Cape Peninsula University of Technology

HEPATO-RENAL, CARDIAC AND REPRODUCTIVE EFFECTS OF *PHYLLANTHUS AMARUS* LEAF EXTRACT IN STREPTOZOTOCIN-INDUCED DIABETIC MALE WISTAR RATS

by

FOLORUNSO ADEWALE OLABIYI

Thesis submitted in fulfilment of the requirements for the degree

Doctor of Philosophy in Biomedical Science

in the Faculty of Health and Wellness Sciences

at the Cape Peninsula University of Technology

Supervisor: Professor OO Oguntibeju

Co-supervisor: Dr YG Aboua

Bellville

June 2020

DECLARATION

I, Folorunso Adewale Olabiyi, declare that the content of this thesis represents my unaided work and that the thesis has not previously been submitted for academic examination towards any qualification. All sources of my information are appropriately acknowledged as references. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.

01:08:2020

Signed Date

ABSTRACT

Indigenous knowledge about natural products forms a basis for medicinal plants research. Natural products are useful in traditional applications; hence, a need to conduct robust scientific research to determine their effects and possible toxicity on different biological cells, tissues, organs, and systems. Importantly, it is evident that useful information on the dosage, efficacy and possible beneficial or harmful effects of some medicinal plants on biological models are lacking. In this thesis, we reported on a single-dosage in vitro antioxidant, antidiabetic, skin depigmentation, cytotoxicity and phytoestrogenic potentials of Phyllanthus amarus (PA) extract. Also, we provided a detailed account of the effects of Phyllanthus amarus aqueous extract on the liver, kidney, heart, and testes in diabetes-induced male Wistar rats. Likewise, we carried out in-vitro antioxidant and the anti-tyrosinase evaluation of the extracts using enzyme-linked immunosorbent assay methods. We evaluated the inhibitory action of alpha-glucosidase and alpha-amylase in the aqueous and the methanol extracts. We also performed cytotoxicity and phytoestrogenicity tests on TM4 (Sertoli) and MCF-7 (breast cancer) cell lines using colourimetric MTT assay, cell counts and E-screen assay, respectively. At the same time, the in-vivo assessment of Phyllanthus amarus aqueous extract was done in male rats using fructose: streptozotocin diabetes model, whereby we administered PA (200mg/kgbwt, 400 mg/kgbwt) as well as GLIBEN (glibenclamide) 0.2mgkgbwt per day to the animals for four weeks (28 days).

In this study, we noted increased phytochemicals in the methanolic extract. Also, we observed more radical cation scavenging (TEAC) action in the aqueous fraction. Methanolic ORAC results produced a significant ($p < 0.0001$) result compared to the aqueous extracts. Besides, PA extract produced a significant inhibition in the α-glucosidase activity. The hexane, as well as the aqueous extracts, gave considerable tyrosinase inhibition ($p < 0.05$) exhibiting half-maximal inhibitory concentrations of 116.08 and 129.25 µg/mL, correspondingly. PA aqueous extract at concentrations between 0.01 and 10 µg/mL produced the highest activity of mitochondrial dehydrogenase. In this instance, the TM4 cell numbers significantly increased versus the untreated control. More so, exposure of Sertoli cells to higher extract concentrations ranging between 100 and 1000 µg/mL resulted in compromised viability. Furthermore, the aqueous extract produced a significant proliferative effect on MCF-7 cells; hence, we confirmed its estrogenic activity.

In our in vivo study, we noted that the animals showed persistent bodyweight loss except in the healthy control up until the 21st day of the experiment. The absolute and relative liver weights of untreated diabetic animals versus the healthy controls showed a significant finding ($p < 0.0001$).

Similarly, the absolute and relative testicular weight of untreated diabetic rats and diabetic rats + PA 200mg/kgbwt produced a significant result ($p < 0.05$). The PA 400mg/kgbwt similarly increased serum insulin compared to the group that received glibenclamide versus untreated diabetic control and healthy control ($p > 0.05$). PA 200mg/kgbwt significantly lowered serum nitric oxide versus the diabetic controls ($p < 0.0001$) while GLIBEN 0.2mg/kgbwt significantly reduced serum nitric oxide versus the normal and diabetic controls, PA 200mg/kgbwt as well as PA 400mg/kgbwt ($p < 0.0001$). PA 200mg/kgbwt, PA 400mg/kgbwt with GLIBEN 0.2mg/kgbwt significantly brought down serum myeloperoxidase (MPO) activity versus the diabetic controls ($p < 0.0001$). Both PA 200mg/kgbwt, as well as PA 400 mg/kgbwt, reduced H_2O_2 levels versus diabetic control ($p > 0.05$). They significantly reduced malondialdehyde (MDA) levels versus diabetic control ($p < 0.0001$). PA 400mg/kgbwt significantly raised glutathione peroxidase (GPx) activity similarly to the diabetic control ($p < 0.0001$). PA 200mg/kgbwt significantly brought down serum triglyceride levels correspondingly to the diabetic non-treated animals ($p < 0.0001$). PA 200mg/kgbwt and PA 400mgkgbwt caused increased liver GPx activity. They reduced the rats' heart, kidney, and liver lipid peroxidation similarly to glibenclamide versus the untreated diabetic rats, normalised alkaline phosphatase (ALP) with aspartate aminotransferase (AST) activities, respectively versus untreated diabetic rats. In contrast, the PA 200mg/kgbwt normalised the gamma-glutamyl aminotransferase (GGT) activity versus the diabetic untreated diabetic rats. The diabetic groups produced significant hypoproteinemia ($p < 0.0001$) versus the untreated healthy rats. Moreover, the PA 200mg/kgbwt improved serum testosterone similarly to glibenclamide versus their diabetic control counterparts and similarly raised serum estradiol compared to diabetic control. Nevertheless, these increases were not significant ($p > 0.05$). It also significantly improved sperm count correspondingly to glibenclamide ($p = 0.0033$) versus diabetic control and the PA 400mg/kgbwt group. Histological findings suggest that PA 200mg/kgbwt and PA 400mg/kgbwt put a halt to the progressive destruction (necrosis) of the islets of Langerhans, hence its probable ability to stimulate endogenous β-cell proliferation. PA 400mg/kgbwt ameliorated the renal lesions that showed in the diabetic group. This dose of the extract did not have any noticeable protective effect on the hepatocytes. Besides, PA did not ameliorate the lesions observed in the diabetic heart. Moreover, PA 200mg/kgbwt reduced seminiferous tubular diameter and an expanded interstitium, suggestive of its potential to improve reproductive functions through enhanced spermatogenesis.

We thus conclude that PA exhibited high antioxidant potentials by being able to inhibit ABTS radical, reduce ferric ion and scavenge oxygen radicals. Both Phyllanthus amarus Hexane and aqueous extracts have a favourable tyrosinase inhibition versus reference kojic acid. Besides,

PA extracts possess a significant inhibitory effect on α-glucosidase. PA extract at lower concentrations elicited Sertoli cell proliferation, probably because of the phytoestrogenicity of Phyllanthus amarus extract, brought about via its active principles, namely, phyllanthin and hypophyllanthin, thus, served as an excellent anticancer agent against breast cancer. One could also suggest that aqueous PA extract may improve reproductive functions through increasing spermatogenesis and decreasing testicular free radicals in diabetic rats. Finally, Phyllanthus amarus possesses an ameliorative effect on complications precipitated by diabetes.

ACKNOWLEDGEMENTS

To God, be all the glory for the success of this doctoral journey.

My unreserved gratitude goes to Professor OO Oguntibeju (supervisor) and Dr YG Aboua (co-supervisor) for their patience, guidance, and support. Thanks to our collaborators, postgraduate colleagues and friends who contributed immensely to the research output and the write up of this thesis.

I sincerely appreciate the ABUAD management who recognised the importance of higher training; hence, my release from work to study and the start-up fellowship as their support and contribution. Worthy of mention is the constant encouragement of Professor Israel Orubuloye (the chairman board of trustees), the Provost, College of Medicine and Health Sciences, Afe Babalola University, Ado-Ekiti, the head of the department, Medical Laboratory Science, and colleagues. All are appreciated.

Special thanks to all faculty members of the Department of Biomedical Sciences, Faculty of Health and Wellness Sciences, CPUT for their countless help and assistance.

CPUT also deserve appreciation on this successful journey for providing the appropriate environment, research facilities, financial support, and infrastructure.

To my mentors and pastors all over, thanks for guidance all these years.

God bless you all.

DEDICATION

This thesis is dedicated to the glory of the almighty GOD, and to my late parents who taught me that education is the best legacy any parent can give to a child.

PREFACE

This report is written according to the author guidelines of the journal each manuscript was submitted or meant for submission, based on the article format of CPUT. There are seven divisions in the report.

Chapter one gives the introduction and what motivated the study, as well as the aim and objectives we set to achieve.

Chapter two reviewed relevant literature on the work previously done in the subject area and to find out how and where the current work could contribute to the body of knowledge in the field. This chapter forms part of a published book by **Apple Academic Press, Incorporated, the USA.**

Chapter three reported on how Phyllanthus amarus extracts exhibited antioxidant, tyrosinase inhibition and cytotoxicity. The manuscript was published in "The Natural Products Journal".

Chapter four reported on the inhibitory activity of aqueous plant extract on α-glucosidase and how it modulates glycaemic indices and oxidative stress inside the diabetic rats' pancreas, by explaining a mechanism of antidiabetic action. The manuscript was prepared for submission to **Life Sciences (ELSEVIER, INC)** for publication.

In chapter five, we answered the question that bothers on whether the plant extract reduces the severity of derangement in lipids, oxidative stress, hepatic, renal and cardiac complications in streptozotocin-diabetic rats. The manuscript was prepared for submission to **BIOMEDICINES** journal for publication.

Chapter six focuses on how our aqueous extract ameliorates sperm abnormality and oxidative stressed diabetic rats' testes via phytoestrogenic as well as radical-scavenging actions. The manuscript was also prepared for submission to **ANDROLOGIA** journal for consideration.

Lastly, **chapter seven** generally discusses the key findings from this study, and we thus recommend the conclusion of the entire research and the highlighted areas for future studies.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

GLOSSARY

ANOVA	Analysis of variance
AQ	Aqueous
CPUT	Cape Peninsula University of Technology
DM	Diabetes mellitus
DMSO	Dimethyl sulphoxide
DPPH	2,2-diphenyl-1-picrylhydrazyl
ED	Erectile dysfunction
FHI	Forestry herbarium Ibadan
FRAP	Ferric reducing antioxidant power
g/kgbwt.	Gram per kilogramme body weight
gm	Gram
GPx	Glutathione peroxidase
GSH	Reduced glutathione
GST	Glutathione-S-transferase
H&E	Haematoxylin and eosin
IBF	Ibuprofen
IDF	International diabetes federation
MCF-7	Michigan cancer foundation-7
MEOH	Methanol
mg/kgbwt.	Milligram per kilogramme body weight
mg/kg BWT	Milligram per kilogramme body weight
MPO	Myeloperoxidase
MTT	[3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide]
NO	Nitric oxide

ORAC	Oxygen reducing antioxidant capacity
OS	Oxidative stress
PA	*Phyllanthus amarus*
REC	Research ethics committee
ROS	Reactive oxygen species
SEM	Standard error of mean
SOD	Superoxide dismutase
SRC	Standard rat chow
STZ	Streptozotocin
T1D	Type 1 diabetes
T2D	Type 2 diabetes
TEAC	Trolox equivalent antioxidant capacity
TM4	Mouse Sertoli cells
WHO	World health organisation

CHAPTER ONE: INTRODUCTION

1.1. Diabetes Mellitus

Diabetes mellitus (DM) is metabolic disarray of many aetiologies, typified by hyperglycaemia and derangements in carbohydrate, lipid, and protein metabolism, either because of the abnormality in insulin synthesis or insulin action (WHO, 1999; Adefegha & Oboh, 2012). This disorder presents symptoms like thirst, urine frequency, impaired vision, and weight loss. Besides, in complicated cases of diabetes mellitus, ketoacidosis or non-ketotic hyperosmolar state could come up, resulting in a reduced state of consciousness, complete unconsciousness and even death when an effective treatment is lacking. This disorder is fast becoming a global epidemic. Findings from the current ninth edition of the IDF Diabetes Atlas confirmed that diabetes is leading as a global health emergency of this century (International Diabetes Federation, 2019b).

Consequently, diabetes is accorded a top ten cause of mortality, and the number of patients with T2D is rising both in developed and developing countries. As such, low- and middle-income countries contributed eighty per cent (International Diabetes Federation, 2019b), with an estimated global prevalence of 9.3%. Also, the IDF reported around 463 million diabetics worldwide, and this figure may go up to 700 million by the year 2045 (International Diabetic Federation, 2019[a]). The report indicates that about USD 760 billion was spent on diabetes representing around 10% of health expenditure, worldwide. The disease burden will give rise to a substantial socio-economic impact, as annual diabetes and associated complications-related expenses exceed USD 100 billion (Jarald et al., 2008; CDC, 2020; Bide & Padalkar, 2020). There are four broad categories of diabetes, namely: a. prediabetes, b. insulin-dependent diabetes mellitus, c. Non-insulin-dependent diabetes and d. diabetes due to pregnancy.

In the current study, the attention was on T2D male Wistar rats that drank 10% fructose for 14 days and were subsequently administered streptozotocin, thereby creating a model of T2D.

T2D arises when the pancreas is unable to synthesise enough insulin, or the body ineffectively utilises the insulin because of the occurrence of insulin resistance (Ferrannini, 1998; Khodabandehloo et al., 2016). Therefore, hyperglycaemia that characterises T2D because of low insulin production or impaired insulin action on the body cells serves as an indicator of diabetes in the animal model under investigation (Maritim et al., 2003; Karalliedde & Gnudi, 2016).

There exist a relationship linking hyperglycaemia, oxidative stress with diabetic complications. Study shows that hyperglycaemia elicits oxidative stress by releasing free radicals. During normal physiological conditions, networks of endogenous antioxidant defences, comprising of both enzymatic and non-enzymatic antioxidants, guards the body by protection from harmful effects of these free radicals. However, these defences become weakened during diabetes, exacerbates by chronic hyperglycaemia, and produce reactive oxygen species (Omotayo, 2012; Bonini, Dull, & Minshall, 2014; Thakur et al., 2018). The higher free radicals generated together with a decline in these antioxidant defences (Bonini, Dull, & Minshall, 2014) could cause damage to cellular organelles and enzymes. It has also been found to result in elevated malondialdehyde levels with resultant insulin resistance (Maritim et al., 2003; Khodabandehloo et al., 2016). More so, oxidative stress can potentially promote macrovascular and microvascular complications that affect the kidney due to diabetes and end-stage renal diseases, cardiovascular diseases, dyslipidaemia, and other conditions seen in diabetes. Another complication experienced among people with diabetes includes erectile dysfunction (ED) which affects male reproductive ability in T2D patients (Agbaje et al., 2007; Furukawa et al., 2017) This may be because, the severe hyperglycaemia, with resultant free radicals, causes damage to the blood vessels that supply the cavernous tissue of the penis. Thus, it prevents its enlargement and stiffening that constitutes an erection (Jarald et al., 2008; Castela & Costa, 2016; Al-Oanzi, 2019).

1.2. Antidiabetic Drugs

Current conventional drugs used to treat diabetes fall into the following classes: (a). Glucose-lowering effectors that reduce hepatic glucose release, e.g. metformin (b). Insulin and Insulin secretagogues that increase pancreatic β-cells' insulin secretion, e.g. sulphonylureas; (c). peroxisome proliferator-activated receptor-Ɣ (PPAR-Ɣ) activators which enhance insulin activity, e.g. the thiazolidinediones; (d). α-glucosidase inhibitors that interfere with gut glucose release, thus, prolonging carbohydrate digestion as well as reducing the glucose absorption rate from the gut (Kuo et al., 2010), ultimately reducing the after-meal increase in blood glucose concentrations (Zhu et al., 2013). However, there are deficiencies associated with current therapies. These include hypoglycaemic episodes, a gradual loss in responsiveness to treatment, gastrointestinal problems, oedema, and weight gain (Collins, 2002; Mazzola, 2012; Tahrani et al., 2016). Other limitations of these drugs include high cost, lack of availability and accessibility for rural dwellers. Given the high rate of diabetes and the limitations of current therapies, there is a need to intensify research efforts for alternative therapeutic interventions.

These therapies must have fewer side effects and should be made available and accessible at low cost to everyone, irrespective of one's socio-economic status.

1.3. Medicinal Plants

The use of plant-derived products could be an alternative to improve health care (Malviya et al., 2010; Vanga & Raghavan, 2018). They are locally available; hence, anyone can easily access them regardless of social-economic status (Maghrani et al., 2005; Choy et al., 2014; Mbogori & Mucherah, 2019). In diabetes, some plants-based alternative medications could provide symptomatic relief and help in preventing secondary complications. Some of these plants also assist in β-cells regeneration and in overcoming insulin resistance. Besides, because they could maintain a typical blood glucose concentration, certain herbs have been reported to have antioxidant activity and hypolipidaemic action (Jarald et al., 2008; Joshi & Jain, 2014; Srivastava, 2018). Several plants scientifically validated for their antidiabetic activities while some have undergone clinical trials in many countries. Phytoconstituents in plants that have antidiabetic activity include; polysaccharides, peptides, alkaloids, glycopeptides, triterpenoids, amino acids, steroids, xanthone, flavonoids, lipids, phenolics, coumarins, iridoids, alkyl disulphides, inorganic ions and guanidines (Grover et al., 2002; Mukherjee et al., 2006; Angothu et al., 2010). Our preliminary studies reported on various medicinal plants with antidiabetic properties; few examples of such validated African traditional medicinal plants with antidiabetic activities include Moringa oleifera Lam., Catharanthus roseus (L.), Carica papaya L., *Phyllanthus amarus* Schum & Thonn and Garcinia kola Heckel.

1.4. Phyllanthus amarus

Phyllanthus amarus (PA) is a tropical and subtropical herb of the Asian and some West African countries (Mazumder et al., 2006; Tahseen & Mishra, 2013; Okaiyeto et al., 2018). The plant is known as seed-under-leaf, leaf flower or stone breakers. The Yoruba people of South-Western Nigeria popularly called it Iyin olobe. Research indicates that people use the leaf and seed of PA when boiled in managing diabetes, obesity, and hyperlipidaemia (Adeneye, 2012). Also, in Nigeria, *Phyllanthus amarus* is commonly called dobisowo, ehin olobe or ehin olubisowo among the Yoruba tribe (Samson, 2016). Phytochemical examination showed that Phyllanthus amarus has many phyto-compounds such as polyphenols and tetra-cyclic triterpenoids (Maciel et al., 2007; Patel et al., 2011), flavonoids (Patel et al., 2011; Verma et al., 2014), tannins (Foo, 1993;

Chopade & Sayyad, 2015) lignans (Kassuya et al., 2006; Leite et al., 2006; Maciel et al., 2007; Pereira et al., 2016; Conrado et al., 2020) alkaloids (Houghton et al., 1996; Kurhekar, 2017) and several other phyto-constituents. With high-performance liquid chromatography (HPLC), preliminary investigations on Phyllanthus amarus aqueous, and methanolic extract also showed the presence of lignans such as phyllanthin, hypophyllanthin as well as rutin and quercetin. The plant extract has high antioxidant contents, as shown by Trolox Equivalent Antioxidant Capacity (TEAC), Oxygen Reducing Antioxidant Capacity (ORAC) and Ferric Reducing Antioxidant Power (FRAP) assays. Previous studies have shown that Phyllanthus amarus reduced serum levels of glucose and glycosylated proteins, improve impaired glucose metabolism, and has favourable effects in various disease states (Adedapo et al., 2014; Verma et al., 2014). This plant traditionally exhibited hypolipidaemic, anti-inflammatory, antioxidant, antimicrobial, antifungal, anti-tuberculosis, analgesic and antidiabetic effects (Patel et al., 2011; Verma et al., 2014). Regardless of the various traditional uses of this vital herb, there are inconclusive and inconsistent empirical reports of its effect on male reproductive and cardiovascular systems among people with diabetes. Besides, the mechanisms of its antidiabetic or antihyperglycaemic action remain unclear; hence, the need for the present study.

1.5. Diabetic complications and reproductive functions

Scientific research indicates a significant correlation between inflammation and oxidative stress in diabetic pathologies (Lee et al., 2007; Rivero et al., 2009; Biswas, 2016). Also, hyperglycaemia selectively causes apoptosis in tubules and endothelial cells (Allen et al., 2003), and that Bcl2 group of proteins regulate occurring apoptotic cell death. Also, increase in inflammatory cytokines such as interleukin 6 (IL-6), interleukin 18 (IL-18), interleukin 1 (IL-1), and tumour necrotic factor-alpha (TNF-α) have been reported in diabetic blood (Esposito et al., 2002; Alexandraki et al., 2008; Liu et al., 2016). Importantly, long-standing hyperglycaemia and high oxidative stress levels play significant roles during secondary diabetic complications especially in nephrotoxicity (Huang et al., 2002; Bonini, Dull, & Minshall, 2014; Kachhawa et al., 2017) as well as hepatic injury (Harrison, 2006; Kim et al., 2009). DM can damage the body's nerves, particularly when blood glucose together with blood pressure become concurrently high, resulting in problems with digestion, erectile dysfunction (ED), among others (International Diabetes Federation, 2019a). ED and loss of libido could be a cause of infertility in some males (Lotti & Maggi, 2018). Besides, oxidative stress reported in about 50% of all infertile men, could explain a common pathology observed in them (Tremellen, 2008; Agarwal et al., 2014; Nowicka-

Bauer et al., 2018). Researchers postulated that ROS raised by sperm and seminal leucocytes in seminal fluid produce infertility by two primary mechanisms. Firstly, they destroy the sperm membrane, thereby lowering sperm motility and its ability to fuse with an oocyte. Secondly, ROS alters the sperm deoxyribonucleic acid (DNA), resulting in the passage of abnormal paternal DNA onto the foetus (Bonini, Dull, & Minshall, 2014). More so, men with T2D have an increased rate of low testosterone levels versus age-matched controls (Aboua et al., 2013). In many cross-sectional studies, male testosterone levels inversely correlate several recognised risk factors for T2D aetiology. Besides, obesity, central adiposity, and high fasting plasma insulin (hyperinsulinaemia) are predominant findings. The hyperinsulinaemia often inhibits normal spermatogenesis, thus, leading to a decreased male fertility (Aboua et al., 2013; Davison, 2016; La Nasa, 2018). Most patients with T2D weigh more or obese, resulting in low testosterone levels and increased pro-inflammatory cytokines, inducing dysfunction in blood vessel wall via the nitric oxide pathway. The above process goes on to explain the relationship between T2D, obesity with erectile dysfunction (Brown et al., 2005; Bhasin et al., 2007; Mukherjee et al., 2020). Similarly, low plasma testosterone level relates with cardiovascular disease and hypertension in aetiology (Jones, 2007; Jones, 2010; Traish, 2014; Nikolaidou et al., 2015; Fernández-Miró et al., 2016; Rovira-Llopis et al., 2017). Also, impaired spermatogenesis correlates with DM (Brown et al., 2005; Bhasin et al., 2007; Jangir & Jain, 2014; Miao et al., 2018). Another complication of DM is skin disorders with diabetic extracutaneous complications, including neuropathy, nephropathy, and retinopathy, that is common among a sizeable number of diabetic patients (Demirseren et al., 2013; Schons, 2018).

