolic Extract and Its Fractions on Type 2 Diabetes and Its Complications

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Type 2 diabetes is the most prominent of all diabetes types, contributing to global morbidity and mortality. Availability and cost of treatment with little or no side effect especially in developing countries, remains a huge burden. This has led to the search of affordable alternative therapies especially from medicinal plants. In this study, the antidiabetic effect of the methanolic extract, dichloromethane (DCM), butanol (BuOH) and aqueous fractions of *Clerodendrum volubile* leaves were investigated in type 2 diabetic rats for their effect on glucose homeostasis, serum insulin level and hepatic biomarkers, lipid profile, pancreatic redox balance and Ca^{2+} levels, and β-cell distribution and function. The DCM was further fractionated to isolate the active compounds, biochanin and 5,7,4′-trimethoxykaempferol. They were investigated for their toxicity and ADMET properties, α-glucosidase and angiotensin I converting enzyme (ACE) inhibitory activities *in silico*. There were significant ($p < 0.05$) decrease in blood glucose, cholesterol, LDL-C, vLDL-C, triglyceride, AST and ALT levels in all treated groups, with DCM fraction showing the best activity. All treated rats showed significantly ($p < 0.05$) improved anti-oxidative activities. Treatment with the DCM fraction led to significant ($p < 0.05$) increased serum insulin and pancreatic Ca^{2+} levels, as well as improved β-cell distribution and function. DCM fraction also showed improved glucose tolerance. DCM fraction dose-dependently inhibited ACE activity. The toxicity class of the isolated compounds was predicted to be 5. They were also predicted to be potent inhibitors of cytochrome P (CYPs) 1A2, 2D6 and 3A4. They docked well with α-glucosidase and ACE. These results indicate the therapeutic potential of

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Anti-diabetic effect of the ethyl acetate fraction of *Clerodendrum volubile*: protocatechuic acid suppresses phagocytic oxidative burst and modulates inflammatory cytokines

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ABSTRACT

The antidiabetic effects of the ethyl acetate (EtOAc) fraction of *Clerodendrum volubile* leaves was investigated in this study. EtOAc was also fractionated to isolate the active compounds. The structure of the isolated compound (Protocatechuic acid) was established using ^{1}H and ^{13}C NMR spectroscopies and mass spectrometry. Protocatechuic acid was investigated for its anti-oxidative burst in polymorphonuclear neutrophils (PMNs) and macrophages. It was also docked with α–glucosidase and TNF-α. Acute treatment with EtOAc fraction of *Clerodendrum volubile* leaves significantly ($p < 0.05$) decreased blood glucose level and hepatic biomarkers, and significantly ($p < 0.05$) increased serum insulin level and β–cell function. It had little or no effect on serum lipid profile and atherogenic indices. Protocatechuic acid significantly ($p < 0.05$) suppressed phagocytic oxidative burst and docked well with α–glucosidase and TNF-α. These results indicate the therapeutic effect of EtOAc fraction of *C. volubile* on type 2 diabetes and its complications, which can be attributed to the main bioactive compound, protocatechuic acid.

1. Introduction

Diabetes is rapidly becoming a major scourge in African communities with change in life style and increasing urbanization being the primary factor. In 2015, 14.2 million cases of diabetes were reported in Africa [1], which has been predicted to rise to 34.2 million in 2040, indicating a more than 100% increment in next 25 years [1]. This increase is a major drain on the health resources of sub-Saharan Africa with low income coupled with other infectious diseases [2]. Diabetes is a complex metabolic disease characterized by inability of the pancreatic β – cells to secret insulin as seen in type 1 diabetes or the insulin secreted is not utilized as seen in type 2 diabetes leading to progressive impairment of glucose tolerance and hyperglycemia [3].