1.6. Animal model of type 2 diabetes (Subramoniam, 2016)

In T2D studies, some workers developed different animal models to monitor disease progression and treatment (Kumar et al., 2014). In this study, we created T2D together with insulin resistance (IR) in a rat model fed with 10% fructose. In this model, we created T2D/IR in male Wistar rats, whereby we allowed the animals to drink 10% fructose water for 2 weeks freely. After which we administered a once-off intraperitoneal injection of streptozotocin (55mg/kgbwt in freshly prepared 0.1M cold citrate buffer (pH 4.5) following overnight fasting. The animal model in this study exhibited T2D symptoms that mimic human diabetes due to the occurrence of insulin resistance that partially killed the pancreatic β-cells and reducing their population (Islam and Wilson, 2012). Studies also revealed that diets high in fructose results in the development of T2D symptoms which includes hyperlipidaemia, hypertension, hepatic insulin resistance, β-cell

dysfunction, and elevated serum liver enzymes activities (Wilson & Islam, 2012; Mohammed et al., 2016).

This thesis, therefore, reported on the effects of Phyllanthus amarus extract in T2D animal model.

1.7. Justification

Previously, Phyllanthus amarus extract exhibits antidiabetic action in a graded dose of 200 mg/kgbwt and 400 mg/kgbwt per day in alloxan-diabetic rats. The 400mg/kgbwt dose was considered a sufficient concentration (Adedapo et al., 2014). However, there is dire need to elucidate its potential to modulate the complex responses associated with oxidative stress, inflammation, apoptosis, and male reproduction. More so, there is insufficient empirical data on its possible beneficial effects on the above biological processes. Besides, we found no study till present that have extensively documented such effect; hence, this is the first study to examine the activities of *Phyllanthus amarus* extract on various organs in one study as an entity. The present study, therefore, investigated 200mg and 400mg extract/kgbwt/day in fructose-fed and medium-dose streptozotocin-diabetic male Wistar rats.

1.8. Significance of the study

The results of the current study are going to tremendously benefit further research initiatives, traditional medicine, and the pharmaceutical industry. *Phyllanthus amarus* extract potentially provides an improvement in the antioxidant and oxidative stress status in the diabetic rats' liver and kidney (Saad et al., 2017). It also possesses the potential to ameliorate cardiovascular risks in severe hyperglycaemia. Besides, the study provided a piece of empirical information on the beneficial or toxicity of the extract on male reproductive function. We have published outputs from the research and presented some at both local and international conferences. Manuscripts were also submitted in peer review, high impact factor journals for publication.

1.9. Aim and specific objectives

Aim: The research investigated possible mechanisms of the antihyperglycaemic effect of Phyllanthus amarus and evaluated its potential to attenuate renal, hepatic, cardiac and reproductive complications caused by diabetes mellitus.

Objectives of the present study include:

- To screen Phyllanthus amarus leaf extract for its phytochemical composition.
- To assess the antioxidant and antidiabetic activities of the extract.
- To determine the impact of the extract on glycaemic indices, oxidative stress, hepatic with renal functions of diabetic and non-diabetic male Wistar rats.
- To examine the effects of PA extract on the cardiac function of diabetic and non-diabetic male Wistar rats.
- To evaluate the phytoestrogenic activity of the extract and assess its effects on the reproductive functions of diabetic and healthy control, male Wistar rats.
- To examine a possible positive or negative effects of the extract on the histology of specific organs.

To achieve the above aim, we divided this study into two parts, as follows:

Part A: In-vitro study that investigated the concentrations of the total polyphenol and other phytochemicals present in the plant extract and their antioxidant capacity. Also, we examined the inhibitory activity of the extract on tyrosinase, α-amylase and α-glucosidase enzymes to determine the mechanism of its depigmentation and antidiabetic actions. More so, we studied the influence of the extract on male reproductive functions using mouse Sertoli (TM4) cell line. Finally, the phytoestrogenic activity of the extract on breast cancer (MCF-7) cell line, was investigated using the E-Screen assay.

Part B: In-vivo study that investigated the effects of a graded dosage of 200mg/kgbwt and 400 mg/kgbwt per day of aqueous plant extract on the hepatic, renal, heart and testicular functions of male Wistar rats.

1.10. Research Questions

- What are the bioactive compounds in Phyllanthus amarus aqueous extracts?
- What are the concentrations of the total polyphenol and individual phytochemicals present in the plant extract?
- What effects does the extract have on male reproductive functions using mouse Sertoli (TM4) cell line? Does the extract exhibit phytoestrogenic activity, using breast cancer (MCF-7) cell line?
- What potential impact does this extract have on the antioxidant capacity of diabetic and non-diabetic male Wistar rats?
- What potential effects does the extract have on biochemical tests such as glycaemic, cardiac, renal, hepatic, and reproductive parameters of diabetic and non-diabetic male Wistar rats?
- What potential effects does the extract have on oxidative stress, inflammatory biomarkers in the heart, kidney, liver, and testes of diabetic and non-diabetic male Wistar rats?

1.11. Hypothesis

H_0: Phyllanthus amarus does not possess antidiabetic properties and will not ameliorate hyperglycaemia induced-tissue damage and oxidative stress created by diabetes on the hepatic, renal, cardiac, and reproductive functions of male Wistar rats.

H_1: Phyllanthus amarus possesses antidiabetic properties and will ameliorate hyperglycaemia induced-tissue damage and oxidative stress created by diabetes on the hepatic, renal, cardiac, and reproductive functions of male Wistar rats.

1.12. Ethical Considerations

The Health and Wellness Sciences Faculty Research Ethics Committee (REC), CPUT, Bellville campus, South Africa approved the ethical application for this study (approval no: CPUT/HW-REC 2016/A1) as well as the Animal Ethics Committee, the University of Ibadan, Nigeria where we carried out the animal study (approval no: UI-ACUREC/19/0017).

References

Aboua, G., Oguntibeju, O. & du Plessis, S.S. 2013. Can lifestyle factors of diabetes mellitus patients affect their fertility? In *Diabetes Mellitus - Insights and Perspectives*. Rijeka. InTech: 95–116.

Adedapo, A., Ofuegbe S. & Oguntibeju, O. 2014. The antidiabetic activities of the aqueous leaf extract of Phyllanthus amarus in some laboratory animals. In *Antioxidant-Antidiabetic Agents and Human Health*. Rijeka. InTech. 115–137.

Adefegha, S.A. & Oboh, G. 2012. In vitro inhibition activity of polyphenol-rich extracts from Syzygium aromaticum (L.) Merr. & Perry (Clove) buds against carbohydrate hydrolysing enzymes linked to type 2 diabetes and Fe2+-induced lipid peroxidation in rat pancreas. *Asian Pacific Journal of Tropical Biomedicine*, 2(10): 774–781.

Adeneye, A.A. 2012. The leaf and seed aqueous extract of Phyllanthus amarus improves insulin resistance diabetes in experimental animal studies. *Journal of Ethnopharmacology*, 144(3): 705–711

Agarwal, A., Virk, G., Ong, C. & du Plessis, S.S. 2014. Effect of oxidative stress on male reproduction. *The World Journal of Men's Health*, 32(1): 1–17.

Agbaje, I.M., Rogers, D.A., McVicar, C.M., McClure, N., Atkinson, A.B., Mallidis, C. & Lewis, S.E.M. 2007. Insulin dependant diabetes mellitus: Implications for male reproductive function. *Human Reproduction*, 22(7): 1871–1877.

Al-Oanzi, Z.H. 2019. Erectile dysfunction attenuation by naringenin in streptozotocin-induced diabetic rats. *Journal of Food Biochemistry*, 43(7): 1–12.

Alexandraki, K.I., Piperi, C., Ziakas, P.D., Apostolopoulos, N. V., Makrilakis, K., Syriou, V.,

Diamanti-Kandarakis, E., Kaltsas, G. & Kalofoutis, A. 2008. Cytokine secretion in long-standing diabetes mellitus type 1 and 2: Associations with low-grade systemic inflammation. *Journal of Clinical Immunology*, 28(4): 314–321.

Allen, D.A., Harwood, S., Varagunam, M., Raftery, M.J. & Yaqoob, M.M. 2003. High glucose-induced oxidative stress causes apoptosis in proximal tubular epithelial cells and is mediated by multiple caspases. *Journal of Federation of American Societies for Experimental Biology*, 17(8): 908–910.

Angothu, S., Lakshmi, S.M. & Kumar, A.S. 2010. A review of medicinal plants potential with antidiabetic activity. *International Journal of Pharmacy and Therapeutics*, 1(1): 15–22.

Bhasin, S., Enzlin, P., Coviello, A. & Basson, R. 2007. Sexual dysfunction in men and women with endocrine disorders. *Lancet*, 369(9561): 597–611.

Bide, P. & Padalkar, A. 2020. Survey on diabetes mellitus and incorporation of bog data, machine learning and IoT to mitigate it. In *6th International Conference on Advanced Computing & Communication Systems*. 1–10.

Biswas, S.K. 2016. Does the interdependence between oxidative stress and inflammation explain the antioxidant paradox? *Oxidative Medicine and Cellular Longevity*, 2016: 17–19.

Bonini, M.G., Dull, R.O. & Minshall, R.D. 2014. Systems Biology of Free Radicals and Antioxidants. *Berlin: Springer*, 1343-1363.

Brown, J.S., Wessells, H., Chancellor, M.B., Howards, S.S., Stamm, W.E., Stapleton, A.E., Steers, W.D., Eeden, S.K. Van Den & McVary, K.T. 2005. Urological complications of diabetes. *Diabetes Care*, 28(1): 177–185.

Castela, Â. & Costa, C. 2016. Molecular mechanisms associated with diabetic endothelial-erectile dysfunction. *Nature Reviews Urology*, 13(5): 266–274.

CDC. 2020. National Diabetes Statistics Report, *2020 Estimate of diabetes and its burden in the United States*. 1-30.

Chopade, A.R. & Sayyad, F.J. 2015. Pain modulation by lignans (Phyllanthin and Hypophyllanthin) and Tannin (Corilagin) rich extracts of Phyllanthus amarus in carrageenan-induced thermal and mechanical chronic muscle hyperalgesia. *Phytotherapy Research*, 29(8): 1202–1210.

Choy, S.Y., Prasad, K.M.N., Wu, T.Y., Raghunandan, M.E. & Ramanan, R.N. 2014. The utilisation of plant-based natural coagulants as future alternatives towards sustainable

water clarification. *Journal of Environmental Sciences*, 26(11): 2178–2189.

Collins, F.M. 2002. *Current treatment approaches to type 2 diabetes mellitus: Successes and shortcomings*. *The American Journal of Managed Care*. 8(16): S460-S471.

Conrado, G.G., Grazzia, N., de Oliveira, A. da S.S., Franco, C.H., Moraes, C.B., Gadelha, F.R., Miguel, D.C. & Garcia, V.L. 2020. Prospecting and identifying Phyllanthus amarus Lignans with Antileishmanial and Antitrypanosomal Activity. *Planta Medica*, 10(1055): 1179–1003.

Davison, G.W. 2016. Exercise and Oxidative Damage in Nucleoid DNA Quantified Using Single Cell Gel Electrophoresis: Present and Future Application. *Human Fertility*, 18(3): 184–193.

Demirseren, D.D., Emre, S., Akoglu, G., Arpacı, D., Arman, A., Metin, A. & Cakır, B. 2013. Relationship between skin diseases and extracutaneous complications of diabetes mellitus: Clinical analysis of 750 patients. *American Journal of Clinical Dermatology*, 15(1): 65–70.

Esposito K, Nappo F, Marfella R, Giugliano G, Giugliano F & Ciotola M. 2002. Inflammatory cytokine concentrations are acutely increased by hyperglycaemia in humans. Role of oxidative stress. *Circulation*. 106:2062-2072.

Fernández-Miró, M., Chillarón, J.J. & Pedro-Botet, J. 2016. Testosterone deficiency, metabolic syndrome and diabetes mellitus. *Medicina Clínica*, 146(2): 69–73.

Ferrannini, E. 1998. Insulin resistance versus insulin deficiency in non-insulin-dependent diabetes mellitus: Problems and prospects. *Endocrine Reviews*, 19(4): 477–490.

Foo, L.Y. 1993. Amariin, a di-dehydrohexahydroxydiphenoyl hydrolysable tannin from Phyllanthus amarus, 33(2): 487–491.

Furukawa, S., Sakai, T., Niiya, T., Miyaoka, H., Miyake, T., Yamamoto, S., Maruyama, K., Ueda, T., Senba, H., Todo, Y., Torisu, M., Minami, H., Onji, M., Tanigawa, T., Matsuura, B., Hiasa, Y. & Miyake, Y. 2017. Diabetic peripheral neuropathy and prevalence of erectile dysfunction in Japanese patients aged <65 years with type 2 diabetes mellitus: The Dogo Study. *International Journal of Impotence Research*, 29(1): 30–34.

Grover, J.K., Yadav, S. & Vats, V. 2002. Medicinal plants of India with antidiabetic potential. *Journal of Ethnopharmacology*, 81(1): 81–100.

Harrison, S.A. 2006. Liver disease in patients with diabetes mellitus. *Journal of Clinical Gastroenterology*, 40(1): 68–76.

Houghton, P.J., Woldemariam, T.Z., O'Shea, S. & Thyagarajan, S.P. 1996. Two securinega-type

alkaloids from Phyllanthus amarus. *Phytochemistry*, 43(3): 715–717.

Huang, C., Kim, Y., Caramori, M.L.A., Fish, A.J., Rich, S.S., Miller, M.E., Russell, G. & Mauer, M. 2002. *Cellular basis of diabetic nephropathy II. The transforming growth factor-β system and diabetics nephropathy lesions in type 1 diabetes*. *Diabetes,* 51:3577-3581.

International Diabetes Federation. 2019a. *Complications in diabetes*. https://www.idf.org/aboutdiabetes/complications.html 20 July 2020.

International Diabetes Federation. 2019b. *IDF Diabetes Atlas Ninth edition 2019*. https://www.idf.org/e-library/epidemiology-research/diabetes-atlas/159-idf-diabetes-atlas-ninth-edition-2019.html. (Accessed: 05/03/2020).

Jangir, R. & Jain, G. 2014. Diabetes mellitus induced impairment of male reproductive functions: A review. *Current Diabetes Reviews*, 10(3): 147–157.

Jarald, E., Joshi, S.B. & Jain, D.C. 2008. Diabetes and herbal medicines. *Iranian Journal of Pharmacology and Therapeutics*, 7(1): 97–106.

Jones, T.H. 2007. Testosterone associations with erectile dysfunction, Diabetes, and Metabolic Syndrome. *European Urology, Supplements*, 6(16): 847–857.

Jones, T.H. 2010. Testosterone deficiency: A risk factor for cardiovascular disease? *Trends in Endocrinology and Metabolism*, 21(8): 496–503.

Joshi, S.C. & Jain, P.K. 2014. A review on the hypolipidaemic and antioxidant potential of some medicinal plants. *World Journal of Pharmacy and Pharmaceutical Sciences*, 3(11): 357–380.

Kachhawa, K., Agrawal, D., Rath, B. & Kumar, S. 2017. Association of lipid abnormalities and oxidative stress with diabetic nephropathy. *Journal of Integrated Nephrology and Andrology*, 4(1): 3–9.

Karalliedde, J. & Gnudi, L. 2016. Diabetes mellitus, a complex and heterogeneous disease, and the role of insulin resistance as a determinant of diabetic kidney disease. *Nephrology Dialysis Transplantation*, 31(2): 206–213.

Kassuya, C.A.L., Silvestre, A., Menezes-de-Lima, O., Marotta, D.M., Rehder, V.L.G. & Calixto, J.B. 2006. Antiinflammatory and antiallodynic actions of the lignan niranthin isolated from Phyllanthus amarus. Evidence for interaction with platelet-activating factor receptor. *European Journal of Pharmacology*, 546(1–3): 182–188.

Khodabandehloo, H., Gorgani-Firuzjaee, S., Panahi, G. & Meshkani, R. 2016. Molecular and cellular mechanisms linking inflammation to insulin resistance and β-cell dysfunction. *Translational Research*, 167(1): 228–256.

Kim, J.Y., Lee, S.H., Song, E.H., Park, Y.M., Lim, J.Y., Kim, D.J., Choi, K.H., Park, S.I., Gao, B. & Kim, W.H. 2009. A critical role of STAT1 in streptozotocin-induced diabetic liver injury in mice: Controlled by ATF3. *Cellular Signalling*, 21(12): 1758–1767.

Kumar, C., Kumar, R. & Nehar, S. 2014. Induction of type-II diabetes by high fructose diet and a low dose of intraperitoneal injection of streptozotocin in albino rats. *International Journal for Pharmaceutical Research Scholars*, 3: 196–202.

Kuo, P., Bellon, M., Wishart, J., Smout, A.J., Holloway, R.H., Fraser, R.J., Horowitz, M., Jones, K.L. and Rayner, C.K., 2010. Effects of metoclopramide on duodenal motility and flow events, glucose absorption, and incretin hormone release in response to intraduodenal glucose infusion. *American Journal of Physiology-Gastrointestinal and Liver Physiology*, *299*(6), G1326-G1333.

Kurhekar, J.V. 2017. Alkaloids - the healers in medicinal plants. *International Journal of Research in BioSciences*, 6(4): 33–39.

La Nasa, J.A. 2018. Obesity, Hypogonadism, and Male Fertility-The Role of Diet and Environment. In *Bioenvironmental Issues Affecting Men's Reproductive and Sexual Health*, 107–119.

Lee, Y.A., You, J.K., Eun, J.C. & Yokozawa, T. 2007. Ameliorative effects of proanthocyanidin on oxidative stress and inflammation in streptozotocin-induced diabetic rats. *Journal of Agricultural and Food Chemistry*, 55(23): 9395–9400.

Leite, D.F.P., Kassuya, C.A.L., Mazzuco, T.L., Silvestre, A., De Melo, L. V., Rehder, V.L.G., Rumjanek, V.M. & Calixto, J.B. 2006. The cytotoxic effect and the multidrug resistance reversing action of lignans from Phyllanthus amarus. *Planta Medica*, 72(15): 1353–1358.

Liu, C., Feng, X., Li, Qi, Wang, Y., Li, Qian & Hua, M. 2016. Adiponectin, TNF-α and inflammatory cytokines and risk of type 2 diabetes: A systematic review and meta-analysis. *Cytokine*, 86: 100–109.

Lotti, F. & Maggi, M. 2018. Sexual dysfunction and male infertility. *Nature Reviews Urology*, 15(5): 287–307.

Maciel, M.A.M., Cunha, A.F. & Dantas, T.N.C. 2007. NMR Characterization of Bioactive Lignans

from Phyllanthus amarus Schum &. *Annals of Magnetic resonance*, 6(3): 76–82.

Maghrani, M., Zeggwagh, N.A., Michel, J.B. & Eddouks, M. 2005. The antihypertensive effect of Lepidium sativum L. in spontaneously hypertensive rats. *Journal of Ethnopharmacology*, 100(1–2): 193–197.

Malviya, N., Jain, S. & Malviya, S. 2010. Antidiabetic potential of medicinal plants. *Acta Poloniae Pharmaceutica - Drug Research*, 67(2): 113–118.

Maritim, A.C., Sanders, R.A. & Watkins, J.B. 2003. Diabetes, oxidative stress, and antioxidants: A review. *Journal of Biochemical and Molecular Toxicology*, 17(1): 24–38.

Mazumder, A., Mahato, A. & Mazumder, R. 2006. The antimicrobial potentiality of Phyllanthus amarus against drug-resistant pathogens. *Natural Product Research*, 20(4): 323–326.

Mazzola, N. 2012. *Review of current and emerging therapies in type 2 diabetes mellitus. American Journal of Managed Care, 18(1): S17-S26.*

Mbogori, T. & Mucherah, W. 2019. Nutrition Transition in Africa: Consequences and Opportunities. *Global Journal of Transformative Education*, 1(1): 5–10.

Miao, X., Xiao, B., Shui, S., Yang, J., Huang, R. & Dong, J. 2018. Metabolomics analysis of serum reveals the effect of Danggui Buxue Tang on fatigued mice induced by exhausting physical exercise. *Journal of Pharmaceutical and Biomedical Analysis*, 151: 301–309.

Mohammed, A., Koorbanally, N.A. & Islam, M.S. 2016. Antidiabetic effect of Xylopia aethiopica (Dunal) A. Rich. (Annonaceae) fruit acetone fraction in a type 2 diabetes model of rats. *Journal of Ethnopharmacology*, 180: 131–139.

Mukherjee, D., Koushik Sen, Gupta, S., Chowdhury, P., Majumder, S. & Guha, P. 2020. Role of androgens in cardiovascular diseases in men: A comprehensive review. In *Chemistry and Biological Activity of Steroids*, 1–24.

Mukherjee, P.K., Maiti, K., Mukherjee, K. & Houghton, P.J. 2006. Leads from Indian medicinal plants with hypoglycemic potentials. *Journal of Ethnopharmacology*, 106(1): 1–28.

Nikolaidou, B., Nouris, C., Lazaridis, A., Sampanis, C. & Doumas, M. 2015. Diabetes Mellitus and Erectile Dysfunction. In *Erectile Dysfunction in Hypertension and Cardiovascular Disease*. Springer International Publishing: 119–128.

Nowicka-Bauer, K., Lepczynski, A., Ozgo, M., Kamieniczna, M., Fraczek, M., Stanski, L., Olszewska, M., Malcher, A., Skrzypczak, W. & Kurpisz, M.K. 2018. Sperm mitochondrial

dysfunction and oxidative stress as possible reasons for isolated asthenozoospermia. *Journal of Physiology and Pharmacology*, 69(3): 403–417.

Omotayo, O. 2012. Oxidative stress in diabetes mellitus: Is there a role for hypoglycemic drugs and/or antioxidants? In *Oxidative Stress and Diseases*, 217–246.

Okaiyeto, K., Nwodo, U.U., Mabinya, L. V. & Okoh, A.I. 2018. A review on some medicinal plants with hepatoprotective effects, 12(24): 168–199.

Patel, J.R., Tripathi, P., Sharma, V., Chauhan, N.S. & Dixit, V.K. 2011. Phyllanthus amarus: Ethnomedicinal uses, phytochemistry and pharmacology: A review. *Journal of Ethnopharmacology*, 138(2): 286–313.

Pereira, R.G., Garcia, V.L., Nova Rodrigues, M.V. & Martínez, J. 2016. Extraction of lignans from Phyllanthus amarus Schum. & Thonn using pressurised liquids and low-pressure methods. *Separation and Purification Technology*, 158: 204–211.

Rivero, A., Mora, C., Muros, M., García, J., Herrera, H. & Navarro-González, J.F. 2009. Pathogenic perspectives for the role of inflammation in diabetic nephropathy. *Clinical Science*, 116(6): 479–492.

Rovira-Llopis, S., Bañuls, C., de Marañon, A.M., Diaz-Morales, N., Jover, A., Garzon, S., Rocha, M., Victor, V.M. & Hernandez-Mijares, A. 2017. Low testosterone levels are related to oxidative stress, mitochondrial dysfunction and altered subclinical atherosclerotic markers in type 2 diabetic male patients. *Free Radical Biology and Medicine*, 108: 155–162.

Saad, B., Zaid, H., Shanak, S. & Kadan, S. 2017. Anti-diabetes and anti-obesity medicinal plants and phytochemicals: safety, efficacy and action mechanisms. 1-257. *Springer Cham*. DOI: 10.1007/978-3-319-54102-0

Samson, A.M. 2016. *In vivo antiplasmodial potential of combinations of three Nigerian antimalarial plants*. Ladoke Akintola University of Technology, Ogbomoso. 1-67.

Schons, K.R.R. 2018. Cutaneous Manifestations in Diabetes Mellitus. In *Dermatology in Public Health Environments*. Cham: Springer International Publishing: 719–738.

Srivastava, R. 2018. The lipid-lowering activity of some medicinal plants: A review of the literature. *Biomedical Journal of Scientific & Technical Research*, 9(1): 1–4.

Subramoniam, A. 2016. *Plants with anti-diabetes mellitus properties*. CRC Press. DOI: 10.1201/b19653

Tahrani, A.A., Barnett, A.H. & Bailey, C.J. 2016. Pharmacology and therapeutic implications of current drugs for type 2 diabetes mellitus. *Nature Reviews Endocrinology*, 12(10): 566–592.

Tahseen, M.A. & Mishra, G. 2013. Ethnobotany and diuretic activity of some selected Indian medicinal plants : A scientific review. *The Pharma Innovation*, 2(3): 109–121.

Thakur, P., Kumar, Ashwini & Kumar, Awanish. 2018. Targeting oxidative stress through antioxidants in diabetes mellitus. *Journal of Drug Targeting*, 26(9): 766–776.

Traish, A.M. 2014. Adverse health effects of testosterone deficiency (TD) in men. *Steroids*, 88: 106–116.

Tremellen, K. 2008. Oxidative stress and male infertility: A clinical perspective. In: *Studies on Men's Health and Fertility*. Totowa, NJ: Humana Press: 243–258.

Vanga, S.K. & Raghavan, V. 2018. How well do plant-based alternatives fare nutritionally compared to cow's milk? *Journal of Food Science and Technology*, 55(1): 10–20.

Verma, S., Sharma, H. & Garg, M. 2014. Phyllanthus amarus: A review. *Journal of Pharmacognosy and Phytochemistry*, 3(2): 18–22.

WHO. 1999. *Definition, diagnosis, and classification of diabetes mellitus and its complications*. Report of a WHO Consultation. 1-49.

Wilson, R.D. & Islam, M.S. 2012. *Fructose-fed streptozotocin-injected rat: An alternative model for type 2 diabetes*. *Pharmacological Reports*, 64,129-139.

Zhu, X., Huang, W. & Qi, H. 2013. GPR119 Agonists: A Novel Strategy for Type 2 Diabetes Treatment. In *Diabetes Mellitus - Insights and Perspectives*. Rijeka. InTech. 59–82.

CHAPTER TWO: LITERATURE REVIEW

Therapeutic potentials of medicinal plants of African origin for diabetes mellitus treatments and management

FOLORUNSO A. OLABIYI[1,3], YAPO G. ABOUA[2] & OLUWAFEMI O. OGUNTIBEJU[1].

[1]Phytomedicine and Phytochemistry Research Group, Oxidative Stress Research Centre, Department of Biomedical Science, Cape Peninsula University of Technology, Bellville Campus, South Africa. Mobile: +27616058403, Email: folabiyi@gmail.com.

[2]Medical Laboratory Science Programme, Department of Health Sciences, Namibia University of Science and Technology, Windhoek, Namibia. Mobile: +264816989127, Email: yaboua@nust.na

[3]Department of Medical Laboratory Science, Afe Babalola University, PMB 5454 Ado-Ekiti, Nigeria.

*Correspondence to Oluwafemi O. Oguntibeju; oguntibejuo@cput.ac.za, M: +27-846331187

NB: Manuscript from this chapter formed part of a book chapter published by *Apple Academic Press, Inc., the USA.*

*"**F. A Olabiyi**, Y.G Aboua, O.O Oguntibeju (2018): Therapeutic potentials of selected medicinal plants for the management of diabetes mellitus: In Innovation in Plant Science for Better Health: From soil to fork. Megh R. Goyah & Ademola O. Ayeleso (Eds). Bioactive compounds of Medicinal Plants: Properties and Potentials for Human Health. Apple Academic Press, Inc., the USA. Pp 187-218"*.

2.1. Introduction

From creation, there has been a wide array of plants that are for various purposes; some are for food, medication, and some gave oxygen that sustains life. Bigger plants or trees serve as windbreakers. In some instances, they might serve as erosion control, especially the legumes. The ornamental plants with flowers provide aesthetic structures to beautify an environment. The benefits of plants in health promotion and maintenance are gaining more attention, such that there is a thin line of demarcation between food and medicine. For example, *Allium sativum* (garlic) is a popular medicinal plant, often used to make food tastier. Besides, it is among the best natural antibiotics known in traditional medicine(Simonetti, 1990; Houshmand et al., 2013; Shakurfow et al., 2015; Hussein et al., 2017; Sharma et al., 2018). It is imperative to note that plants are essential and unique for their role in public health. Scientists have shown that edible plants with antioxidant properties, especially the *Brassicaceae* such as cabbage (*Brassica oleracea L*) or some Liliaceae, like garlic and onion, have the potential to prevent the appearance of some degenerative diseases like cancer or other diseases of the cardiovascular system (Rupp, 2011; Luo et al., 2019). Also, lycopene, a flavonoid that is abundant in tomatoes as well as watermelon, can prevent or retard the advancement of cancers (Khan et al., 2008; Kim et al., 2014; Ilahy et al., 2019). Likewise, apples (*Malus domestica* Borkh) and watermelon (*Cucumis melo*), because of their anti-inflammatory and antioxidant content guards the digestive system against colorectal cancer (Guaâdaoui et al., 2015). More so, oranges (*Citrus sinensis*), lemon (*Citrus limonum*) together with other citrus fruits are abundant in ascorbic acid (or vitamin

C) and pectin. They can protect the digestive system against diseases such as cancer (Mazed et al., 2011; Sharma et al., 2018).

People use herbal preparations to treat numerous diseases. For instance, in developed countries like the United States (U.S), about 37% of her population uses medicinal plants to manage specific disease condition (Farnsworth & Soejarto, 1991; Craft et al., 2015) and in the past, physicians prescribe medication that contains approximately 25% of plant-derived active ingredients. Besides, the number of appointments with providers of traditional medicine in the U.S is more than the number of meetings with all primary care physicians (WHO, 1999[a]; Kong et al., 2003; Brown, 2017). Similarly, in Pakistan, approximately 52% of respondents, attested to their using complementary and alternative medicine to treat their ailments (Shaikh et al., 2009). Also, in Japan, between 60% and 70% of allopathic doctors recommend indigenous medications to their clients. Moreover, in China, traditional and alternative medicine represents about 40% of all health care (WHO, 1999; WHO, 2002; Chen et al., 2018).

In recent time, there is a reawakening of attention in the field of herbal medicine or phytomedicine. The reason for this development might be due to better knowledge of plants or probably because of scientific and technological advancements. Modern techniques such as high-performance liquid chromatography (HPLC), gas chromatography (GC), thin-layer chromatography (TLC), atomic absorption spectrophotometry (AAS), X-ray crystallography and nuclear magnetic resonance spectroscopy (NMR) abounds to characterise biological extracts fully. Another probable reason is the increasingly adverse effects of orthodox medication, treatment failure rates and the exorbitant cost of the medicines that compel individuals to find alternative or complementary medicine. In this study, therefore, we aimed at examining some work previously done on selected medicinal plants, to clarify the importance of further evidence-based research on *Phyllanthus amarus* as our plant of interest to provide a piece of detailed scientific knowledge, mainly, on its antidiabetic, antihyperglycaemic and antioxidant potentials.

2.2. Diabetes Mellitus

Diabetes mellitus (DM) is a derangement in carbohydrate, protein as well as lipids metabolism resulting in excess blood glucose. The resultant glucose is underutilised, thereby leading to a condition called hyperglycaemia (Burtis et al., 2008; Kumar et al., 2016). Research indicates that if one fails to treat DM, affected individuals may suffer acute life-threatening hyperglycaemic states, such as ketone acidosis or unconsciousness due to the high osmolality. Failure to quickly

treat the disease predisposes the patient to specific complications, e.g. vision impairment that could lead to blindness, renal failure, nerve damage and atherosclerosis resulting in a stroke, gangrene, or coronary artery disease (Burtis et al., 2008). Notable evidence of diabetes mellitus includes bodyweight loss, polyphagia or (excess hunger), polydipsia (or excess) thirst and polyuria (or frequency of urination). Karuna et al. (2011) suggested that chronic hyperglycaemia results from diabetes mellitus due to defects in insulin secretion or action. On examining the seriousness of this disorder and its complications, cardiovascular disease (CVD) is the leading cause of the illness and death among people with diabetes with 65% of deaths traceable to heart disease or stroke (Lopez Hernandez, 2013). Myocardial infarction (MI) is a high-risk condition of the cardiovascular system due to diabetes mellitus. Possible mechanisms of action include insulin resistance, changes in endothelial functions, dyslipidaemia, chronic inflammation and release of mediators of inflammation, procoagulability and impaired fibrinolysis (Patel et al., 2016).

The derangements emanating from chronic, elongated hyperglycaemia, as shown by uncontrollable diabetes mellitus, can result in blood vessels and peripheral nerves damage; this may positively raise the possibility of a heart attack. Moreover, hyperlipidaemia, especially, cholesterol, triglycerides and high atherogenic index are important risk factors for cardiovascular diseases particularly among people with diabetes (Martín-Timón et al., 2014; Quispe et al., 2016; Reiner, 2017). A study shows that people with diabetes have an increased rate of atherosclerotic vascular disease (Paneni et al., 2013; Fetterman et al., 2016). Research suggests that endothelial injury may mark the aetiology of atherosclerosis with platelet adhesion and aggregation at the site of injury. Moreover, a report shows that among people with diabetes, evidence of endothelial dysfunction abounds. The authors concluded that smooth muscle cell proliferation plays a crucial role in the pathological discovery in atherosclerosis. Besides, the accumulation of lipids in the atherosclerotic lesion present as intracellular and extracellular esterified cholesterol (Kruth, 2001; Grebe & Latz, 2013; Aluganti Narasimhulu et al., 2016). Failure to control diabetes results in lipids deposition in large vessels because of elevated plasma low-density lipoprotein cholesterol (LDL) levels and decreased plasma high-density lipoprotein cholesterol (HDL) levels (Colwell et al., 1981; Linton et al., 2019). The condition described usually occur in some people with diabetes with thrombosis. The anomaly of endothelial, platelet, smooth muscle, lipoprotein, and coagulation characteristics may contribute to the problem of increased atherosclerosis among people with diabetes. Therefore, a vivid insight into what is responsible for this process to aid in designing appropriate preventive, therapeutic approach is highly imperative.

Furthermore, available records show that one of the complications called diabetic nephropathy (DN) is responsible for the disease and premature death seen in patients with insulin-dependent DM (Karnib & Ziyadeh, 2010; Ahmad, 2015; Keri et al., 2018). Rao et al. (2015) established DN as the leading cause of chronic kidney disease and end-stage renal failure globally. They reported that DN patients experienced more oxidative stress than T2D without DN, thus, concluding that elevated malondialdehyde levels and decreased glutathione peroxidase activity seen in DN are due to oxidative stress while high glycated haemoglobin (HbA1c) and microalbuminuria levels observed in DN are as a result of prolonged hyperglycaemia. Elevated total protein levels often seen in DN might be because of more protein catabolism, but their high urea and creatinine levels resulted from kidney dysfunctions.

2.3. Prevalence of Diabetes Mellitus

Globally, about 463 million people aged 20–79 years are currently diabetic. This figure represents 9.3% of the world's population with the disease. The number will predictably increase to 578 million (10.2%) by 2030 and to 700 million (10.9%) by 2045 (International Diabetes Federation, 2019). There is an astronomical progression when comparing these values to the global report published by the WHO on estimating the worldwide prevalence of diabetes to increase from 2.8% in 2000 to 4.4% by the year 2030 (Roglic et al., 2005). By implication, the global population of people with diabetes was forecasted to rise from 171 million in 2000 to 366 million in 2030 (Roglic et al., 2005; Whiting et al., 2011; Al-Shahrani et al., 2012; Bhat et al., 2016). An approximate prevalence of diabetes in adults has risen from 151 million in 2000 (4.6% of the global population at that time) to 463 million (9.3%) (IDF, 2019). Also, Shaw et al. (2010) projected that the number of adults affected (285 (6.4%) million adults in 2010) would rise to about 439 million (7.7%) by the year 2030. In contrast, some investigators estimated that the prevalence of diabetes in Africa is 1% in rural areas and this value ranges from 5% to 7% in urban sub-Saharan Africa (Dalal et al., 2011). Moreover, Nigeria, the most populous country in Africa (World Facts, 2018), accounting for 15.98% or one-sixth of the continent's population (World population prospects, 2012) have a population of about 195 million people. In Nigeria, approximately 82,869,000 adults (20-79 years) have diabetes, with a prevalence of 1.9% (International Diabetes Federation, 2015[a]). This prevalence represented more than 1.56 million adult cases of diabetes in Nigeria in 2015, but it is now 2,743,800 in 2020 with a prevalence of 3% (International Diabetes Federation, 2020[a]). The surge in the prevalence of diabetes mellitus among adults in Nigeria is suggestive of the gradual adoption of western lifestyles, urbanisation,

increasing ageing populations, more sedentary lifestyles, and unhealthy diets, including high sugar intakes, as this often leads to insulin resistance. On the other hand, South Africa, with an average population of 53 million and total adult (20-79 years) people of about 32,756,000 has diabetes prevalence of 7% (International Diabetes Federation, 2015[b]). The total number of cases of adults (20-79 years) that have diabetes in South Africa stood at 2,286,000 in 2015 which have now moved to 4,581,200 with a prevalence of 12.8% in 2020 (International Diabetes Federation, 2020[b]). DM is most prevalent among the Indian population in South Africa, ranging between 11% to 13%. The Indians are presumably having strong genetic tendencies for diabetes, followed by 8-10% among the coloured community, then between 5-8% among the blacks and about 4% among the white community (Distiller, 2014).

2.4. Pathological process of Diabetes

Damage to β-cells of the pancreas arising through cell-mediated immune destruction cause T1D (Kawasaki et al., 2004; Boldison & Wong, 2016; Zheng et al., 2017) the resultant effect of which is absolute insulin deficiency (Erejuwa, 2012). In most patients, the T-cells mediate the destruction with TD1 account for about 5-10% of all newly diagnosed diabetes mellitus (American Diabetes Association, 2011; Tremblay & Hamet, 2019). In contrast, insulin resistance (IR) and β-cell dysfunction are pathological defects in patients with T2D. IR is the inability of insulin or its reduced ability to act on the peripheral tissue, thereby preventing their glucose uptake. This process may be the primary underlying pathological process in diabetes. Again, β-cell dysfunction occurs when the pancreas is unable to secrete enough insulin to account for the insulin resistance. T2D is the most prevalent form of diabetes mellitus, constituting between 90% and 95% of all diabetic cases (American Diabetes Association, 2011; Murea et al., 2012; Sosale et al., 2014; Gheibi et al., 2017). Available evidence shows that insulin resistance is the primary defect that precedes the derangement in insulin secretion and clinical diabetes (Brown et al., 2016). Although the debate is still on, however, T2D is an extremely heterogeneous disorder with no available cause to explain the progression from typical glucose tolerance to diabetes (Brown et al., 2005; Karalliedde & Gnudi, 2016).

2.5. Diabetes mellitus and Oxidative Stress

Research showing complications connected to diabetes, also link oxidative stress, that is precipitated by the generation of free radicals (Matough et al., 2012; Khan et al., 2015; Yaribeygi

et al., 2019). These free radicals are reactive compounds, naturally generated by the body cells. They elicit positive effects on the immune system or adverse effects on lipids, proteins, or DNA oxidation (Sung et al., 2013). To minimise these deleterious effects, an organism requires complex protection known as the antioxidant system. This system consists of antioxidant enzymes such as catalase, glutathione peroxidase, superoxide dismutase and non-enzymatic antioxidants like vitamin E [tocopherol], vitamin A [retinol], vitamin C [ascorbic acid], glutathione and uric acid (Finaud et al., 2006; Ighodaro & Akinloye, 2018). When free radical production counterbalances antioxidant defences, it leads to a condition called oxidative stress (Sies et al., 2017). Oxidative stress could denote a disturbance in the pro-oxidant/antioxidant balance in favour of the former. Furthermore, oxidative stress ensues when the generation of reactive oxygen species (ROS) overwhelms the antioxidant defence mechanisms, thus, leading to cellular damage (Tremellen, 2008; Silva et al., 2010; Kandola et al., 2015; Fatima, 2018). The imbalance between these oxidants and antioxidants in favour of the oxidants that potentially lead to damage forms the basis of oxidative stress (Sies, 2000; Sies, 2015; Ighodaro & Akinloye, 2018). More so, ROS and reactive nitrogen species (RONS) are generated by several biochemical and cellular processes, with antioxidant defences there to neutralise their adverse effects, and oxidative stress is occurring from the imbalance between RONS production and antioxidant protection (Liguori et al., 2018). To better understand the pathological process, critical roles played by oxidative stress in diabetic complication was reported (Niedowicz & Daleke, 2005; Sagoo & Gnudi, 2018). Oxidative stress is thus, viewed as a recognized pathogenetic mechanism in diabetic complications. Research shows that hyperglycaemia is a widespread cause of enhanced free radical levels, whereas oxidative stress involvement in glycaemic regulation needs resolution (Ceriello, 2000). Furthermore, a scientific report indicates that hyperglycaemia induces oxidative stress among diabetic subjects (De Carvalho Vidigal et al., 2012; Rochette et al., 2014; Arif et al., 2018). It is important to note that during oxidative stress, oxidative phosphorylation produced ROS. In another study involving oxidative stress and myocardial injury in diabetic heart, the investigators pointed out that diabetes mellitus increases the risk of developing cardiovascular disease five-fold (Brown et al., 2006; Ansley & Wang, 2013).

2.6. Diabetes and Skin infections

Elevated blood glucose and derangements of carbohydrate and lipid metabolism with resultant long-term systemic complications characterise diabetes mellitus (Al-Mutairi, 2006;

Chattopadhyay et al., 2020). Scientific evidence indicates that the incidence of cutaneous disorders in diabetes varies between 30% and 71% according to different authors (Quatrano & Loechner, 2012). Research suggests that cutaneous manifestations of DM generally appear after the development of the disease but maybe the first presenting signs or may even precede the primary disease manifestations by some years in some people with diabetes. Research also suggests that DM tops the list of endocrine disorders, affecting about 8.3% of the US population (Centres for Disease Control, 2011). Besides, skin disorders among diabetic patients are quite common and diverse, and the condition was reportedly present in about 79.2% among people with diabetes in the U.S (Demirseren et al., 2014). Poor glycaemic control may account for the various skin diseases and extracutaneous complications observed in diabetes mellitus. Currently, the global burden of DM has increased to an epidemic proportion, and the most significant future increase is going to occur in Africa (Kibirige et al., 2019).

Moreover, a report shows that vitamin D deficiency, caused by malnutrition, chronic infections (e.g. TB, HIV) and dark skin pigmentation may explain the increased chances of developing DM unique to sub-Saharan Africa (Prentice et al., 2009; Kibirige et al., 2019). However, the pathogenesis of skin disorder observed in diabetes is still not clear. Some of the diseases include conditions associated with insulin resistance, e.g. *acanthosis Nigricans*, which is likely the most readily recognised skin manifestation of diabetes (Kalus et al., 2012). It is a hyperpigmented velvety thickening of skin folds, presenting predominantly in the neck, axilla, and groin areas. Eruptive xanthoma (EX) is another skin lesion presented among people with diabetes. EX is not common and occurs more often in patients with poorly controlled T2D. It occurs on the buttocks, elbows, and knees as sudden onset crops of yellow papules with an erythematous base (Paron & Lambert, 2000; Banerjee, 2017). Some cutaneous infections associated with diabetes include candidiasis, dermatophytosis, and bacterial infections (Duff et al., 2015).

2.7. Diabetes and Phytoestrogens

Phytoestrogens are polyphenols with similar structures to the endogenous oestrogen. As shown in Table 2.1 below, two main types of phytoestrogens are widely recognised; namely isoflavones and lignans (Kurzer & Xu, 1997; Dixon, 2004; Prakash & Gupta, 2011; Rishi, 2019). Animal

experimentation and pre-clinical models have strongly suggested that phytoestrogens may have antidiabetic properties via oestrogen-dependent and oestrogen-independent pathways (Talaei & An, 2015).

Proper nutrition is beneficial in the control of T2D (Avignon et al., 2012). Dated back to the prehistoric era, the incidence of T2D is relatively low in a traditional Asian community. Some workers hypothesised that a regular Asian diet, with high consumption of whole grains, various vegetables and fruits and small portions of meat products, contains lots of protective components against the development of T2D. Among others, soybean and soy products as the unique element of traditional Asian diet have generated much interest due to considerable difference in its consumption compared to a Western diet (Kwon et al., 2010). Although there are several potential beneficial compounds such as soy protein, dietary fibre, monounsaturated and polyunsaturated fat, vitamins and minerals in soybean and soy products, one group of polyphenols abundant in soy products, isoflavones, is suggestive for diabetes prevention and management (Bhathena & Velasquez, 2002; Kwon et al., 2010).