Type 2 diabetes has been recognized as a common diabetes since more than 90 or almost 95% people are suffering from this type of diabetes [1]. It is mostly characterized by insulin resistance with concomitantly reduced pancreatic β–cell function [4]. Subclinical chronic inflammation has been implicated as one of the pathogenic factors underlying insulin resistance [5]. Increased metabolic activities of islet cells on elevation of blood glucose level causes an elevated production of Reactive Oxygen Species (ROS) [4,5]. This has been attributed to increase respiratory oxidative burst upon stimulation of neutrophils and macrophages [6]. This in turn leads to the release of inflammatory cytokines and

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Original article

Pectolinarigenin from the leaves of *Clerodendrum volubile* shows potent immunomodulatory activity by inhibiting T – cell proliferation and modulating respiratory oxidative burst in phagocytes

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ABSTRACT

There have been increasing interest in the use of plant-derived substance as immunomodulators for the treatment and management of inflammatory ailments. *Clerodendrum volubile*, a leafy vegetable is known for its folkloric applications in the treatments of several inflammatory related ailments, but with little scientific evidence. This study reports the isolation, structure elucidation and *in vitro* immunomodulatory potentials of pectolinarigenin from *C. volubile* leaves. The immunomodulatory potentials of the crude methanolic extract and fractions [*n*-hexane (Hex), dichloromethane (DCM), ethyl acetate (EtOAc) and n – butanol (BuOH)] were investigated on whole blood, neutrophil and macrophage phagocytic respiratory burst using luminol-amplified chemiluminescence technique. DCM fraction showed higher inhibitory activity on respiratory burst, indicating high suppressive immunomodulatory potency. The DCM fraction was further fractionated using a gravity column chromatography loaded with silica gel. The column was eluted with mixtures of Hex and DCM (92.5:7.5) in increasing order of polarity up to Hex: DCM (88:12) to afford 5,7-Dihydroxy-6,4′-dimethoxyflavone (pectolinarigenin). The structure of the compound was established using data obtained from ^{1}H- and ^{13}C NMR spectroscopies and mass spectrometry. The isolated flavone was investigated for its inhibitory activity of neutrophil phagocytes respiratory burst as well as T – Cell proliferation. The compound exhibited significant activities (at $p < 0.05$) indicating high suppressive immunomodulatory potency. The potent suppressive effect of pectolinarigenin on polymorphonuclear neutrophils (PMNs) respiratory oxidative burst and T – cell proliferation suggests an immunomodulatory potential and pathway of the flavonoid.

1. Introduction

There have been increasing interests in the role of medicinal plants as immunomodulators. This is due to the presence of notable phytochemicals that have been implicated in the modulation of phagocytes such as monocytes, macrophages, and neutrophils [1]. These phagocytes release large volumes of reactive oxygen species (ROS) upon stimulation by pathogens through a

Abbreviations: ^{13}C NMR, Carbon-13 nuclear magnetic resonance; ^{1}H NMR, proton nuclear magnetic resonance; BuOH, n – butanol; CL, chemiluminescence; COSY, Correlation Spectroscopy; CPM, counts per minute; DCM, Dichloromethane; DMSO, Dimethyl Sulfoxide; EI-MS, electron ionization mass spectrometry; EtOAc, Ethyl Acetate; Hex, n-hexane; HMBC, Heteronuclear multiple-bond correlation; HMQC, heteronuclear multiple-quantum Correlation; MHS, modified Hank's solution; MPO, myeloperoxidase; MTT3, -(4, 5-dimethyl thiazole-2-yl)-2, 5-diphenyltetrazolium bromide; NOESY, nuclear overhauser effect spectroscopy; PMNs, polymorphonuclear neutrophils; ROS, reactive oxygen species.

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Concentrated hot water-infusion of *phragmanthera incana* improves muscle glucose uptake, inhibits carbohydrate digesting enzymes and abates Fe^{2+}-induced oxidative stress in hepatic tissues

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ABSTRACT

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

1. Introduction

Diabetes mellitus (DM) has, over the years, become one of the leading threats to human health [1]. Available data reveal that the prevalence of diabetes has increased from 4.7% in 1980 to 8.5% in 2014 [1,2]. The International Diabetes Federation (IDF) projected a 55% increase of prevalence by 2032, when at least 592 million people will be living with diabetes [3].

Diabetes is a chronic disorder of energy metabolism characterized by hyperglycemia and glucose intolerance due to the deficiency of insulin as seen in type 1 diabetes, or impaired insulin action, or both as seen in type 2 diabetes (T2D) [4]. Among all types of diabetes, T2D is the most common one, owing to urbanization and changes from traditional to westernized diets [2]. Chronic hyperglycemia in T2D has been linked to increase in free radicals or impaired antioxidant defense mechanism in the body, resulting to oxidative stress [5]. Oxidative stress has been implicated in the progression and pathogenesis of T2D, leading to micro- and macro-vascular complications [6].