Table 2.1: Chemical classification of the most widely investigated phytoestrogens and their major dietary sources (Rice & Whitehead, 2006)

Flavones	**Flavanones**	**Isoflavones**	**Coumestans**	**Lignans[a]**	**Stilbenes**
Apigenin	flavanone	Geinstein	Coumestrol	Enterolactone	Resveratrol
Quercetin	Naringenin	Biochanin A	Enterodiol		
Chrysin	8-Prenyl-naringenin	Diadzein	7-Hydroxyflavone		Formononetin
Mostly red/yellow fruits and vegetables	Citrus fruits, hops	Legumes, soybean and clover	Bean shoot, alfalfa clover	Most cereal, fruits and vegetables	Grape skins, red wine seed ---

[a]Enterolactone and entero diol are metabolized in the gut from the plant lignans secoldolariciresinol and matairesinol

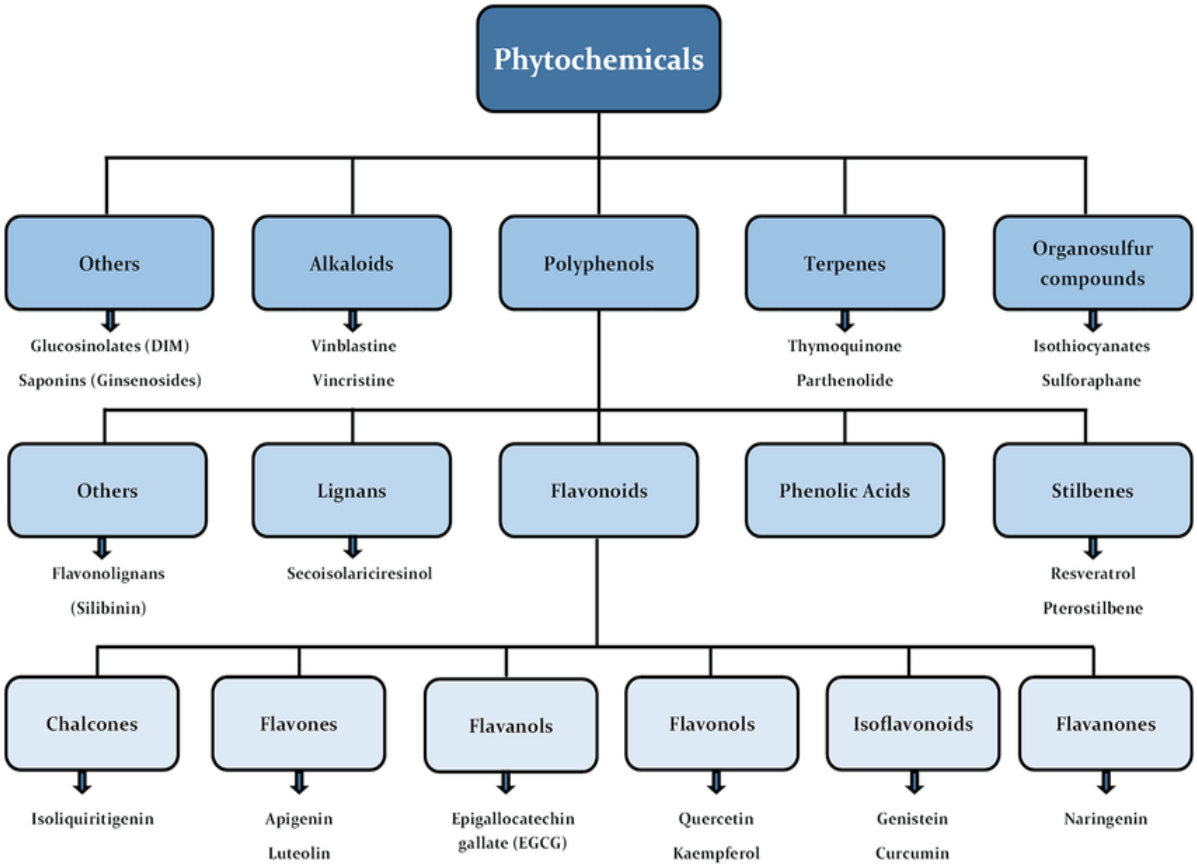


Figure 2.1: Classification of Phytochemicals

Adapted from Classification of Phytochemicals involved in breast cancer chemoprevention (Younas et al., 2018) Available via licence: Creative Commons by Non-Commercial 3.0

2.8. Some Common African Medicinal Plants with Antidiabetic activities

The tables (2.2 – 2.6) below summarise some selected African medicinal plants scientifically proven for their ability to demonstrate antidiabetic activity using an animal model as well as humans based on their origin from various parts of the continent (Mohammed et al., 2014). However, for the present study, our focus is on *Phyllanthus amarus*.

Table 2.2: Selected Medicinal plants of West African origin with Antidiabetic activities

No	Scientific name	Common name	Parts studied	References

1	Allium cepa	Onion	Bulb	(Eyo et al., 2011; Ozougwu, 2011)
	Allium sativum		Bulb	(Eyo et al., 2011; Ojo et al., 2012)
2.	Anacardium occidental	Cashew	Leaf	(Fagbohun & Odufunwa, 2010; Ukwenya et al., 2012)
3.	Azadirachta indica	Neem	Leaf	(Koffuor et al., 2011; Akpan et al., 2012)
4	Carum carvi	Caraway	Fruit	(Ene et al., 2006; Ene et al., 2007)
5.	Gongronema latifolium	Amaranth globe, Utazi	Root/stem	(Nwanjo et al., 2006; Orok et al., 2012; Adebajo et al., 2013)
6.	Hibiscus sabdariffa L.	Red sorrel	Calyces	(Sini et al., 2011; Ademiluyi & Oboh, 2013)
7.	Indigofera pulchra L.	Indigofera	Leaf	(Tanko et al., 2009)
8.	Moringa oleifera	Horseradish	Leaf	(Edoga et al., 2013)
9.	Nauclea latifolia	Bishop's head	Root/stem/ Leaf	(Effiong et al, 2013; Yessoufou et al., 2013)
10.	Ocimum gratissimum	African/clove basil	Leaf	(Egesie et al., 2006; Morakinyo et al., 2011; Onaolapo et al., 2011)
11	Parkia biglobosa jacq	African locust bean	Seed	(Odetola et al., 2006)
12	Phyllanthus amarus	Stonebreaker/gulf leaf flower	Root/stem/ Leaf	(Lawson-Evi et al., 2011; Mbagwu et al., 2011; Owolabi et al., 2011; Adeneye, 2012)
13	Picralima nitida	Picralima	Pulp/seed	(Aguwa et al., 2001; Igboasoiyi et al., 2007)
14.	Vernonia amygdalina	Bitter leaf	Leaf	(Akpaso et al., 2011; Atangwho et al., 2012; Modu et al., 2013)
16.	Zingiber officinale	Ginger	Rhizome	(Morakinyo et al., 2011; Arikawe et al., 2013)
17.	Telfairia occidentalis Hook f.	Fluted pumpkin	Seed	(Eseyin et al., 2007)

Table 2.3: Selected Medicinal plants of North African origin with Antidiabetic activities

No	Scientific name	Common name	Parts studied	References
1.	Ajuga iva L.	Herb ivy	Whole plant	(El-Hilaly et al., 2006; Hamden et al., 2008)
2.	Allium cepa	Onion	Bulb	(El-Soud & Khalil, 2010; Eldin et al., 2010)
3.	Balanites aegyptiaca	Desert date /Hegleg	Fruits	(Zaahkouk et al., 2003)
4.	Carum carvi	Caraway	Fruit/oil	(Eddouks et al., 2004; Lemhadri et al., 2006; Dawidar et al., 2010)
5.	Chamaemelum nobile	Chamomile	Aerial parts	(Lemhadri et al., 2007)
6.	Curcumin longa L.	Curcuma	Root	(Mohamed et al., 2009)
7.	Morus alba	White mulberry	Leaf/Root/bark	(El-Beshbishy et al., 2006; El-Sayyad et al., 2011)
8.	Nigella sativa	Black seed	Seed	(Mohamed et al., 2009; Benhaddou-Andaloussi et al., 2011)
9.	Lepidium sativum L.	Peppergrass	Stem	(Eddouks et al., 2005)
10.	Ziziphus spira Christi	Christ's Thorn Jujube	Leaf	(M.Hussein et al., 2006; Michel et al., 2011)

Table 2.4: Selected Medicinal plants of Southern African origin with Antidiabetic activities

No	Scientific name	Common name	Parts studied	References

1.	Aloe ferox Mill.	Bitter aloe	Leaf	(Loots et al., 2011)
2.	Artemisia afra jacq	African wormwood	Leaf	(Afolayan & Sunmonu, 2011; Afolayan & Sunmonu, 2013)
3.	Momordica charantia L.	Bitter melon	Whole plant	(Dzeufient et al., 2007)
4.	Bryophyllum pinnatum Lam.	Good luck/ life plant	Leaf	(Ojewole, 2005)
5.	Camellia sinensis L.	White tea		(Islam, 2011)
6.	Catharanthus roseus L.G Don	Madagascar periwinkle	Leaf	(Ojewole & Adewunmi, 2000)
7.	Hypoxis hemerocallidea	African potato	Corm	(Mahomed & Ojewole, 2003; Ojewole, 2006)
8.	Raphia gentiliana De wild		Fruit	(Mpiana et al., 2013)
9.	Sclerocarya birrea A. Rich Hochst	Jelly plum	Stem/bark	(Musabayane et al., 2006; Gondwe et al., 2008)
10.	Sutherlandia frutescens R.	Cancer brush	Leaf	(Chadwick et al., 2007; MacKenzie et al., 2009)

Table 2.5: Selected Medicinal plants of Central African origin with Antidiabetic activities

No	Scientific name	Common name	Parts studied	References
1.	Anacardium occidentale L.	Cashew	Leaf	(Sokeng et al., 2007)
2.	Bersama agleriana	Winged bersama	Leaf	(Nyah Njike et al., 2005; Pierre et al., 2012)
3.	Citrullus lanatus Thunb.	Watermelon	Seed	(Teugwa et al., 2013)
4.	Cucurbita moschata	Butternut squash	Pods	(Teugwa et al., 2013)

	Duchesne ex Poir.			
5.	Dichrostechys glomerata chiov	Chinese lantern	Seed	(Kuate et al., 2011)
6.	Dracena arborea Wild	Dragon tree	Root	(Wankeu-Nya et al., 2013)
7.	Kalanchoe crenata Andr Haw	Never ride	Whole plant	(Kamgang et al., 2008)
8.	Lagenaria siceraria L.	Bottle gourd	Seed	(Teugwa et al., 2013)
9.	Sclerocarya birrea A. Rich. Hochst.	Jelly plum	Stem bark	(Dimo et al., 2007; Makom et al., 2010)
10.	Telfairia occidentalis Hook. f.	Fluted pumpkin	Seed	(Loots et al., 2011)

Table 2.6: Selected Medicinal plants of East African origin with Antidiabetic activities

No	Scientific name	Common name	Parts studied	References
1.	Aspilla pluriseta schweinf	Dwarf aspilia	Root	(Piero et al., 2011)
2.	Bidens pilosa L.	Spanish needle	Leaf	(Piero et al., 2011)
3.	Catha edulis vahl	Bushman's tea	Root	(Piero et al., 2011)
4.	Erythrina abyssinica Lam.	Red-hot poker	Stem bark	(Piero et al., 2011)
5.	Ficus sycomorus L.	Fig-mulberry	Stem bark	(Njagi et al., 2012)
6.	Moringa stenopetala Baker F.	Cabbage tree	Leaf	(Nardos et al., 2011)
7.	Pappea capensis Eckl. & Zeyh.	Jacket plum	Leaf/ Stem bark	(Karau et al., 2012)
8.	Strychnos henningsii Gilg.	Red bitter berry	Leaf	(Piero et al., 2011)

2.8.1. Origin of Phyllanthus amarus plant

Phyllanthus amarus (PA) is a medicinal plant of traditional Hindu alternative medicine in southern India; it is also available in the Philippines, Cuba (Verma et al., 2014) and some parts of West African countries such as Nigeria (Adeneye, 2012) and Togo (Lawson-Evi et al., 2011). In Nigeria, *Phyllanthus amarus* is called, ‘’dobisowo’’, ‘’ehin olobe’’ or ‘’ehin olubisowo’’ among the Yoruba tribe, and ‘’ngwu’’ by the Igbo tribe and ‘’Buchi oro’’ by the Asaba people (Samson, 2016). The plant is one of the Euphorbiaceae popularly used traditionally to manage several diseases. It is known as carry-me seed or stone breaker.

Figure 2.2: *Phyllanthus amarus* plant

2.8.1.1. Ethnobotanical and Traditional Uses of *Phyllanthus amarus*

''Phyllanthus amarus is traditionally useful to treat medical conditions such as diarrhoea, dysentery, dropsy, jaundice, intermittent fevers, urinogenital disorders and skin disorders such as wounds, scabies, sores, ulcers, itching, oedema, ringworms, and tubercular ulcers, scabby and crusty lesions'' (Verma et al., 2014). Its efficacy on the urinogenital system is assumed to be because of its antiurolithic property meant to treat ''renal stone, gallstone, other kidney-related problems, appendicitis and prostate problems'' (Khatoon et al., 2006; Sen & Batra, 2013; Ushie et al., 2013). Due to its positive effect to treat gastrointestinal disorders, it is employed to treat dysfunctions such as dyspepsia, colic, diarrhoea, constipation, and dysentery. Furthermore, people use this herb for lots of gynaecological problems like leucorrhoea, menorrhagia and mammary abscess (Verma et al., 2014). In south-western Nigeria, local people prepare the leaf and seed extract of PA as water decoction for the local management of DM, obesity as well as hyperlipidaemia (Adeneye, 2012).

2.8.1.2. Biological and Pharmacological Properties of *Phyllanthus amarus*

The activities listed below establish the therapeutic values of *Phyllanthus amarus* herb.

2.8.1.2.1. Anticancer activity

In vitro and in vivo anticancer potentials (EL-Garawani et al., 2020) of hypophyllanthin and phyllanthin isolated from *Phyllanthus amarus* against breast cancer was investigated. The researchers tested the in vitro anticancer action of the plant against two cell lines (MCF-7 and MDA-MB-231) using colourimetric MTT assay technique. On the other hand, the study used Sprague-Dawley rats with N-methyl-N-nitrosourea to induce mammary cancer to evaluate the In vivo anticancer activity. The study revealed a dose-dependent inhibition on the cell lines, as evident in the calculated half-maxima (IC_{50}) values. The weight of the tumours in the hypophyllanthin and phyllanthin-treated groups was significantly lower ($p < 0.001$) compared to the untreated N-methyl-N-nitrosourea-induced cancer group. Findings from this investigation indicated that the isolated lignan compounds, hypophyllanthin and phyllanthin demonstrated significant anticancer activities against breast cancer, both in vitro and in vivo (Parvathaneni et al., 2014). Another study investigated the physicochemical properties, antioxidant and cytotoxic

activities of crude extracts and fractions from *Phyllanthus amarus* using spectrophotometric method. The outcome from the survey indicate that *Phyllanthus amarus* methanolic (MEOH) extract had lower residual moisture (7.40%) and water (0.24%) contents. There was a higher amount of saponins, phenolics, flavonoids, and proanthocyanidins than those of the *Phyllanthus amarus* aqueous (PAQ) extract. The antioxidant activity of MEOH extract was significantly higher ($p < 0.05$) than those of the PAQ extract, MEOH fractions and phyllanthin. The investigators also observed higher cytotoxic activity of MEOH extract using MTT colourimetric assay on selected cell lines in comparison to the PAQ extract and MEOH fractions. There was a significantly lower ($p < 0.05$) cytotoxic potential of the PAQ extract than those of 50 nM gemcitabine and a saponin-enriched extract from quillajia bark but was markedly higher than that of phyllanthin at 2µg/mL. Findings from this study suggest that the *Phyllanthus amarus* extract is a potential source for the development of natural antioxidant products and could serve as a novel anticancer drug (Nguyen et al., 2017).

2.8.1.2.2. Antioxidative potential

The antioxidant property of 70% aqueous: ethanol extract of *Phyllanthus amarus* roots and its ether-soluble, ethyl acetate-soluble, and aqueous fractions were investigated by various in vitro assays. The root extracts showed higher 2,2-diphenyl-1-picrylhydrazyl (DPPH), hydroxyl, superoxide, and nitric oxide radical scavenging and reducing power activity. Among all the samples examined, the ethyl acetate-soluble fraction demonstrated the highest radical scavenging activity and total phenolics content. LCMS/MS analysis of the ethyl acetate-soluble fraction identified twenty- eight different phenolic compounds. Most of the compounds exist as their glycosides, and many of these compounds were gallic acid derivatives. The analytical method also identified free epicatechin and gallic acid in the ethyl acetate-soluble fraction. The study suggested that *Phyllanthus amarus* root is a potent antioxidant and could serve for the prevention of diseases related to oxidative stress (Maity et al., 2013).

Another study evaluated the effects of *Phyllanthus amarus* (PA) on oxidative stress damage, inflammation, together with soreness in muscle after a moderate-intensity exercise (Roengrit et al., 2015). In this study, twelve men randomly took part in two of a three-day cycle with a one-week washout period. At the commencement day, participants consumed two capsules of PA or

placebo control (CONTL) before 20 minutes of cycling. Followed, they consumed four tablets on the same day after exercise and six capsules per day for the next two days. Blood samples were collected before, immediately after exercise, 24 hours, and 48 hours post-exercise. The researchers measured pain tolerance at both legs. Findings show that plasma ascorbic acid levels (Vit. C) in the PA group were higher than those in the CONTL group post-exercise. At 48 hours after exercise, Vit. C levels increased in the PA group, but the CONTL group were lower than the pre-exercise levels. However, plasma levels of creatine kinase (CK) were increased in both groups post-exercise compared to that before the exercise. The neutrophil count increased immediately after exercise than the pre-exercise levels in the CONTL group. They concluded that acute supplementation with PA improves antioxidant status after a single session of moderate-intensity training (Roengrit et al., 2015).

2.8.1.2.3. Actions on Kidney Stones & Uric Acid

A study to investigate the antilithic effect of orthosiphon grandiflorus, Hibiscus sabdariffa and *Phyllanthus amarus* extracts on known risk factors of calcium oxalate stones in rats was conducted. In this study, thirty (30) male Wistar rats were divided into five (5) groups. Controls were fed with standard diet while the remaining groups received a 3% glycolate diet for four (4) weeks to induce hyperoxaluria. One glycolate group served as the untreated group while they administered others with oral extract of Orthosiphon grandiflorus, Hibiscus sabdariffa and *Phyllanthus amarus* (Woottisin et al., 2011) at a dose of 3.5 mg daily. 24-hrs' urine and blood samples were collected. They harvested the Kidneys for histology. They also measured calcium and oxalate renal tissue content. The group fed with Hibiscus sabdariffa showed significantly decreased serum oxalate and glycolate, and higher oxalate urinary excretion. However, the *Phyllanthus amarus* group showed increased considerably urinary citrate compared to the untreated group. Histology revealed less calcium oxalate crystal deposition in the kidneys of Hibiscus sabdariffa and *Phyllanthus amarus* treated rats than in the untreated rats. Renal tissue calcium content in the rats also was significantly lower than untreated rats. The parameters in the Orthosiphon grandiflorus treated group were comparable to those in the untreated group. Hibiscus sabdariffa and *Phyllanthus amarus* decreased kidney calcium crystal deposition. The

authors concluded that the antilithic effect of Hibiscus sabdariffa might, therefore, be associated with lower oxalate retention in the kidney and higher excretion into urine while that of *Phyllanthus amarus* may depend on increased urinary citrate. On the contrary, administering Orthosiphon grandiflorus had no antilithic (Woottisin et al., 2011).

2.8.1.2.4. Antinociceptive Activity

A randomised crossover (double-blind) experiment to investigate the antioxidant and antinociceptive effects of Phyllanthus amarus on improving exercise recovery in sedentary men was done. Twelve (12) men participated in a two -3-day phases separated by a 1-week washout. On the first day of the experiment, randomly divided participants ingested two capsules of either PA (PA group) or placebo (PL group) 20 minutes before a single bout of cycling at high intensity for 20 minutes followed by four capsules (two capsules after lunch and dinner), and six capsules/day for the next two days. They collected blood samples before, immediately after, 24 and 48 hours after the exercise. They also measured pain threshold at the mid-thigh on both legs. Results indicate that malondialdehyde concentration in the PA group was lower than that in the PL group ($p < 0.05$) 48 hours after high-intensity exercise. Ascorbic acid (Vit. C) concentration was higher in the PA than in the PL group ($p < 0.05$) immediately after high-intensity exercise. Threshold pain in both legs in the PA group was higher than in the PL group 24 and 48 hours after a high-intensity workout. The study found no significant differences in creatine kinase, leukocyte counts or inflammation between groups. The authors concluded that acute PA supplementation reduced oxidative stress and muscle soreness induced by high-intensity exercise (Roengrit et al., 2014).

2.8.1.2.5. Antimicrobial activity

A bacterial infection is responsible for many anomalies in human health globally, leading to high mortality in low-income countries. On the other hand, diabetes caused by excess glucose in the

blood (hyperglycaemia) remains a problem in Africa, resulting in partial paralysis or stroke as this often triggers blood pressure. A study evaluated the antibacterial and in vitro antidiabetic activities of Phyllanthus amarus. Antibacterial activity on bacterial strains such as Staphylococcus aureus, Pseudomonas aeruginosa, Campylobacter jejuni, Streptococcus pyogenes, Bacillus subtilis, Salmonella typhi, Klebsiella pneumonia (clinical isolate) and Vancomycin-resistant enterococci (VRE). They carried out the antibacterial activity by agar well diffusion while susceptibility test was performed by Kirby Bauer's et al. method. The investigators further subjected the leaf extract to the inhibitory effect of glucose utilisation. Results indicate that most of the microbes showed significant diameter zones of inhibition against the extract at various concentrations. The minimum inhibitory concentration (MIC) and minimum bacterial concentration (MBC) further confirmed the potency of the aqueous extract on selected bacteria. In vitro antidiabetic evaluation of leaf extract showed a concentration-dependent activity. The authors concluded that the aqueous leaf extract of Phyllanthus amarus possesses a significant antibacterial and inhibitory effect on glucose diffusion in vitro thus confirming its traditional claim in treating bacterial infection and diabetes (Ukwubile & Odugu, 2018).

2.8.1.2.6. Antileptospiral Activity

A study attempted to use herbal medicine to cure leptospirosis either by direct killing or by inhibiting the growth of leptospira. In this study, researchers used a methanolic and aqueous extract of whole plants of Eclipta alba and *Phyllanthus amarus* to assess the mechanism(s) of its antileptospiral and antioxidant activity. The study evaluated the antioxidant activity and radical scavenging activity of methanolic and aqueous extracts of selected plant materials against 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical and 2,2'azinobis-(3-ethylbenzthiazoline- 6-sulfonic acid) (ABTS) activity. The study also investigated the DNA damaging effect on leptospira. Finally, extracts were tested at different concentration in their respective solvent systems by Tube Dilution Technique (TDT) and Micro Dilution Technique (MDT) tests. Both tube dilution again evaluated the antileptospiral activity of Eclipta alba and P. amarus, and microdilution technique. The results showed a better inhibitory action against various serogroups of

Leptospira. The study compared the DPPH free radical scavenging effect of the extract with standard antioxidant ascorbic acid. IC_{50} values were 75 µg/ml for extract, and 45 µg/ml for ascorbic acid and the methanol extracts of both plants had good activity, and IC_{50} values were 55 µg/ml and 30 µg/ml in ABTS assay. The plant extracts were found to be very effective against leptospirosis, hence, their possible use to control Leptospirosis (Chandan et al., 2012)

2.8.1.2.7. Anticonvulsant Activity

A study investigated the *Phyllanthus amarus* anticonvulsant effect on maximal electroshock-induced seizures (MES) and pentylenetetrazole (PTZ)-induced seizures. The study evaluated the aqueous and ethanolic leaves and stems extracts of *Phyllanthus amarus* (70 mg/kg body weight) for their anticonvulsant effect on MES and PTZ-induced seizures in Swiss albino rats. The study reported the latency of tonic convulsions and the number of animals protected from tonic convulsions. Findings also revealed that the aqueous and ethanolic extracts of the leaves and stems of P. amarus (70 mg/kg body weight) significantly ($p < 0.001$) abolished the hind limb extension induced by MES. The same dose also considerably ($p < 0.001$) protected the animals from PTZ- induced tonic convulsions. The data suggest that the aqueous and ethanolic extracts of *Phyllanthus amarus* may produce its anticonvulsant effects via non-specific mechanisms since it abolished the hind limb extension induced by MES as well as delayed the latency of seizures made by PTZ. (Manikkoth et al., 2011).

2.8.1.2.8. Antidiabetic Activity

To test the antidiabetic action of *Phyllanthus amarus* (PA), a study investigated the aqueous and hydroalcoholic extract of the plant. Fasted rats (12 hours) were made diabetic by a single intraperitoneal injection of 120 mg per kg of alloxan monohydrate. Two doses (500 and 1000 mg per kg body weight) extracts of *Phyllanthus amarus* were administered orally to the diabetic rats. The untreated healthy control group receives distilled water only. After fifteen days' treatment, the body weight gain, blood glucose level, serum insulin, total cholesterol, triglycerides and malondialdehyde were evaluated. At the doses tested, the aqueous and hydroalcoholic extract

of *Phyllanthus amarus* significantly lowered blood glucose level after 15 days of administration. The aqueous extract reduced body weight gain compared to the hydroalcoholic extract. Contrarily, the hydroalcoholic extract reduced the serum malondialdehyde level. However, there was an increased serum insulin level in a group treated with extracts compared to the diabetic control group. In all the groups, the serum total cholesterol and triglycerides are similar. This study demonstrated the potential antidiabetic property of aqueous and hydroalcoholic extract of *Phyllanthus amarus*, thus justifying its traditional use (Lawson-Evi et al., 2011). A similar study investigated an oral antihyperglycemic action of ethanolic extract of PA at 150-600 mg/kg/day in untreated controls and 10% sucrose–induced insulin-resistant rats by estimating fasting blood glucose, insulin and Insulin resistance indices(Adeneye, 2012). The study measured body weight, serum lipids and atherogenic index. Results revealed that PA could effectively control glucose level in diabetes mellitus via improvement in insulin resistance, thus confirming the ethnomedicinal use of the herb in the local treatment of diabetes mellitus. A study also looked at the antidiabetic activities of soft drink extract (SDExt) of the plant in rats. The study used standard phytochemical assays to test for the phytoactive compounds. The investigators conducted acute toxicity in mice to determine safe doses for this plant extract and to assess the antidiabetic activities of the SDExt of the plant. They induced diabetes mellitus in rats using alloxan, whereas glibenclamide at 0.2mg/kg served as a standard drug in this study. The results show that the doses of extract used in the study caused a significant reduction in blood glucose concentration, cholesterol, and triglyceride levels of diabetic rats. The histopathology of the liver of the diabetic non-treated and glibenclamide-treated groups revealed a widespread vacuolar change in the hepatocytes with no visible lesion seen in the kidney and pancreas of extract-treated and glibenclamide-treated groups. There was no lesion in the liver of the SDExt-treated group. The authors concluded that their findings might have established the traditional basis for the use of PA as an antidiabetic agent with its pharmacological activities attributed to the presence of flavonoids and other phenolics contained in the plant. At the doses investigated, SDExt also seems safer than glibenclamide even though the latter is more potent (Adedapo & Ofuegbe, 2014).

Furthermore, a study investigated the benefits of aqueous extract of *Phyllanthus amarus* (PAAExt) on insulin resistance, and oxidative stress in high fructose (HF) fed male Wistar rats. HF diet (66% of fructose) and PAAExt (200 mg/kg body weight/day) were administered concurrently to the rats for 60 days. After sixty days, the fructose-fed rats showed weight gain, increased blood glucose and insulin levels, impaired glucose tolerance, impaired insulin sensitivity, dyslipidaemia, hyperleptinemia, and hypoadiponectinemia ($p < 0.05$). Co-administration of PAAExt with HF diet significantly ameliorated all these alterations. Regarding hepatic antioxidant status, higher lipid peroxidation and protein oxidation brought down reduced glutathione (GSH) levels and the activities of enzymatic antioxidants. The PAAExt treatment significantly attenuated the histopathological changes like mild to severe distortion of the typical architecture with the prominence and widening of the liver sinusoids in the HF diet-fed rats. These findings show that PAAExt is beneficial in improving insulin sensitivity and prevents metabolic syndrome and hepatic oxidative stress in fructose-fed rats(Putakala et al., 2017[a]).

2.8.1.2.9. Anti-Inflammatory Activity

A study evaluated the anti-inflammatory and analgesic potential of *Phyllanthus amarus* as these phenomena have assumed a global dimension because of their implications in a variety of diseases. The study investigated a methanol extract of the leaves of *Phyllanthus amarus* for its anti-inflammatory and analgesic activities in Wistar rats and mice using carrageenan-induced inflammation as well as histamine-induced oedema. The analgesic effect was determined using an acetic acid writhing method and formalin-induced paw lick test. The standard phytochemical analysis identified the actual contents responsible for its biological effects. The extract at 100 and 200mg/kg body weight reduced significantly ($p < 0.05$) the formation of oedema induced by histamine and carrageenan. In the acetic acid-induced writhing test, the extract showed good analgesic activity when compared to the control group. In the formalin-induced paw lick test, the extract at 100mg and 200mg/kg weight doses exhibited good analgesic activity when compared to the untreated control group. Ibuprofen at 10mg/kg weight doses served as the reference drug in this experiment. Results suggest that methanol extract of the leaves of *Phyllanthus amarus* has excellent anti-inflammatory and analgesic potential. These biological effects

exhibited by the extract of this plant may be attributed to the presence of flavonoids and other phenols contained therein (Ofuegbe et al., 2014).

2.8.1.2.10. Protective potential on the liver, kidney, and heart

A study investigated the hepatoprotective effect of methanolic extract of the leaf of *Phyllanthus amarus* (PA) against ethanol-induced oxidative damage in adult male Wistar rats. PA (250 and 500mg/kg/day) and ethanol (5g/kg/day, 20% w/v) was administered orally to the animals for four weeks and three weeks, respectively. The ethanol treatment markedly decreased the level of reduced glutathione (GSH), superoxide dismutase (SOD) and catalase (CAT) in the liver, which was significantly enhanced by PA treatment. Glutathione-S transferase (GST), increased after chronic ethanol administration, was reduced considerably by PA treatment in the liver. Also, PA significantly increased the activities of hepatic aminotransferases as well as alkaline phosphatase (ALP), with a concomitant markedly reduced plasma aminotransferases activity in the ethanol-challenged rats. The amount of lipid peroxidation increased after chronic ethanol administration, was significantly reduced in the liver by PA co-treatment. Results showed that PA leaf extract could protect the liver from oxidative damage caused by ethanol possibly by decreasing the rate of lipid peroxidation and increasing the antioxidant defence mechanism in rats (Faremi et al., 2008).

Some other authors reviewed the hepatoprotective activity of PA plant and its probable mechanisms of action. Besides, several studies have indicated that this plant possesses remarkable hepatoprotective and antihepatotoxic potentials against liver toxicity models such as acetaminophen, alcohol, and carbon tetrachloride. The suggested mechanism relates principally to the phytochemical lignan - phyllanthin, which exhibit antioxidant effect against the oxidative stress generated by the hepatotoxins(Hassan & Masud, 2018).

In yet another study, renal damage associated with a lifestyle of inactivity (sedentary) and consumption of high-sugar diets was on the increase. The study investigated the nephroprotective activity of aqueous extract of *Phyllanthus amarus* (PAAEt) against high fructose diet (HFD) induced renal damage (RD) in Wistar rats. High fructose diet (66% fructose) alone and in combination with PAAEt (200mg/kgbwt/day) was given simultaneously to HFD group(F) and HFD+Treatment(F+T) rats respectively for 60 days. The study quantified functional markers of renal tissue such as urea, uric acid, and creatinine levels in plasma on the initial and final days of the experiment. It also evaluated the activities of aminotransferases, glucose-6-

phosphatase (G-6-P), and fructose-1, 6- bisphosphatase and aldose reductase and sorbitol dehydrogenase in the kidney. It measured oxidative stress (OS) markers such as lipid peroxidation and protein oxidation and antioxidants status in renal tissue. Examined also was the renal histopathological changes. Co-treatment with PAAEt to group F+T prevented the rise in the levels of functional markers and elevated activities of the aminotransferases, gluconeogenic and polyol pathway enzymes of group-F ($p < 0.05$). The treatment also prevented OS developed in group-F by elevated stress markers, and depletion of antioxidants in groupF+T. Treatment group-F+T also protected the observed histological changes in group-F. Normal rats + treatment (C+T) group showed no histological changes in the same way with normal rats without treatment (group-C). Thus, PAAEt effectively alleviated fructose diet-induced renal damage which may be due to its antioxidant activity. The authors concluded that this plant could be used as an adjuvant therapy for the prevention and management of HFD induced renal damage (Mallaiah et al., 2014).

Another study observed that the increased number of populations with stroke/heart attack is due to a sedentary lifestyle and consumption of high-sugar diets. This study investigated the cardio-protective activity of aqueous extract on *Phyllanthus amarus* (PAAE) against high-fructose (HF) diet-induced cardiac damage in Wistar rats. The research clearly shows the cardioprotection efficacy of PAAE against HF-diet induced oxidative stress in rats (Putakala et al., 2017[b]).

2.8.1.2.11. Antiviral activity

A study to investigate the anti-hepatitis C virus (Anti-HCV) potential of *Phyllanthus amarus* was conducted. In this study, the investigators tested the inhibitory effect of different plant extracts against NS3 and NS5B enzymes of the hepatitis C virus. Methanolic extracts from various plant materials and their inhibitory effects on the viral enzymes were evaluated by *in vitro* enzymatic assay techniques. The study investigated the impact of the extract on viral RNA replication by using TaqMan Real-Time reverse transcriptase-polymerase chain reaction (RT – PCR). Findings from this study showed that *Phyllanthus amarus* root (PAR) extract depicts a significant inhibition of HCV-NS3 protease enzyme, whereas *Phyllanthus amarus* leaf (PAL) extract showed considerable inhibition of NS5B in *vitro*.

Further, the PAR and PAL extract significantly inhibited replication of HCV monocistronic replicon ribonucleic acid (RNA) and HCV H77S viral RNA in HCV cell culture

system. However, both PAR and PAL extracts did not show cytotoxicity in Huh7 cells in the MTT assay. Furthermore, the addition of PAR and interferon-alpha (IFN-α) showed an additive effect in the inhibition of HCV RNA replication. Results suggest the possible molecular basis of the inhibitory activity of PA extract against HCV which would help in optimization and subsequent development of specific antiviral agent using *Phyllanthus amarus* as a potent natural source (Ravikumar et al., 2011).

2.8.1.2.12. Antityrosinase activities of *Phyllanthus amarus*

Ethnobotanical studies showed that *Phyllanthus amarus* is useful to treat various disorders such as skin ulcers, sores, swelling and itchiness, wounds, bruises, scabies, oedematous swellings, tubercular ulcers, ringworm and crusty lesions (Verma et al., 2014). However, there is no data found on its antityrosinase activities, which is the therapeutic basis of most body creams used to treat hyperpigmentation and other skin lesions. In our preliminary study, we evaluated the antioxidant, tyrosinase inhibitory activities and cytotoxicity of *Phyllanthus* amarus. The study revealed that the hexane and aqueous extracts of Phyllanthus amarus demonstrated a significant tyrosinase inhibition ($p < 0.05$) with their half-maximal inhibitory concentration equals 116.08 and 129.25 µg/mL respectively, but its methanolic extract shows no significant effect (Olabiyi et al., 2020).

References

Adebajo, A.C., Ayoola, M.D., Odediran, S.A., Aladesanmi, A.J., Schmidt, T.J. & Verspohl, E.J. 2013. Evaluation of ethnomedical claim III: Anti-hyperglycemic activities of gongronema latifolium root and stem. *Journal of Diabetes*, 5(3): 336–343.

Adedapo, A. & Ofuegbe, S. 2014. The evaluation of the hypoglycemic effect of soft drink leaf extract of Phyllanthus amarus (Euphorbiaceae) in rats. *Journal of Basic and Clinical Physiology and Pharmacology*, 25(1): 47–57.

Ademiluyi, A.O. & Oboh, G. 2013. Aqueous extracts of roselle (Hibiscus sabdariffa Linn.) varieties inhibit α-amylase and α-glucosidase activities in vitro. *Journal of Medicinal Food*, 16(1): 88–93.

Adeneye, A.A. 2012. The leaf and seed aqueous extract of Phyllanthus amarus improves insulin resistance diabetes in experimental animal studies. *Journal of Ethnopharmacology*, 144(3): 705–711.

Afolayan, A.J. & Sunmonu, T.O. 2011. Artemisia afra Jacq. ameliorates oxidative stress in the pancreas of streptozotocin-induced diabetic Wistar rats. *Bioscience, Biotechnology, and Biochemistry*, 75(11): 2083–2086.

Afolayan, A.J. & Sunmonu, T.O. 2013. Protective role of Artemisia afra aqueous extract on tissue antioxidant defence systems in streptozotocin-induced diabetic rats. *African journal of traditional, complementary, and alternative medicines*, 10(1): 15–20.

Aguwa, C., Ukwe, C., Inya-Agha, S. & Okonta, J. 2001. Antidiabetic effect of Picralima nitida aqueous seed extract in an experimental rabbit model. *Journal of Natural Remedies*, 1(2): 135–139.

Ahmad, J. 2015. Management of diabetic nephropathy: Recent progress and future perspective. *Diabetes and Metabolic Syndrome: Clinical Research and Reviews*, 9(4): 343–358.

Akpan, H., Ekaidem, I., Usoh, I. & Ebong, P. 2012. Effect of aqueous extract of Azadirachta indica (Neem) leaves on some indices of pancreatic function in alloxan-induced diabetic Wistar rats. *Pharmacologia*, 3: 420–425.

Akpaso, M.I., Atangwho, I.J., Akpantah, A., Fischer, V.A., Igiri, A.O. & Ebong, P.E. 2011. Effect

of combined leaf extracts of Vernonia amygdalina (Bitter Leaf) and Gongronema latifolium (Utazi) on the pancreatic β-Cells of streptozotocin-induced diabetic rats. *British Journal of Medicine and Medical Research*, 1(1): 24–34.

Al-Mutairi, N. 2006. Skin diseases seen in diabetes mellitus. *Bulletin of the Kuwait Institute for Medical Specialization*, 5: 30–39.

Al-Shahrani, M., A., Hassan, A., Al-Rubeaan, K.A., Sharqawi, A.H. Al & Ahmad, N.A. 2012. *Effects of diabetes education program on metabolic control among Saudi type 2 diabetic patients. Pakistan Journal of Medical Sciences,* 28(5).

Aluganti Narasimhulu, C., Fernandez-Ruiz, I., Selvarajan, K., Jiang, X., Sengupta, B., Riad, A. & Parthasarathy, S. 2016. Atherosclerosis - do we know enough already to prevent it? *Current Opinion in Pharmacology*, 27: 92–102.

American Diabetes Association. 2011. *Diagnosis and classification of diabetes mellitus*. Diabetes Care, **34** (1): 62-69.

Ansley, D.M. & Wang, B. 2013. Oxidative stress and myocardial injury in the diabetic heart. *Journal of Pathology*, 229(2): 232–241.

Arif, Z., Neelofar, K., Arfat, M.Y., Zaman, A., Tarannum, A., Parveen, I., Ahmad, S., Khan, M.A., Badar, A. & Islam, S.N. 2018. Hyperglycemia induced reactive species trigger structural changes in human serum albumin of type 1 diabetic subjects. *International Journal of Biological Macromolecules*, 107: 2141–2149.

Arikawe, A.P., Daramola, A.O., Olatunji-Bello, I.I. & Obika, L.F. 2013. Insulin, pioglitazone and Zingiber officinale administrations improve proliferating cell nuclear antigen immunostaining effects on diabetic and insulin-resistant rat testis. *Journal of Experimental and Clinical Medicine*, 30(1): 49–55.

Atangwho, I.J., Ebong, P.E., Eyong, E.U., Asmawi, M.Z. & Ahmad, M. 2012. Synergistic antidiabetic activity of Vernonia amygdalina and Azadirachta indica: Biochemical effects and possible mechanism. *Journal of Ethnopharmacology*, 141(3): 878–887.

Avignon, A., Hokayem, M., Bisbal, C. & Lambert, K. 2012. Dietary antioxidants: Do they have a role to play in the ongoing fight against abnormal glucose metabolism? *Nutrition*, 28(7–8): 715–721.

Banerjee, A. 2017. Dermatological Emergencies. In *Emergency Clinical Diagnosis*. Springer, Cham. 143-156.

Benhaddou-Andaloussi, A., Martineau, L., Vuong, T., Meddah, B., Madiraju, P., Settaf, A. & Haddad, P.S. 2011. The in vivo antidiabetic activity of Nigella sativa Is mediated through activation of the AMPK pathway and increased muscle glut4 content. *Evidence-Based Complementary and Alternative Medicine*, 2011: 1–9.

Bhat, T.A., Dhar, S.A., Dar, T.A., Naikoo, M.A., Naqqash, M.A., Bhat, A. & Butt, M.F. 2016. The musculoskeletal manifestations of type 2 diabetes mellitus in a Kashmiri population. *International Journal of Health Sciences*, 10(1): 55–66.

Bhathena, S.J. & Velasquez, M.T. 2002. The beneficial role of dietary phytoestrogens in obesity and diabetes. *American Journal of Clinical Nutrition*, 76(6): 1191–1201.

Boldison, J. & Wong, F.S. 2016. Immune and Pancreatic β Cell Interactions in Type 1 Diabetes. *Trends in Endocrinology and Metabolism*, 27(12): 856–867.

Brown, R.J., Araujo-Vilar, D., Cheung, P.T., Dunger, D., Garg, A., Jack, M., Mungai, L., Oral, E.A., Patni, N., Rother, K.I. & Von Schnurbein, J. 2016. The diagnosis and management of lipodystrophy syndromes: a multi-society practice guideline. *The Journal of Clinical Endocrinology & Metabolism*, *101*(12), 4500-4511.

Brown, A.C. 2017. An overview of herb and dietary supplement efficacy, safety and government regulations in the United States with suggested improvements. Part 1 of 5 series. *Food and Chemical Toxicology*, 107: 449–471.

Brown, J.R., Edwards, F.H., O'Connor, G.T., Ross, C.S. & Furnary, A.P. 2006. The diabetic disadvantage: Historical outcomes measures in diabetic patients undergoing cardiac surgery-the pre-intravenous insulin era. *Seminars in Thoracic and Cardiovascular Surgery*, 18(4): 281–288.

Brown, J.S., Wessells, H., Chancellor, M.B., Howards, S.S., Stamm, W.E., Stapleton, A.E., Steers, W.D., Eeden, S.K. Van Den & McVary, K.T. 2005. Urological complications of diabetes. *Diabetes Care*, 28(1): 177–185.

Burtis, C.A., Ashwood, E. & Bruns, D. 2008. *Carbohydrates.* Tietz Fundamentals of Clinical Chemistry, New Delhi. Elsevier. 22: 380.

Centres for Disease and Control (CDC). 2011. *Centres for Disease Control and Prevention. National diabetes fact sheet: national estimates and general information on diabetes and prediabetes*.

Ceriello, A. 2000. Oxidative stress and glycemic regulation. *Metabolism: Clinical and*

Experimental, 49(2 SUPPL. 1):27–29.

Chadwick, W.A., Roux, S., van de Venter, M., Louw, J. & Oelofsen, W. 2007. Anti-diabetic effects of Sutherlandia frutescens in Wistar rats fed a diabetogenic diet. *Journal of Ethnopharmacology*, 109(1):121–127.

Chandan, S., Umesha, S. & Balamurugan, V. 2012. Antileptospiral, antioxidant and DNA damaging properties of Eclipta alba and Phyllanthus amarus. *Journal of Veterinary Science & Technology*, 1(4):1–8.

Chattopadhyay, S., Samanta, S., Ganguly, S., Banerjee, J. & Chanda, R. 2020. Treatment of diabetic mellitus (DM), a Long term endocrine metabolic disorder: A review. *Sustainable Humanosphere*, 16(1):416–424.

Chen, Y.L., Zhao, C., Zhang, L., Li, B., Wu, C.H., Mu, W., Wang, J.Y., Yang, K.H., Li, Y.P., Chen, C. & Wang, Y.Y. 2018. Toward evidence-based Chinese medicine: status quo, opportunities, and challenges. *Chinese journal of integrative medicine*, *24*(3), 163-170.

Colwell, J.A., Lopes-Virella, M. & Halushka, P. V. 1981. Pathogenesis of atherosclerosis in diabetes mellitus. *Diabetes Care*, 4(1):121–133.

Craft, R., McClure, K.C., Corbett, S., Ferreira, M.P., Stiffarm, A.M. & Kindscher, K. 2015. Ethnic differences in medicinal plant use among University students: a cross-sectional survey of self-reported medicinal plant use at two Midwest Universities. *BMC Complementary and Alternative Medicine*, 15(1):1–10.

Dalal, S., Beunza, J.J., Volmink, J., Adebamowo, C., Bajunirwe, F., Njelekela, M., Mozaffarian, D., Fawzi, W., Willett, W., Adami, H.O. & Holmes, M.D. 2011. Non-communicable diseases in sub-Saharan Africa: What we know now. *International Journal of Epidemiology*, 40(4):885–901.

Dawidar, A.M., Abdel-Mogib, M., Abou-Elzahab, M.M., Berghot, M.A., Mahfouz, M., El-Ghorab, A.H. & Hussien, K. 2010. Effect of photo-oxygenation on biological activities of some commercial Egyptian essential oils. *Revista Latinoamericana de quimica*, 38(3): 168–179.

De Carvalho Vidigal, F., Guedes Cocate, P., Pereira, L.G. & De Cássia, R.G.A. 2012. The role of hyperglycemia in the induction of oxidative stress and inflammatory process. *Nutricion Hospitalaria*, 27(5):1391–1398.

Demirseren, D.D., Emre, S., Akoglu, G., Arpacı, D., Arman, A., Metin, A. & Cakır, B. 2014. Relationship between skin diseases and extracutaneous complications of diabetes mellitus:

Clinical analysis of 750 patients. *American Journal of Clinical Dermatology*, 15(1): 65–70.

Dimo, T., Rakotonirina, S. V., Tan, P. V., Azay, J., Dongo, E., Kamtchouing, P. & Cros, G. 2007. Effect of Sclerocarya birrea (Anacardiaceae) stem bark methylene chloride/methanol extract on streptozotocin-diabetic rats. *Journal of Ethnopharmacology*, 110(3): 434–438.

Distiller, L. 2014. Prevalence of diabetes in South Africa | Health24. *Health24*.

Dixon, R.A. 2004. Phytoestrogen. *Annual Review of Plant Biology*, 55(1): 225–261.

Duff, M., Demidova, O., Blackburn, S. & Shubrook, J. 2015. Cutaneous manifestations of diabetes mellitus. *Clinical Diabetes*, 33(1): 40–48.

Dzeufient, P.D.D., Y.Ohandja, D., Tedong, L., Asongalem, E.A., Dimo, T. & Kamtchouing, S.D.S.P. 2007. Antidiabetic effect of Ceiba pentandra extract on streptozotocin-induced non-insulin-dependent diabetic (NIDDM) rats. *African journal of traditional, complementary, and alternative medicines*, 4(1): 47–54.

Eddouks, M., Lemhadri, A. & Michel, J.B. 2004. Caraway and caper: Potential anti-hyperglycaemic plants in diabetic rats. *Journal of Ethnopharmacology*, 94(1): 143–148.

Eddouks, M., Maghrani, M., Zeggwagh, N.A. & Michel, J.B. 2005. Study of the hypoglycaemic activity of Lepidium sativum L. aqueous extract in normal and diabetic rats. *Journal of Ethnopharmacology*, 97(2): 391–395.

Edoga, C.O., Njoku, O.O., Amadi, E.N. & Okeke, J.J. 2013. Blood sugar lowering effect of Moringa oleifera Lam in albino rats. *International Journal of Science and Technology*, 3(1): 88–90.