Most oral synthetic antidiabetic drugs have been targeted towards the control of hyperglycemia, thus, limiting its progression to secondary complications [7]. These drugs exert their actions by improving pancreatic β-cell function, reducing glucotoxicity, decreasing glucose reabsorption in the kidney, increasing insulin sensitivity in the muscle and liver, decreasing hepatic glucose production, and increasing gastric emptying following increasing GLP-1 secretion [8].

Plant-derived natural products are gaining more and more popularity in the management of diabetes not only due to their lower cost, but also for having negligible or no side effects [9]. The ability of polyphenols, terpenoids, alkaloids, saponins, flavonoids, and glycosides from plants to scavenge free radicals or chelate active metals to attenuate their redox action have been beneficial to human health [10]. Some medicinal plants have been employed in the folkloric treatment and management of diabetes and its complications from time immemorial. Their antidiabetic properties have also been demonstrated in

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Azadirachta indica inhibits key enzyme linked to type 2 diabetes *in vitro*, abates oxidative hepatic injury and enhances muscle glucose uptake *ex vivo*

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ABSTRACT

The progression of secondary complications in type 2 diabetes (T2D) has been linked to oxidative stress caused by hyperglycemia. Therefore, the control of hyperglycemia is the main target in the treatment of diabetes. The present study investigated the scavenging and ameliorative potentials of different fractions of *Azadirachta indica (A. indica)* ethanol stem bark extract in Fe^{2+}-induced oxidative injury in hepatic tissue as well as their ability to inhibit enzymes linked to diabetes and in enhancing muscle glucose uptake *via* some *in vitro* and *ex vivo* experimental models. The results revealed that the butanol fraction of the extract showed a significantly ($p < 0.05$) higher DPPH scavenging activity than the other fractions (IC_{50} 0.0154 μg/mL), while the aqueous fraction showed the highest FRAP activity (IC_{50} 25.32 μg/mL). Although all the fractions ameliorated Fe^{2+}-induced oxidative injury in hepatic tissue by significantly reducing malondialdehyde (MDA) concentration in a dose dependent manner, the butanol fraction showed the highest activity in this regard. In addition, the activities of catalase and superoxide dismutase (SOD) were significantly improved by the butanol and dichloromethane fractions. Butanol and ethyl acetate fractions showed the highest inhibitory effect on α-glucosidase (IC_{50} 0.23 μg/mL) and α-amylase (IC_{50} 14.79 μg/mL) activities, respectively. Although all the fractions significantly improved glucose uptake in psoas muscle with or without insulin, the butanol fraction showed the highest activity (GU_{50} 6.22 μg/mL) in this regard. Gas chromatography–mass spectroscopy (GC–MS) analysis of the fractions revealed the presence of sistosterol, stigmasterol, campestrol, squalene, nimbiol among others. Molecular docking of some of these compounds with AMP-activated protein kinase (α-AMPK), α-amylase and α-glucosidase showed a positive interaction. These results suggest that the butanol and ethyl acetate fractions of *A. indica* may have bioactive compounds with antidiabetic potentials.

1. Introduction

The prevalence of diabetes has increased over the years, making it a major global public health challenge, affecting approximately 415 million people worldwide. This number is expected to increase to 645 million by the year 2040 [1]. There are predominantly two types of diabetes, namely type 1 and type 2. Type 2 diabetes (T2D) accounts for more than 90% of all diabetic cases and is characterized by hyperglycemia and insulin insensitivity leading to pancreatic beta cell dysfunction overtime. When insulin response is low (insulin resistance), glucose uptake in skeletal muscle is markedly reduced [2]. Experimental and clinical evidences have shown that hyperglycemia is one of the major causative factors that is responsible for increasing reactive oxygen species (ROS) production which induces oxidative stress and ultimately secondary complications of diabetes [3–7]. Thus, the agents with glycemic control ability are the modulators of diabetes associated oxidative stress and complications.

Currently available anti-diabetic drugs for T2D are associated with potential adverse effects such as hypoglycemia [8], urinary bladder cancer, weight gain, fluid retention, osteoporosis and cardiovascular complications [9,10]. Hence, there is a need to search for alternative remedy with lesser or no side effect.

The use of medicinal plant as alternative therapy with minimum side effect has been widely explored [11]. *A. indica*, also known as

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