Effiong, G.S., Mgbeje, B.I., Igile, G.O., Atangwho, J.I., Eyong, E.U. & Ebong, P.E. 2013. Antioxidant enzymes activity and hormonal changes following administration of ethanolic leaves extracts of nauclea latifolia and gongronema latifolium in streptozotocin induced-diabetic rats. *European Journal of Medicinal Plants*, 3(2): 297–309.

Egesie, U.G., Adelaiye, A.B., Ibu, J.O. & Egesie, O.J. 2006. Safety and hypoglycaemic properties of aqueous leaf extract of Ocimum gratissimum in streptozotocin-induced diabetic rats. *Nigerian Journal of Physiological Sciences*, 21(1–2): 31–35.

El-Beshbishy, H.A., Singab, A.N.B., Sinkkonen, J. & Pihlaja, K. 2006. Hypolipidemic and antioxidant effects of Morus alba L. (Egyptian mulberry) root bark fractions supplementation in cholesterol-fed rats. *Life Sciences*, 78(23): 2724–2733.

El-Garawani, I., Emam, M., Elkhateeb, W., El-Seedi, H., Khalifa, S., Oshiba, S., Abou-Ghanima, S. and Daba, G., 2020. In vitro antigenotoxic, antihelminthic and antioxidant potentials based on the extracted metabolites from Lichen, Candelariella vitellina. *Pharmaceutics*, *12*(5), p.477.

El-Hilaly, J., Tahraoui, A., Israili, Z.H. & Lyoussi, B. 2006. Hypolipidemic effects of acute and sub-chronic administration of an aqueous extract of Ajuga iva L. whole plant in normal and diabetic rats. *Journal of Ethnopharmacology*, 105(3): 441–448.

El-Sayyad, H.I.H., El-Sherbiny, M.A., Sobh, M.A., Abou-El-Naga, A.M., Ibrahim, M.A.N. & Mousa, S.A. 2011. Protective effects of Morus alba leaves extract on ocular functions of pups from diabetic and hypercholesterolemic mother rats. *International Journal of Biological Sciences*, 7(6): 715–728.

El-Soud, N.A. & Khalil, M. 2010. Antioxidative effects of allium cepa essential oil in streptozotocin-induced diabetic rats. *Macedonian Journal of Medical Sciences*, 3(4): 344–351.

Eldin, I.M.T., Ahmed, E.M. & Abd, E.H.M. 2010. A preliminary study of the clinical hypoglycemic effects of Allium cepa (Red Onion) in type 1 and type 2 diabetic patients. *Environmental Health Insights*, 4: 71–77.

Ene, A.C., Bukbuk, D.N. & Ogunmola, O.O. 2006. Effect of different doses of black caraway (Carum carvi l)oil on the level of serum creatinine in alloxan-induced diabetic rats. *Journal of Medical Sciences*, 6(4): 701–703.

Ene, A.C., Nwankwo, E.A. & Samdi, L.M. 2007. Alloxan-Induced Diabetes in Rats and the Effects of Black Caraway (Carum carvi L.) Oil on Their Body Weights. *Research Journal of Medicine and Medical Sciences*, 2(2): 48–52.

Erejuwa, O. 2012. Oxidative stress in diabetes mellitus: Is there a role for hypoglycemic drugs and/or antioxidants? In *Oxidative Stress and Diseases*. 217–246.

Eseyin, O.A., Ebong, P., Ekpo, A., Igboasoiyi, A. & Oforah, E. 2007. Hypoglycemic effect of the seed extract of Telfairia occidentalis in rat. *Pakistan Journal of Biological Sciences*, 10(3): 498–501.

Eyo, J.E., Ozougwu, J.C. & Echi, P.C. 2011. Hypoglycaemic effects of allium cepa, Allium sativum and Zingiber officinale aqueous extracts on alloxan-induced diabetic rattus novergicus. *Physiology and Endocrinology*, 19(3): 121–126.

Fagbohun, T.R. & Odufunwa, K.T. 2010. Hypoglycemic effect of methanolic extract of Anacardium occidentale leaves in alloxan-induced diabetic rats. *Nigerian Journal of physiological sciences*, 25(1): 87–90.

Faremi, T., Suru, S. & Fafunso, M. 2008. Hepatoprotective potentials of Phyllanthus amarus against ethanol-induced oxidative stress in rats. *Food and Chemical Toxicology*, 46(8): 2658–2664.

Farnsworth, N. & Soejarto, D. 1991. Global importance of medicinal plants in the conservation of medicinal plants. *The Conservation of Medicinal Plants*, 26: 26–51.

Fatima, S. 2018. Role of reactive oxygen species in male reproduction. In *Novel Prospects in Oxidative and Nitrosative Stress*. 71–91.

Fetterman, J.L., Holbrook, M., Westbrook, D.G., Brown, J.A., Feeley, K.P., Bretón-Romero, R., Linder, E.A., Berk, B.D., Weisbrod, R.M., Widlansky, M.E., Gokce, N., Ballinger, S.W. & Hamburg, N.M. 2016. Mitochondrial DNA damage and vascular function in patients with diabetes mellitus and atherosclerotic cardiovascular disease. *Cardiovascular Diabetology*, 15(53): 1–7.

Finaud, J., Lac, G., Filaire, E. & Biologie, L. 2006. Oxidative Stress Relationship with Exercise and Training. *Sports Medicine*, 36(4): 327–358.

Gheibi, S., Kashfi, K. & Ghasemi, A. 2017. A practical guide for induction of type-2 diabetes in rat: Incorporating a high-fat diet and streptozotocin. *Biomedicine and Pharmacotherapy*, 95(24): 605–613.

Gondwe, M., Kamadyaapa, D.R., Tufts, M., Chuturgoon, A.A. & Musabayane, C.T. 2008. Sclerocarya birrea [(A. Rich.) Hochst.] [Anacardiaceae] stem-bark ethanolic extract (SBE) modulates blood glucose, glomerular filtration rate (GFR) and mean arterial blood pressure (MAP) of STZ-induced diabetic rats. *Phytomedicine*, 15(9): 699–709.

Grebe, A. & Latz, E. 2013. *Cholesterol crystals and inflammation*. *Current rheumatology reports*, **15**(3): 313.

Guaâdaoui, A., Bouhtit, F., Cherfi, M. & Hamal, A. 2015. The preventive approach of biocompounactives (2): A review in recent advances in common fruits. *International Journal of Nutrition and Food Sciences*, 4(2): 189–207.

Hamden, K., Ayadi, F., Jamoussi, K., Masmoudi, H. & Elfeki, A. 2008. The therapeutic effect of phytoecdysteroids rich extract from Ajuga iva on alloxan-induced diabetic rats liver, kidney

and pancreas. *BioFactors*, 33(3): 165–175.

Hassan, M.A. & Masud, I.A. 2018. An overview of the hepatoprotective potentials of Phyllanthus amarus. *Journal of Pharmacognosy and Phytochemistry*, 7(2): 2777–2782.

Hussein, H.J., Hameed, I.H. & Hadi, M.Y. 2017. A review: Anti-microbial, anti-inflammatory effect and cardiovascular effects of garlic: Allium sativum. *Research Journal of Pharmacy and Technology*, 10(11): 4069–4078.

Igboasoiyi, A.C., Essien, E.E., Eseyin, O.A. & Ubam, G. 2007. Screening of the seed of Picralima nitida for hypoglycaemic activity. *Pakistan Journal of Biological Sciences*, 10(5): 828–830.

Ighodaro, O.M. & Akinloye, O.A. 2018. First-line defence antioxidants-superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX): Their fundamental role in the entire antioxidant defence grid. *Alexandria Journal of Medicine*, 54(4): 287–293.

Ilahy, R., Tlili, I., Siddiqui, M.W., Hdider, C. & Lenucci, M.S. 2019. Inside and beyond colour: Comparative overview of functional quality of tomato and watermelon fruits. *Frontiers in Plant Science*, 10(769): 1–26.

International Diabetes Federation. 2015[a]. Diabetes in Nigeria. www.idf.org/membership/afr/nigeria.(Accessed: 06/05/2019)

International Diabetes Federation. 2015[b]. Diabetes in South Africa. www.idf.org/membership/afr/southafrica.(Accessed: 06/05/2019).

International Diabetes Federation. 2019. *IDF Diabetes Atlas. Ninth edition 2019*. https://www.idf.org/e-library/epidemiology-research/diabetes-atlas/159-idf-diabetes-atlas-ninth-edition-2019.html. (Accessed: 05/03/2020).

International Diabetes Federation. 2020[a]. https://idf.org/our-network/regions-members/africa/members/20-nigeria.html(Accessed: July 24, 2020).

International Diabetes Federation. 2020[b]. https://idf.org/our-network/regions-members/africa/members/25-south-africa.html (Accessed: July 24, 2020).

Islam, M.S. 2011. Effects of the aqueous extract of white tea (Camellia sinensis) in a streptozotocin-induced diabetes model of rats. *Phytomedicine*, 19(1): 25–31.

Kalus, A.., Chien, A.. & Olerud, J.. 2012. Diabetes mellitus and other endocrine diseases. *In Fitzpatrick's Dermatology in General Medicine*. 1840–1869.

Kamgang, R., Youmbi Mboumi, R., Foyet Fondjo, A., Fokam Tagne, M.A., Mengue N'dillé, G.P.R. & Ngogang Yonkeu, J. 2008. The antihyperglycaemic potential of the water-ethanol extract of Kalanchoe crenata (Crassulaceae). *Journal of Natural Medicines*, 62(1): 34–40.

Kandola, K., Bowman, A. & Birch-Machin, M.A. 2015. Oxidative stress - A key emerging impact factor in health, ageing, lifestyle and aesthetics. *International Journal of Cosmetic Science*, 37(2): 1–8.

Karalliedde, J. & Gnudi, L. 2016. Diabetes mellitus, a complex and heterogeneous disease, and the role of insulin resistance as a determinant of diabetic kidney disease. *Nephrology Dialysis Transplantation*, 31(2): 206–213.

Karau, G.M., Njagi, E.N.M., Machocho, A.K., Wangai, L.N. & Kamau, P.N. 2012. Hypoglycemic activity of aqueous and ethyl acetate leaf and stem bark extracts of Pappea capensis in alloxan-induced diabetic BALB/c mice. *British Journal of Pharmacology and Toxicology*, 3(5): 251–258.

Karnib, H.H. & Ziyadeh, F.N. 2010. The cardiorenal syndrome in diabetes mellitus. *Diabetes Research and Clinical Practice*, 89(3): 201–208.

Karuna, R., Bharathi, V.G., Reddy, S.S., Ramesh, B. & Saralakumari, D. 2011. Protective effects of Phyllanthus amarus aqueous extract against renal oxidative stress in Streptozotocin-induced diabetic rats. *Indian Journal of Pharmacology*, 43(4): 414–418.

Kawasaki, E., Abiru, N. & Eguchi, K. 2004. Prevention of type 1 diabetes: From the viewpoint of β cell damage. *Diabetes Research and Clinical Practice*, 66: 27–32.

Keri, K.C., Samji, N.S. & Blumenthal, S. 2018. Diabetic nephropathy: newer therapeutic perspectives. *Journal of Community Hospital Internal Medicine Perspectives*, 8(4): 200–207.

Khan, A.N., Khan, R.A., Ahmad, M. & Mushtaq, N. 2015. Role of antioxidant in oxidative stress and diabetes mellitus. *Journal of Pharmacognosy and Phytochemistry*, 3(6): 217–220.

Khan, N., Afaq, F. & Mukhtar, H. 2008. Cancer chemoprevention through dietary antioxidants: progress and promise. *Antioxidants and Redox Signaling*, 10(3): 475–510.

Khatoon, S., Rai, V., Rawat, A.K.S. & Mehrotra, S. 2006. Comparative pharmacognostic studies of three Phyllanthus species. *Journal of Ethnopharmacology*, 104(1–2): 79–86.

Kibirige, D., Lumu, W., Jones, A.G., Smeeth, L., Hattersley, A.T. & Nyirenda, M.J. 2019. Understanding the manifestation of diabetes in sub-Saharan Africa to inform therapeutic

approaches and preventive strategies: a narrative review. *Clinical Diabetes and Endocrinology*, 5(2): 2–8.

Kim, C.H., Park, M.K., Kim, S.K. & Cho, Y.H. 2014. Antioxidant capacity and anti-inflammatory activity of lycopene in watermelon. *International Journal of Food Science and Technology*, 49(9): 2083–2091.

Koffuor, G., Amoatengor, P., Okai, C. & Fiagbe, N. 2011. Hypoglycemic effects of whole and fractionated Azadirachta Indica (Neem) seed oils on alloxan-induced diabetes in New Zealand white rabbits. *Journal of the Ghana Science Association*, 13(1): 117–127.

Kong, J.M., Goh, N.K., Chia, L.S. & Chia, T.F. 2003. Recent advances in traditional plant drugs and orchids. *Acta Pharmacologica Sinica*, 24(1): 7–21.

Kruth, H.S. 2001. Lipoprotein cholesterol and atherosclerosis. *Current Molecular Medicine*, 1(6): 633–653.

Kuate, D., Etoundi, B., Ngondi, J. & Oben, J. 2011. Effects of dichrostachys glomerata spice on cardiovascular diseases risk factors in normoglycemic and type 2 diabetic obese volunteers. *Food Research International*, 44: 1197–1202.

Kumar, V., Bhatt, P.C., Kaithwas, G., Rashid, M., Al-abbasi, F.A., Khan, J.A.J., Anwar, F. & Verma, A. 2016. α-Mangostin mediated pharmacological modulation of hepatic carbohydrate metabolism in diabetes-induced Wistar rat. *Beni-Suef University Journal of Basic and Applied Sciences*, 5(3): 255–276.

Kurzer, M.S. & Xu, X. 1997. Dietary Phytoestrogens. *Annual Review of Nutrition*, 17: 353–381.

Kwon, D., Daily, J., Kim, H. & Park, S. 2010. Antidiabetic effects of fermented soybean products on type 2 diabetes. *Nutrition Research*, 30: 1-13.

Lawson-Evi, P., Eklu-Gadegbeku, K., Agbonon, A., Aklikokou, K., Creppy, E. & Gbeassor, M. 2011. Antidiabetic activity of Phyllanthus amarus schum and thonn (Euphorbiaceae) on alloxan-induced diabetes in male Wistar rats. *Journal of Applied Sciences*, 11(16): 2968–2973.

Lemhadri, A., Burcelin, R., Sulpice, T. & Eddouks, M. 2007. Chamaemelum nobile L. aqueous extract represses endogenous glucose production and improves insulin sensitivity in streptozotocin-induced diabetic mice. *American Journal of Pharmacology and Toxicology*, 2(3): 116–122.

Lemhadri, A., Hajji, L., Michel, J.B. & Eddouks, M. 2006. Cholesterol and triglycerides lowering

activities of caraway fruits in normal and streptozotocin-diabetic rats. *Journal of Ethnopharmacology*, 106(3): 321–326.

Liguori, I., Russo, G., Curcio, F., Bulli, G., Aran, L., Della-Morte, D., Gargiulo, G., Testa, G., Cacciatore, F., Bonaduce, D. & Abete, P. 2018. Oxidative stress, aging, and diseases. *Clinical Interventions in Aging*, 13: 757–772.

Linton, M., Yancey, P., Davies, S., Jerome, W., Linton, E., Song, W., Doran, A. & Vickers, K. 2019. The role of lipids and lipoproteins in atherosclerosis. *In Endotext [Internet]*. MDText. com, Inc.www.endotext.org (Accessed: 15/06/2020).

Loots, D.T., Pieters, M., Islam, M.S. & Botes, L. 2011. Antidiabetic effects of aloe ferox and aloe greatheadii var. Davyana leaf gel extracts in a low-Dose streptozotocin diabetes rat model. *South African Journal of Science*, 107(7–8): 1–6.

Lopez Hernandez, M.A. 2013. Hyperglycemia and Diabetes in Myocardial Infarction. In *Diabetes Mellitus - Insights and Perspectives*. Rijeka. InTech: 169–192.

Luo, C., Xu, X., Wei, X., Feng, W., Huang, H., Liu, H., Xu, R., Lin, J., Han, L. & Zhang, D. 2019. Natural medicines for the treatment of fatigue: Bioactive components, pharmacology, and mechanisms. *Pharmacological Research*, 148: 104409.

MacKenzie, J., Koekemoer, T., Venter, M. van de, Dealtry, G. & Roux, S. 2009. Sutherlandia frutescens limits the development of insulin resistance by decreasing plasma free fatty acid levels. *Phytotherapy Research*, 23(4): 1609–1614.

Mahomed, I. & Ojewole, J. 2003. Hypoglycemic effect of Hypoxis hemerocallidea corm (African potato) aqueous extract in rats. *Methods and Findings in Experimental Clinical. Pharmacology*, 25: 617–623.

Maity, S., Chatterjee, S., Variyar, P.S., Sharma, A., Adhikari, S. & Mazumder, S. 2013. Evaluation of antioxidant activity and characterization of phenolic constituents of Phyllanthus amarus root. *Journal of Agricultural and Food Chemistry*, 61(14): 3443–3450.

Makom, I., Frigerio, F., Casimir, M., Ngueguim, F.., Dongo, E., Kamtchouing, P., Dimo, T. & Maechler, P. 2010. Sclerocarya birrea (Anacardiaceae) stem-bark extract corrects glycaemia in diabetic rats and acts on beta-cells by enhancing glucose-stimulated insulin secretion. *The Journal of Endocrinology*, 205(1): 79–86.

Mallaiah, P., Sudhakara, G., Srinivasulu, N., Sasi Bhusana Rao, B. & Saralakumari, D. 2014. Preventive effect of Phyllanthus amarus on high fructose diet-induced renal damage in

male Wistar rats. *Journal of Experimental and Applied Animal Science*, 1(2): 186–198.

Manikkoth, S., Deepa, B., Joy, A.E. & Rao, S.N. 2011. Anticonvulsant activity of Phyllanthus amarus in experimental animal models. *International Journal of Applied Biology and Pharmaceutical Technology*, 2(4): 144–149.

Martín-Timón, I., Sevillano-Collantes, C., Segura-Galindo, A. & Cañizo-Gómez, F.J. del. 2014. Type 2 diabetes and cardiovascular disease: Have all risk factors the same strength? *World Journal of Diabetes*, 5(4): 444–470.

Matough, F.A., Budin, S.B., Hamid, Z.A., Alwahaibi, N. & Mohamed, J. 2012. The role of oxidative stress and antioxidants in diabetic complications. *Sultan Qaboos University Medical Journal*, 12(1): 5–18.

Mazed, M.A., Linda, Y., Mazed, S. & Linda, Y. 2011. Nutritional supplement for the prevention of cardiovascular disease, Alzheimer's disease, diabetes, and regulation and reduction of blood sugar and insulin resistance. 2: 1–15.

Mbagwu, H.O.C., Jackson, C., Jackson, I., Ekpe, G., Eyaekop, U. & Essien, G. 2011. Evaluation of the hypoglycemic effect of aqueous extract of Phyllanthus amarus in alloxan-induced diabetic albino rats. *International Journal for Pharmaceutical and Biomedical Research*, 2(3): 158–160.

M.Hussein, H., M.El-Sayed, E. & A.Said, A. 2006. Antihyperglycemic, antihyperlipidemic and antioxidant effect of Zizyphus spina christi and Zizyphus jujuba in alloxan diabetic rats. *International Journal of pharmacology*, 2(5): 563–570.

Michel, C.G., Nesseem, D.I. & Ismail, M.F. 2011. Anti-diabetic activity and stability study of the formulated leaf extract of Zizyphus spina-christi (L.) Wild with the influence of seasonal variation. *Journal of Ethnopharmacology*, 133(1): 53–62.

Modu, S., Adeboye, E., Maisaratu, A. & Mubi, B. 2013. Studies on the administration of Vernonia amygdalina Del.(Bitter leaf) and glucophage on blood glucose level of alloxan–Induced diabetic rats. *International Journal of Medicinal Plant and Alternative Medicine*, 1: 13–19.

Mohamed, A.M., EL-Sharkawy, F.Z., Ahmed, S.A.A., Aziz, W.M. & Badary, O.A. 2009. Glycemic control and therapeutic effect of Nigella sativa and Curcuma longa on rats with streptozotocin-induced diabetic hepatopathy. *Journal of Pharmacology and Toxicology*, 4(2): 45–57.

Mohammed, A., Ibrahim, M. & Islam, M. 2014. African medicinal plants with antidiabetic

potentials: A Review. *Planta Medica*, 80(5): 354–377.

Morakinyo, A.O., Akindele, A.J. & Ahmed, Z. 2011. Modulation of antioxidant enzymes and inflammatory cytokines: Possible mechanism of the anti-diabetic effect of ginger extracts. *African Journal of Biomedical Research*, 14(3): 195–202.

Mpiana, P.T., Masunda, T.A., Longoma, B.F., Tshibangu, D.S.T. & Ngbolua, K.N. 2013. Anti-hyperglycemic activity of raphia gentiliana De wild. (Arecaceae). *European Journal of Medicinal Plants*, 3(2): 233–240.

Murea, M., Ma, L. & Freedman, B.I. 2012. Genetic and environmental factors associated with type 2 diabetes and diabetic vascular complications. *Review of Diabetic Studies*, 9(1): 6–22.

Musabayane, C., Gondwe, M., Kamadyaapa, D., Moodley, K. & Ojewole, J.A. 2006. The effects of Sclerocarya birrea stem-bark aqueous extract on blood glucose, kidney and cardiovascular function in rats. *In 197th Meeting of the Society for Endocrinology*, 12: 36.

Nardos, A., Makonnen, E. & Debella, A. 2011. Effects of crude extracts and fractions of moringa stenopetala (Baker f) cufodontis leaves in normoglycemic and alloxan-induced diabetic mice. *African Journal of Pharmacy and Pharmacology*, 5(20): 2220–2225.

Nguyen, V., Sakoff, J. & Scarlett, C. 2017. Physicochemical properties, antioxidant and cytotoxic activities of crude extracts and fractions from Phyllanthus amarus. *Medicines*, 4(42): 1–15.

Niedowicz, D.M. & Daleke, D.L. 2005. The role of oxidative stress in diabetic complications. *Cell Biochemistry and Biophysics*, 43(2): 289–330.

Njagi, J., Piero, M., Ngeranwa, J. & Njagi, E. 2012. Assessment of the antidiabetic potential of Ficus sycomorus on alloxan-induced diabetic mice. *International Journal of Diabetes Research*, 1(4): 47–51.

Nwanjo, H.U., Okafor, M.C. & Oze, G.O. 2006. Anti-lipid peroxidative activity of Gongronema latifolium in streptozotocin-induced diabetic rats. *Nigerian journal of physiological sciences*, 21(1–2): 61–65.

Nyah Njike, G., Watcho, P., Benoît Nguelefack, T. & Kamanyi, A. 2005. Hypoglycaemic activity of extracts of the leaves of Bersama engleriana in rats. *African Journal of Traditional, Complementary and Alternative Medicine*, 2(3): 215–221.

Odetola, A.A., Akinloye, O., Egunjobi, C., Adekunle, W.A. & Ayoola, A.O. 2006. Possible antidiabetic and antihyperlipidaemic effect of fermented Parkia biglobosa (JACQ) extract in

alloxan-induced diabetic rats. *Clinical and Experimental Pharmacology and Physiology*, 33(9): 808–812.

Ofuegbe, S., Adedapo, A. & Adeyemi, A. 2014. Anti-inflammatory and analgesic activities of the methanol leaf extract of Phyllanthus amarus in some laboratory animals. *Journal of Basic and Clinical Physiology and Pharmacology*, 25(2): 175–180.

Ojewole, J.A. & Adewunmi, C.O. 2000. Hypoglycaemic effects of methanolic leaf extract of Catharanthus roseus (Linn.) G. Don (Apocynaceae) in normal and diabetic mice. *Acta Medical Biology*, 48: 55–58.

Ojewole, J.A.O. 2005. Antinociceptive, anti-inflammatory and antidiabetic effects of Bryophyllum pinnatum (Crassulaceae) leaf aqueous extract. *Journal of Ethnopharmacology*, 99(1): 13–19.

Ojewole, J.A.O. 2006. Antinociceptive, anti-inflammatory and anti-diabetic properties of Hypoxis hemerocallidea Fisch. & C.A. Mey. (Hypoxidaceae) corm ['African Potato'] aqueous extract in mice and rats. *Journal of Ethnopharmacology*, 103(1): 126–134.

Ojo, R.J., Memudu, A.E., Akintayo, C.O. & Akpan, I.S. 2012. Preventive effect of Allium sativum on alloxan-induced diabetic rat. *Journal of Agricultural and Biological Science*, 7(8): 609–612.

Olabiyi, F.A., Aboua, Y.G., Popoola, O.K., Monsees, T.K. & Oguntibeju, O.O. 2020. Evaluation of antioxidant, antityrosinase activities and cytotoxic effects of Phyllanthus amarus extracts. *The Natural Products Journal*, 10(2): 130–138.

Onaolapo, A.Y., Onaolapo, O.J. & Adewole, S.A. 2011. Ethanolic extract of Ocimum grattissimum leaves (Linn.) rapidly lowers blood glucose levels in diabetic Wistar rats. *Macedonian Journal of Medical Sciences*, 4(4): 351–357.

Orok, U., Eneji, E., Luke, O., Eyo, R., Sampson, E., Iwara, I. & Oko, O. 2012. Effect of ethanolic root and twig extracts of Gongronema latifolium (utazi) on kidney function of streptozotocin-induced hyperglycemic and normal Wistar rats. *Journal of Medicine and Medical Sciences*, 3(5): 291–296.

Owolabi, O.A., James, D.B., Anigo, K.M., Iormanger, G.W. & Olaiya, I.I. 2011. The combined effect of aqueous extracts of Phyllanthus amarus and vitex doniana stem bark on blood glucose of streptozotocin (STZ) induced diabetic rats and some liver biochemical parameters. *Journal of Pharmacology and Toxicology*, 2(3): 143–147.

Ozougwu, J.C. 2011. Anti-Diabetic effects of Allium cepa (onions) aqueous extracts on alloxan-induced diabetic rattus novergicus. *Journal of Medicinal Plants Research*, 5(7): 1134–1139.

Paneni, F., Beckman, J.A., Creager, M.A. & Cosentino, F. 2013. Diabetes and vascular disease: Pathophysiology, clinical consequences, and medical therapy: Part I. *European Heart Journal*, 34(31): 2436–2446.

Paron, N. & Lambert, P.W. 2000. Cutaneous manifestations of diabetes mellitus. *Primary Care*, 27(2): 371–383.

Parvathaneni, M., Battu, G.R., Gray, A.. & Gummalla, P. 2014. Investigation of the anticancer potential of hypophyllanthin and phyllanthin against breast cancer by in vitro and in vivo methods. *Asian Pacific Journal of Tropical Disease*, 4: 71–76.

Patel, T.P., Rawal, K., Bagchi, A.K., Akolkar, G., Bernardes, N., Dias, D. da S., Gupta, S. & Singal, P.K. 2016. Insulin resistance: an additional risk factor in the pathogenesis of cardiovascular disease in type 2 diabetes. *Heart Failure Reviews*, 21(1): 11–23.

Piero, N.M., N Joan, M., Cromwell, K.M., Joseph, N.J., Wilson, N.M., Daniel, M., Peter, G.K. & N, N.E. 2011. Hypoglycemic activity of some Kenyan plants traditionally used to manage diabetes mellitus in Eastern province. *Journal of Diabetes & Metabolism*, 2(8): 1–6.

Pierre, W., Gildas, A.J.H., Ulrich, M.C., Modeste, W.N., Benoît, N.T. & Albert, K. 2012. Hypoglycemic and hypolipidemic effects of Bersama engleriana leaves in nicotinamide/streptozotocin-induced type 2 diabetic rats. *BMC Complementary and Alternative Medicine*, 12(264): 1–6.

Prakash, D. & Gupta, C. 2011. *Role of Phytoestrogens as Nutraceuticals in Human Health*. Phytochemicals of Nutraceutical Importance. *Biotechnology: An Indian Journa*l. 5: 1-8.

Prentice, A., Schoenmakers, I., Jones, K.S., Jarjou, L.M.A. & Goldberg, G.R. 2009. Vitamin D deficiency and its health consequences in Africa. *Clinical Reviews in Bone and Mineral Metabolism*, 7(1): 94–106.

Putakala, M., Gujjala, S., Nukala, S., Bongu, S.B.R., Chintakunta, N. & Desireddy, S. 2017[a]. Cardioprotective effect of Phyllanthus amarus against high fructose diet-induced myocardial and aortic stress in a rat model. *Biomedicine and Pharmacotherapy*, 95: 1359–1368.

Putakala, M., Gujjala, S., Nukala, S. & Desireddy, S. 2017[b]. Beneficial effects of Phyllanthus amarus against high fructose diet-induced insulin resistance and hepatic oxidative stress in male Wistar rats. *Applied Biochemistry and Biotechnology*, 183(3): 744–764.

Quatrano, N. & Loechner, K.. 2012. Dermatologic manifestations of endocrine disorders. *Current Opinion in Paediatrics*, 24(4): 487–493.

Quispe, R., Martin, S.. & Jones, S.. 2016. Triglycerides to high-density lipoprotein–cholesterol ratio, glycemic control and cardiovascular risk in obese patients with type 2 diabetes. *Current Opinion in Endocrinology, Diabetes and Obesity*, 23(2): 150–156.

Rao, P.P., Sujatha, C., Latha, N.M., Kumar, J.P. & Deepa, M. 2015. Association between microalbuminuria and oxidative stress in diabetic nephropathy. *International Journal of Clinical and Biomedical Research*, 1(2): 96–98. http://www.ijmbr.com/reviewed/3.1.1.pdf.

Ravikumar, Y.S., Ray, U., Nandhitha, M., Perween, A., Raja Naika, H., Khanna, N. & Das, S. 2011. Inhibition of hepatitis C virus replication by herbal extract: Phyllanthus amarus as potent natural source. *Virus Research*, 158: 89–97.

Reiner, Ž. 2017. Hypertriglyceridaemia and risk of coronary artery disease. *Nature Reviews Cardiology*, 14(7): 401–411.

Rice, S. & Whitehead, S.A. 2006. Phytoestrogens and breast cancer - Promoters or protectors? *Endocrine-Related Cancer*, 13(4): 995–1015.

Rishi, R. 2019. Chemistry and mechanism of action of phytoestrogens. *In Phytoestrogens In Functional Foods*: 81–96.

Rochette, L., Zeller, M., Cottin, Y. & Vergely, C. 2014. Diabetes, oxidative stress and therapeutic strategies. *Biochimica et Biophysica Acta - General Subjects*, 1840(9): 2709–2729.

Roengrit, T., Wannanon, P., Prasertsri, P., Kanpetta, Y., Sripanidkulchai, B.O & Leelayuwat, N. 2014. Antioxidant and anti-nociceptive effects of Phyllanthus amarus on improving exercise recovery in sedentary men: A randomized crossover (double-blind) design. *Journal of the International Society of Sports Nutrition*, 11(1): 1–9.

Roengrit, T., Wannanon, P., Prasertsri, P., Kanpetta, Y., Sripanidkulchai, B.O., Wattanathorn, J. & Leelayuwat, N. 2015. Antioxidant effect of Phyllanthus amarus after moderate-intensity exercise in sedentary males: A randomized crossover (double-blind) study. *Journal of Physical Therapy Science*, 27(4): 1181–1186.

Roglic, G., Unwin, N., Bennett, P.H., Mathers, C., Tuomilehto, J., Nag, S., Connolly, V. & King, H. 2005. The burden of mortality attributable to diabetes: realistic estimates for the year 2000. *Diabetes Care*, 28(9): 2130–2135.

Rupp, R. 2011. How carrots won the trojan war. In Curious (but True) Stories of Common

Vegetables. In: Deborah B. Ed. Storey Publishing. ISBN 978 1 60342 968 9.

Sagoo, M.K. & Gnudi, L. 2018. Diabetic nephropathy: Is there a role for oxidative stress? *Free Radical Biology and Medicine*, 116: 50–63.

Samson, A.M. 2016. *In vivo antiplasmodial potential of combinations of three Nigerian antimalarial plants*. Ladoke Akintola University of Technology, Ogbomoso. 1-67.

Sen, A. & Batra, A. 2013. The study of in vitro and in vivo antioxidant activity and total phenolic content of Phyllanthus amarus Schum. & Thonn.: A medicinally important plant. *International Journal of Pharmacy and Pharmaceutical Science*, 5(3): 942–947.

Shaikh, S.H., Malik, F., James, H. & Abdul, H. 2009. Trends in the use of complementary and alternative medicine in Pakistan: A population-based survey. *Journal of Alternative and Complementary Medicine*, 15(5): 545–550.

Shakurfow, F.A.A., Mahmoud, M.B. & Gamal, M.A.B. 2015. Assessment of antimicrobial activity of onion extract (Allium cepa) and garlic (Allium sativum) extracts on Listeria monocytogenes; in vitro study. *Lebda Medical Journal*, 1: 1–5.

Sharma, D., Rani, R., Chaturvedi, M. & Yadav, J.P. 2018. Antibacterial efficacy and gas chromatography-mass spectrometric analysis of bioactive compounds present in different extracts of Allium sativum. *Asian Journal of Pharmaceutical and Clinical Research*, 11(4): 280–286.

Shaw, J.E., Sicree, R.A. & Zimmet, P.Z. 2010. Global estimates of the prevalence of diabetes for 2010 and 2030. *Diabetes Research and Clinical Practice*, 87(1): 4–14.

Sies, H. 2015. Oxidative stress: A concept in redox biology and medicine. *Redox Biology*, 4: 180–183.

Sies, H. 2000. What is oxidative stress? In *Oxidative Stress and Vascular Disease*. 1–8.

Sies, H., Berndt, C. & Jones, D.P. 2017. Oxidative Stress. *Annual Review of Biochemistry*, 86(1): 715–748.

Silva, F.M. da, Marques, A. & Chaveiro, A. 2010. Reactive oxygen species: A double-edged sword in reproduction. *The Open Veterinary Science Journal*, 4(1): 127–133.

Simonetti, G. 1990. *Simon & Schuster's guide to herbs and spices*. Stanley Schuler (ed) New York. Fireside. ISBN 0-671-73489-X

Sini, J.M., Umar, I.A. & Inuwa, H.M. 2011. The beneficial effects of extracts of hibiscus

sabdariffa calyces in alloxan-diabetic rats: Reduction of free-radical load and enhancement of antioxidant status. *Journal of Pharmacognosy and Phytotherapy*, 3(10): 141–149.

Sokeng, S.D., Lontsi, D., Moundipa, P.F., Jatsa, H.B., Watcho, P. & Kamtchouing, P. 2007. Hypoglycemic effect of Anacardium occidentale L . methanol extract and fractions on streptozotocin-induced diabetic rats. *Global Journal of Pharmacology*, 1(1): 1–5.

Sosale, A., Prasanna Kumar, K., Sadikot, S., Nigam, A., Bajaj, S., Zargar, A. & Singh, S. 2014. Chronic complications in newly diagnosed patients with Type 2 diabetes mellitus in India. *Indian Journal of Endocrinology and Metabolism*, 18(3): 355–360.

Sung, C.-C., Hsu, Y.-C., Chen, C.-C., Lin, Y.-F. & Wu, C.-C. 2013. Oxidative Stress and Nucleic Acid Oxidation in Patients with Chronic Kidney Disease. *Oxidative Medicine and Cellular Longevity*, 2013: 1–15.

Talaei, M. & An, P. 2015. Role of phytoestrogens in the prevention and management of type 2 diabetes. *World Journal of Diabetes*, 6(2): 271–283.

Tanko, Y., Abdelaziz, M., Adelaiye, A., Fatihu, M. & Musa, K. 2009. Effects of ethyl acetate portion of Indigofera pulchra leaves extract on blood glucose levels of alloxan-induced diabetic and normoglycemic Wistar rats. *Asian Journal of Medical Science*, 1: 10–14.

Teugwa, C.M., Boudjeko, T., Tchinda, B.T., Mejiato, P.C. & Zofou, D. 2013. Anti-hyperglycaemic globulins from selected Cucurbitaceae seeds used as antidiabetic medicinal plants in Africa. *BMC Complementary and Alternative Medicine*, 13(63): 1–8.

Tremblay, J. & Hamet, P. 2019. Environmental and genetic contributions to diabetes. *Metabolism Clinical and Experimental*, 100: 1–6.

Tremellen, K. 2008. Oxidative stress and male infertility: A clinical perspective. In: *Studies on Men's Health and Fertility*. Totowa, NJ: Humana Press: 243–258. http://link.springer.com/10.1007/978-1-61779-776-7_16.

Ukwenya, V.O., Ashaolu, J.O., Adeyemi, A.O., Akinola, O.A. & Caxton-Martins, E.A. 2012. Antihyperglycemic activities of methanolic leaf extract of Anacardium occidentale (Linn.) on the pancreas of streptozotocin-induced diabetic rats. *Journal of Cell and Animal Biology*, 6(11): 169–174.

Ukwubile, C.A. & Odugu, J.A. 2018. Evaluation of antibacterial and in vitro antidiabetic activities of Phyllanthus amarus Linn. (phyllanthaceae) leaf ethanol extract. *Journal of Bacteriology & Mycology: Open Access*, 6(4): 254–258.

Ushie, O.A., Neji, P.A., Etim, E.E. & Nsor, G.E. 2013. Phytochemical screening and antimicrobial activities of Phyllanthus amarus stem bark extracts. *International Journal of Modern Biology and Medicine*, 3(3): 101–112.

Verma, S., Sharma, H. & Garg, M. 2014. Phyllanthus amarus: A review. *Journal of Pharmacognosy and Phytochemistry*, 3(2): 18–22.

Wankeu-Nya, M., Florea, A., Bâlici, S., Watcho, P., Matei, H. & Kamanyi, A. 2013. Dracaena arborea alleviates ultra-structural spermatogenic alterations in streptozotocin-induced diabetic rats. *BMC Complementary and Alternative Medicine*, 13(71): 1–9.

Whiting, D.R., Guariguata, L., Weil, C. & Shaw, J. 2011. IDF Diabetes Atlas: Global estimates of the prevalence of diabetes for 2011 and 2030. *Diabetes Research and Clinical Practice*, 94(3): 311–321.

WHO. 1999b. *Definition, diagnosis, and classification of diabetes mellitus and its complications*.

WHO. 1999a. Traditional, *Complementary and Alternative Medicines and Therapies. Washington DC, WHO Regional Office for the Americas*.

WHO, 2002. *World Health Organization. 2002. WHO Traditional Medicine Strategy 2002–2005. World Health Organization, Geneva*.

Woottisin, S., Hossain, R.Z., Yachantha, C., Sriboonlue, P., Ogawa, Y. & Saito, S. 2011. Effects of orthosiphon grandiflorus, hibiscus sabdariffa and Phyllanthus amarus extracts on risk factors for urinary calcium oxalate stones in rats. *Journal of Urology*, 185(1): 323–328.

World Facts. 2018. The 10 Most Populated Countries in Africa. *World Atlas*.

World Population Prospects. 2012. *Population Division of the Department of economic and social affairs of the United Nations secretariat*.

Yaribeygi, H., Atkin, S.L. & Sahebkar, A. 2019. A review of the molecular mechanisms of hyperglycemia-induced free radical generation leading to oxidative stress. *Journal of Cellular Physiology*, 234(2): 1300–1312.

Yessoufou, A., Gbenou, J., Grissa, O., Hichami, A., Simonin, A.M., Tabka, Z., Moudachirou, M., Moutairou, K. & Khan, N.A. 2013. Anti-hyperglycemic effects of three medicinal plants in diabetic pregnancy: Modulation of T cell proliferation. *BMC Complementary and Alternative Medicine*, 13: 1–13.

Younas, M., Hano, C., Giglioli-Guivarc'H, N. & Abbasi, B.H. 2018. Mechanistic evaluation of

phytochemicals in breast cancer remedy: Current understanding and future perspectives. *The Royal Society of Chemistry Advances*, 8(52): 29714–29744.

Zaahkouk, S.A.M., Rashid, S.Z.A. & Mattar, A.F. 2003. Anti - diabetic properties of water and ethanolic extracts of Balanites aegyptiaca fruits flesh in senile diabetic rats. *Egyptian Journal of Hospital Medicine*, 10: 90–108.

Zheng, Y., Wang, Z. & Zhou, Z. 2017. MiRNAs: Novel regulators of autoimmunity-mediated pancreatic β-cell destruction in type 1 diabetes. *Cellular and Molecular Immunology*, 14(6):488–496.

CHAPTER THREE: Evaluation of antioxidant, antityrosinase activities and cytotoxic effects of *Phyllanthus amarus* Extracts

Folorunso Adewale Olabiyi[a,4], Yapo Guillaume Aboua[1], Olugbenga Kayode Popoola[2], Thomas Klaus Monsees[3], Oluwafemi Omoniyi Oguntibeju[*b]

[a*b]Oxidative Stress Research Centre, Department of Biomedical Sciences, Cape Peninsula University of Technology, Bellville 7535, South Africa; [1]Department of Health Sciences, Namibia University of Science and Technology, Private Bag 13388 Windhoek, Namibia. [2]Department of Chemistry, Ekiti State University, PMB 5363, Ado-Ekiti, Ekiti State, Nigeria. [3]Department of Medical Biosciences, University of the Western Cape, Bellville, 7535, South Africa. [4]Department of Medical Laboratory Science, Afe Babalola University, P.M.B 5454 Ado-Ekiti, Nigeria.

*Corresponding author: Telephone +27(0)219538495, Fax +27(0)219538490, E-mail: bejufemi@yahoo.co.uk, oguntibejuo@cput.ac.za

Note: Manuscript already published by the Natural Products Journal.

''Folorunso Adewale Olabiyi, *Yapo Guillaume Aboua, Olugbenga Kayode Popoola, Thomas Klaus Monsees, Oluwafemi Omoniyi Oguntibeju (2020). Evaluation of Antioxidant, Antityrosinase Activities and Cytotoxic effects of Phyllanthus amarus Extracts. The Natural Products Journal. 10(2), 130-138. https://doi.org/10.2174/2210315509666190405100745".*

ABSTRACT

Background: In recent time, there has been much attention on the antioxidant and antityrosinase potentials of medicinal plants and their various health benefits. *Phyllanthus amarus* (PA*)* is widely used, but there is a scarcity of empirical data on its in-vitro skin depigmentation activity and cytotoxicity, and its impact on mediators of reactive oxygen species (ROS). In the present study, we evaluated its tyrosinase inhibitory efficacy, antioxidant potentials and cytotoxic activities.

Methods: In this study, quantitative determination of phytochemicals, oxygen reducing antioxidant capacity (ORAC), trolox equivalent antioxidant capacity (TEAC) were carried out on Phyllanthus amarus extracts. Antityrosinase efficacy of the hexane, methanol and aqueous extracts was evaluated with ELISA method. Cytotoxicity studies were done with TM4 (mouse Sertoli) cells using colourimetric MTT assay and cell counts.

Results: Hexane and aqueous extracts demonstrated significant antityrosinase activity ($p < 0.05$) (IC_{50} = 116.08 and 129.25 µg/mL respectively) while its methanolic extract did not give any significant effect. Higher levels of the phytochemicals (total polyphenol, flavonoids and flavonol) were observed in the methanolic fraction of the extract. Also, higher radical cation scavenging (TEAC) activity was observed in the aqueous extract. These values were significant ($p < 0.0001$), whereas ORAC results of the methanol extract showed significantly ($p < 0.0001$) higher oxygen reducing antioxidant potential than the aqueous extract. The aqueous extract displayed the highest mitochondrial dehydrogenase activity at lower concentrations (0.01 to 10 µg/mL). TM4 cell numbers were also significantly higher compared to the untreated control. Sertoli cell viability was compromised after exposure to higher extract concentrations (100 to 1000 µg/mL).

Conclusion: Hexane and aqueous extracts of *Phyllanthus amarus* possess better antityrosinase action when compared to the standard drug (kojic acid). Its high antioxidant potentials by its ability to scavenge oxygen radicals, reduce ferric ion and inhibit ABTS radical may be related to its phytochemicals. Extracts at lower concentrations stimulated Sertoli cell proliferation, which possibly may be due to its phytoestrogenic activities conferred by its active components such as phyllanthin and hypophyllanthin.

Keywords: *Phyllanthus amarus*, tyrosinase inhibition, antioxidant, cytotoxicity, Sertoli cells, oxidative stress

3.1. Introduction

Several plants have therapeutic potentials to ameliorate various pathological conditions: hence, their medicinal uses [1]. Some of these medicinal plants possess abundant antioxidants. Essential sources of antioxidants include fruits and vegetables, nuts, cocoa, whole grains, olive oil, and beverages, such as coffee and tea. These abundant micronutrients in human diet possess protective role against non-communicable, chronic diseases [1]. Due to their antioxidant contents, some of them can scavenge high levels of reactive oxygen species (ROS) and free radicals generated by the body system. An imbalance in the levels of ROS generated against the antioxidants defence system in an organism result in oxidative stress [2], and this can lead to severe cellular, tissue, organ, and system damage. Excess production of ROS can also lead to inflammation, premature ageing disorders, including an uncontrolled formation of dark macromolecular pigments in the outer surface of the skin [3], and several disease conditions, such as cancer, diabetes, and atherosclerosis [4].

Synthetic and conventional drugs are usually prescribed to treat and manage various disease conditions. These drugs have attendant side effects, which may make them intolerable to some patients. On the other hand, phytochemicals, which are natural products, possess various health benefits, they are thus considered safer and healthier [5]. Among others, their antioxidant and tyrosinase inhibitory potentials are now being studied in both the food and cosmetic industries. Tyrosinase is a key enzyme in the biosynthesis of melanin and browning of fruits [6]. Their inhibitors are, therefore, important targets to slow down the process of browning and reduce melanin formation. Also, cosmetic industries use tyrosinase inhibitors, especially, in skin-whitening products and in treating various dermatological disorders due to the accumulation of

an excessive level of epidermal pigmentation, including life-threatening melanoma [7; 8]. In the same vein, antioxidants may help to scavenge the deleterious effects of free radicals which the disease process may generate (9).

Phyllanthus amarus (*P. amarus*) is a herbaceous plant of the Euphorbiaceae family. It can be found in the tropical regions of the world, including Southern India, China, and some Western African countries [10; 11]. Other names of this plant include stone breakers, leaf flower, and seed-under-leaf. It is often referred to as ''Iyin olobe'' by the Yoruba tribe of the South-Western Nigeria, where the water decoction of its leaf and seed, is reputably used for the local management of diabetes, obesity and hyperlipidaemia [12]. Its ethnomedicinal uses include topical application to treat skin ulcers, sores, swelling and itchiness, wounds, bruises, scabies, oedematous swellings, tubercular ulcers, ringworm, and crusty lesions [13]. Phytochemical studies revealed that *P. amarus* has numerous phyto-constituents such as polyphenols and tetracyclic triterpenoids [14], flavonoids [13], tannins [15; 16], lignans [17; 18; 14] and alkaloids [19]. Extracts and certain compounds isolated from *P. amarus* show a wide spectrum of pharmacological activities including antiviral, anti-diabetic, anti-inflammatory, analgesic, anti-tuberculosis, antibacterial, hypolipidaemic, antioxidant, hepatoprotective, nephroprotective and diuretic properties [20;21].

A histopathological report of the *Phyllanthus amarus* aqueous extracts fed to healthy male rats showed marked testicular degeneration with severe disorganization of seminiferous tubules [22]. However, the mechanism of this disorder remains unclear. It is thus, imperative to investigate the effects of the extracts on Sertoli cells as representative of what goes on in the hypothalamic-pituitary-testicular axis. Also, in vitro skin depigmentation activity of this plant as well as its relationship with other mediators of ROS causing diseases has not been investigated. More so, synthetic tyrosinase inhibitors such as kojic acid and hydroquinone have been effectively used as depigmentation agents, but their side effect is gaining increasing attention in the cosmetic industry. They have been found to cause severe skin inflammation; hence, the need to explore the use of natural, plant-derived metabolites to treat skin diseases. The objective of the present study, therefore, was to evaluate the tyrosinase inhibitory, as well as the anti-oxidative and cytotoxic actions of *Phyllanthus amarus* extracts.

3.2. Materials and Methods

3.2.1. Chemicals

4-dimethylaminocinnamaldehyde (DMACA), kojic acid, mushroom tyrosinase, 3,4-dihydroxy-L-phenylalanine (L-DOPA) (MERCK, South Africa) dimethyl sulfoxide (DMSO), Folin-Ciocalteau's phenol reagent, Sodium carbonate (Na_2CO_3), Sodium hydroxide (NaOH), Trolox [6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid], AAPH (2, 2'-azobis-2-methyl-propanimidamide, dihydrochloride), ABTS (2,2'–azino-di-3-ethylbenzthialozine sulphonate) (MERCK, South Africa), Fluorescein. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). DMEM-12(1:1 (1x) culture medium (GIBCO, UK), 10% Foetal Bovine Serum, 1% Penicillin (1000U/mL) and 1% Streptomycin (1000U/mL) (LONZA, Germany) were used. All other chemicals used were of analytical reagents grade.

3.2.1.1. Plant materials

Fresh whole plants of *Phyllanthus amarus* were collected from Afe Babalola University, Ado-Ekiti, Nigeria. The plant was identified and authenticated by a botanist at the taxonomy section of the forestry herbarium, Forestry Research Institute of Nigeria (FRIN), Ibadan; where a voucher specimen was deposited (FHI NO: 110242).

3.2.1.2. Preparation of crude extracts

Shade-dried leaves of *Phyllanthus amarus* Schum and thonn were milled to powder with the aid of a mechanical miller (Mincer 9FQ-28, China) at Afe Babalola University, Ado-Ekiti, Nigeria. Each time, about 63 g of the powder was extracted using 125 mL of 70% methanol by Soxhlet method for 8 hours. The solvent extracts were then filtered and evaporated using a rotary evaporator (Buchi Rotavapor II, Buchi Germany) at 40^{o}C. The crude extracts were shaken with absolute methanol and defatted with n-hexane (3 times), ethyl acetate (3 times) leaving the aqueous fraction. Each fraction was dried under reduced pressure and kept at 4^{o}C until ready for use. The aqueous fraction was lyophilized in a freeze-drying system (VirTis Genesis 25EL, SP Scientific, NY, USA) for 16 hours, and the product was kept at 4^{o}C until ready for further use.

3.2.2. Antioxidant Activities

3.2.2.1. Determination of total polyphenol

Phyllanthus amarus extracts were determined using Folin-Ciocalteau's assay in which total polyphenol content in the extract reacts with the phenol reagent, while gallic acid serves as the standard according to the method described by Waterhouse [23]. 25µL sample/standards were pipetted into a 96-wells plate, followed by 125 µL of 200 mM Folin-Ciocalteau reagent and allowed to stand for 5mins at room temperature. 100 µL of 7.5%$^{w}/_{v}$ Na_2CO_3 was added, and the plates incubated at room temperature for 2 hours before reading.

3.2.2.2. Determination of Flavonoids

Total flavonoid content was determined by a modified aluminium chloride colourimetric method [24]. In this method, appropriate dilutions of sample extracts were reacted with sodium nitrite, which resulted in a flavonoid-aluminium complex formation using aluminium chloride. On a 96-well assay plate, 50 µL diluted sample/standards were pipetted in triplicates. This was followed by the addition of 42 µL of distilled water. 60 µL of sodium nitrite was then added to each well and mixed. After 10 minutes of incubation at room temperature, 60 µL of Aluminium chloride was added to each well, mixed and allowed to stand at room temperature for 5 minutes. 60 µL of Sodium hydroxide was added lastly to each well, and mixed. The absorbance was measured at 510 nm and compared to that of catechin standards

3.2.2.3. Determination of Flavonols

The analysis makes use of quercetin as the standard for measuring flavonol at 360 nm. On a 96-well assay plate, 12.5 µL of diluted samples/standards were pipetted in triplicates. This was followed by addition of 237.5 µL of 2% HCL to each well. Plates were left for 30 minutes at room temperature before absorbance was measured.

3.2.2.4. Determination of Flavanol

Flavanol content of *Phyllanthus amarus* extract was determined using p-dimethylaminocinnamaldehyde procedure [25]. This analysis makes use of 4-dimethylaminocinnamaldehyde (DMACA), which reacts with flavanols to form a characteristic light blue colour that is measured at 640 nm. On a 96-well assay plate, 50 µL of diluted sample/standards were pipetted in triplicates. This was followed by addition of 250 µL of DMACA to all the wells. The plate was incubated at room temperature for 30 minutes before absorbance readings were measured.

3.2.2.5. Determination of Ferric Reducing Antioxidant Power

The Ferric Reducing Antioxidant Power (FRAP) assay uses antioxidants as reductants in a redox-linked colourimetric method [26], employing an easily reduced oxidant present in stoichiometric excess. At low pH, reduction of a ferric tripyridyl triazine (Fe^{2+}-TPTZ) complex to the ferrous form, which has an intense blue colour, can be monitored by measuring the change in absorption at 593 nm. The change in absorbance, therefore, is directly related to the reducing power of the electron-donating antioxidants present in the reaction mixture. On a 96-well assay plate, 10µL of diluted sample/standards were pipetted in triplicates. This was followed by addition of 300 µL of FRAP working reagent to each well. The plate was incubated for 30 mins in a 37^{o}C oven before being read in a microplate.

3.2.2.6. Determination of Oxygen Reducing Antioxidant Capacity

Oxygen reducing antioxidant capacity of *Phyllanthus amarus* extracts were determined by a method of Prior *et al.,* [27]. The assay measures the loss of fluorescence over time due to peroxyl-radical formation by the breakdown of AAPH (2, 2'-azobis-2-methyl-propanimidamide, dihydrochloride). Trolox [6-Hydroxy-2, 5,7,8-tetramethylchroman-2-carboxylic acid], a water-soluble vitamin E analogue, serves as the reference standard. The Oxygen Reducing Antioxidant Capacity (ORAC) assay is a kinetic assay measuring fluorescein decay and antioxidant protection over time. A peroxyl radical (ROO.) is formed from the breakdown of

AAPH (2,2'-azobis-2-methyl-propanimidamide, dihydrochloride) at 37^{o}C. The peroxyl radical can oxidize fluorescein (3',6'-dihydroxy-spiro[isobenzofuran-1[3*H*],9'[9*H*]-xanthen]-3-one) to generate a product without fluorescence. Antioxidants suppress this reaction by a hydrogen atom transfer mechanism, inhibiting the oxidative degradation of the fluorescein signal. The fluorescence signal is measured over 120 minutes by excitation at 485 nm, emission at 538 nm. The concentration of antioxidant in the test sample is proportional to the fluorescence intensity through the course of the assay and is assessed by comparing the net area under the curve to that of a known antioxidant, Trolox. Briefly, in a 1.5 mL eppendorf tubes, a fifty-fold diluted sample in 10% methanol was made, followed by suitable dilutions of Trolox standard to get appropriate concentrations according to the standard operating procedure (SOP). On a 96-well assay black plate, 12 µL of diluted sample/standards were pipetted in triplicates, followed by 138 µL of diluted fluorescein in phosphate buffer (p^{H} 7.4). Plates were incubated at 37^{o}C for 20 minutes inside the Fluorometer (FLUOROSKAN ASCENT by Thermo Electron Corporation, MA, USA). This was followed by the addition of 50 µL AAPH to all the wells. The plates were read inside the machine every minute for 120 minutes.

3.2.2.7. Determination of Trolox Equivalent Antioxidant Capacity

Trolox equivalent antioxidant capacity of the extracts was determined using an improved ABTS radical cation assay of Re et al., [28]. The method was based on the ABTS (2,2'–azino-di-3-ethylbenzthialozine sulphonate) radical cation scavenging assay. Methyl myoglobin activated to its ferryl state by hydrogen peroxide is incubated with ABTS to form a stable, long-lived radical cation, $ABTS^{\cdot+}$

$$HX\text{-}Fe^{3+} + {}^{\cdot}X\text{-}[Fe^{4+}=O]\ {}^{\cdot}X\text{-}[Fe^{4+}=O] + ABTS \rightarrow ABTS^{\cdot+} + HX\text{-}Fe^{3+}$$

Where, $HX\text{-}Fe^{3+}$ = metmyoglobin

${}^{\cdot}X\text{-}[Fe^{4+}=O]$ = ferrylmyoglobin

The radical cation can be monitored by measurement of one of its characteristic absorption maxima at 640, 734 and 820 nm. Antioxidants added to this system can either scavenge the $ABTS^{\cdot+}$ formed or interfere in the radical generating process. In this assay, in a 1.5 mL eppendorf tubes, a five-fold diluted sample in 10% methanol was made, followed by suitable dilutions of Trolox standard. On a 96-well assay plate, 25 µL of diluted sample/standards were pipetted in

triplicates. This was followed by addition of 300 µL of ABTS working reagent to each well. The plate was incubated for 30 minutes at room temperature.

3.2.3. Determination of tyrosinase inhibitory activity

The tyrosinase inhibitory activities of the extract were determined by the method described by Curto et al. [29]. Extracts were dissolved in DMSO (Dimethyl sulphoxide) to a final concentration of 1 mg/mL. Graded doses of 1000 µg/mL, 500 µg/mL, 250 µg/mL, 100 µg/mL, and 50 µg/mL of the extracts were prepared using 50 mmol/L, potassium phosphate buffer (pH 6.5). Kojic acid was used as a control. Briefly, 70 µL of the sample in dimethyl sulfoxide (DMSO) was introduced to each well of a 96-well plate before adding 30 µL of tyrosinase enzyme solution (0.002 mg/mL). After 5 minutes, 2.0 mmol/L of L-tyrosine solution was pipetted into the wells containing sample extracts. The absorbances were taken in a microplate ELISA reader (Multiskan Spectrum by Thermo Electron Corporation, MA, USA) at 492 nm after 30 minutes of incubation. Kojic acid serves as the positive control. The percentage inhibition was calculated as follows:

$$\%\ \boldsymbol{inhibition} = \left\{\frac{\left[\boldsymbol{A_{control} - A_{sample}}\right]}{\boldsymbol{A_{control}}}\right\} \boldsymbol{x\ 100}$$

3.2.4. Cytotoxicity Studies

3.2.4.1. Evaluation of cytotoxicity using Colorimetric MTT assay

The cytotoxicity activities of the extracts were determined by a modified MTT method (Mosmann; Lappalainen et al., [30; 31]. The assay is based on the reduction of yellow MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] by the mitochondrial dehydrogenase enzyme of viable cells to blue formazan crystals.

3.2.4.2. Cell culture

TM4 mouse Sertoli cells were purchased from ATCC (USA, no CRL-1715). TM4 cells were seeded at a density of 4000 cells/ 200µL/well in a 96 well plate. They were allowed to attach and grow for 24 hours in a complete culture medium which comprises of DMEM supplemented with

2.5% foetal bovine serum, 5% horse serum and 1% Penicillin (1000 U/mL) and 1% Streptomycin (1000U/mL antibiotics, to be treated the next day. Extracts were dissolved in 0.1% DMSO (Dimethyl sulphoxide) to a final concentration of 1 mg/mL. Serial dilutions from 1000 µg/mL, to 100 µg/mL, 10 µg/mL, 0.1 µg/mL and 0.01 µg/mL of the extracts were prepared using complete growth DMEM medium. Four wells were used for each concentration of the extracts. Another four wells were used for untreated negative control (0.1% DMSO in DMEM) and four wells for 10% DMSO as a positive control. After incubation for 24 hours, the media were discarded from the plates and cells were then washed with PBS (100 µL / well). 200 µL of fresh complete culture media were added to each well, followed by 20 µL of MTT solution. After that, the plates were kept in the incubator at 37^{o}C, 5% CO_2 and 95% humidified air. After 4 hours' incubation, the supernatant was discarded, and the plate tapped upside down on paper towels to remove all remaining fluids. 100 µL of non-sterile, undiluted DMSO was added to each well to dissolve the blue formazan, and the absorbance was then measured with microplate (MTP) reader (POLAR STAR OMEGA, BMG LABTECH) at a wavelength of 570 nm with a reference wavelength of 690 nm to negate the effect of cell debris and precipitated proteins which may be produced by the dissolving process. The optical density was calculated by subtracting the absorbance of the reference wavelength from the absorbance of the test wavelength. The mitochondrial dehydrogenase activity of TM4 cells, which is a measure of the viability of the cells [32], was calculated as a percentage according to the following equation:

$$\%\, Viability = \left\{\frac{Absorbance\ of\ treated\ cells}{Absorbance\ of\ untreated\ cells}\right\} x100$$

3.2.4.3. Study of the morphological changes

Cells that were seeded at a density of 2000 cells/200µL/well in a 96 well plate were used to analyse morphological changes produced by the plant extracts. Cells were allowed to attach and grow for 24 hours in complete culture medium to be treated with varying concentrations of the extracts for another 24 hours. Cells were observed under an inverted system microscope (200x and 400x magnifications) and microphotographs were taken. At least five random microscopic visible fields for each concentration and compound were analysed for cell counts.

3.2.5. Statistical Analysis

All experiments were carried out at least three times, having similar findings and the representative results were reported. Calculations were performed on Excel. A comparison was done using GraphPad Prism software, version 8.0 (GraphPad Software, San Diego, California, USA). With mean ± SD, $p < 0.001$ and $p < 0.05$ were considered statistically significant.

3.3. Results

As graphically depicted in figure 3.1, showing the polyphenols present in the extracts, findings in this study, revealed a significantly ($p < 0.0001$) higher total polyphenol (1540.98 ± 127.25 vs 1117.17 ± 74.37 mg gallic acid equivalent (GAE)/g sample), flavonoids (632.45 ± 58.54 vs 414.59 ± 35.74 mg catechin equivalent (CE)/g sample) flavonol (824.26 ± 34.62 vs 270.33 ± 138.48 mg quercetin acid equivalent (QAE)/g sample), but rather a significantly ($p < 0.0001$) lower flavanol (63.80 ± 20.56 vs 94.87 ± 15.56 mg catechin equivalent (CE)/g sample) were observed in the methanol fraction of the extract when compared to the aqueous fraction. Also, as represented in figure 3.2, which shows the antioxidant capacity of the extracts, we observed a significantly ($p < 0.0001$) higher ferric reducing antioxidant power (11703.05 ± 570.34 vs 6512.14 ± 385.58 µmol Vit.C/g sample) and radical cation scavenging (TEAC) activity (1430.23 ± 113.56 vs 613.63 ± 47.11 µmol trolox equivalent (TE)/g sample in the aqueous extract as against the methanolic extract. Conversely, the ORAC results of the methanolic extract (10439.37 ± 971.19 µmol trolox equivalent (TE)/g sample, is significantly ($p < 0.0001$) higher than that of the aqueous extracts showing 3614.92 ± 204.25 µmol trolox equivalent (TE)/g sample. Figure 3.3 depicts the results of antityrosinase activities of Hexane, methanol, and aqueous extracts of *Phyllanthus amarus* when compared to reference kojic acid. Our results revealed that hexane and aqueous extracts exhibit significant anti-tyrosinase activity ($p < 0.05$) while its methanolic extract produces no statistically significant finding.

Figures 3.4 to 3.6 show the significant proliferative effect of the extracts as seen at the lower concentrations of the extracts (0.01 µg/mL – 100 µg/mL) when compared with the negative control. The standard ibuprofen drug also exhibits proliferative effects when compared with the negative controls and 1000 µg/mL of all the extracts produced dead, inactive, or non-viable cells in a similar manner to the positive controls.

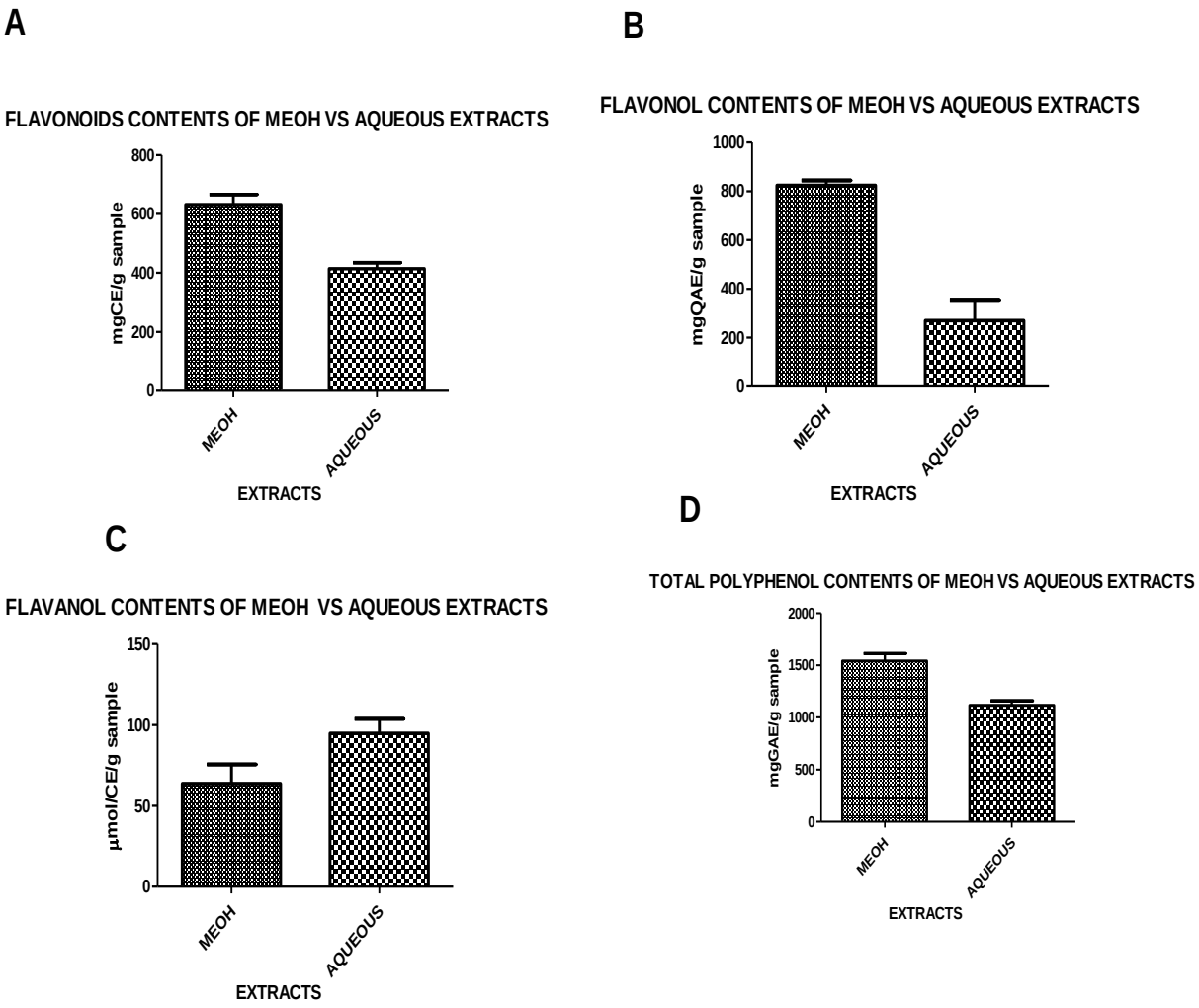


Figure 3.1: Polyphenols content of extracts

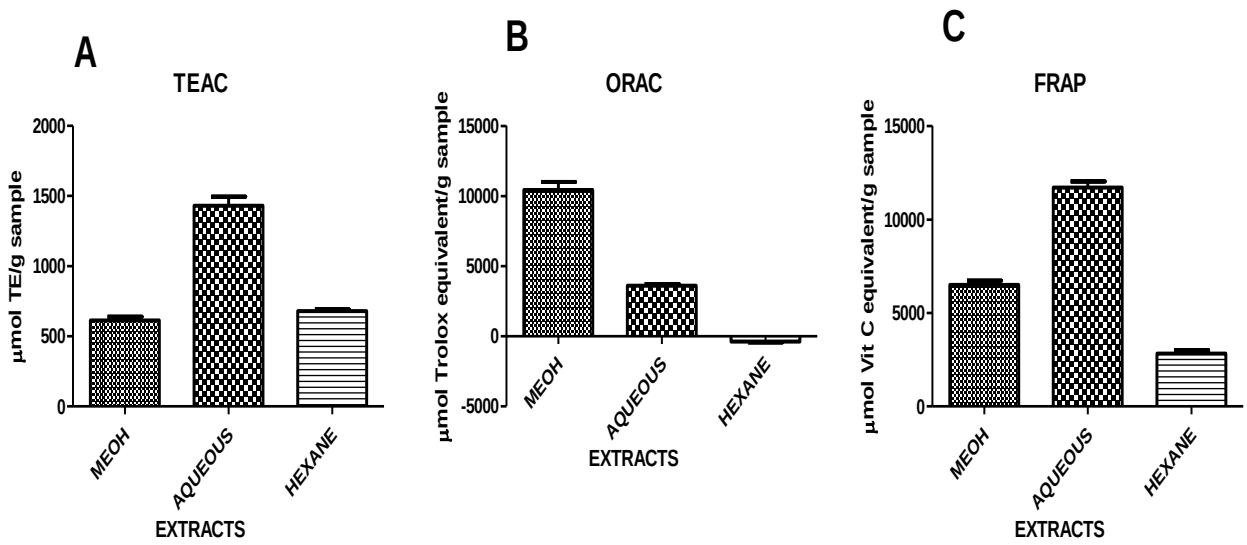


Figure 3.2: Antioxidant capacity of extracts

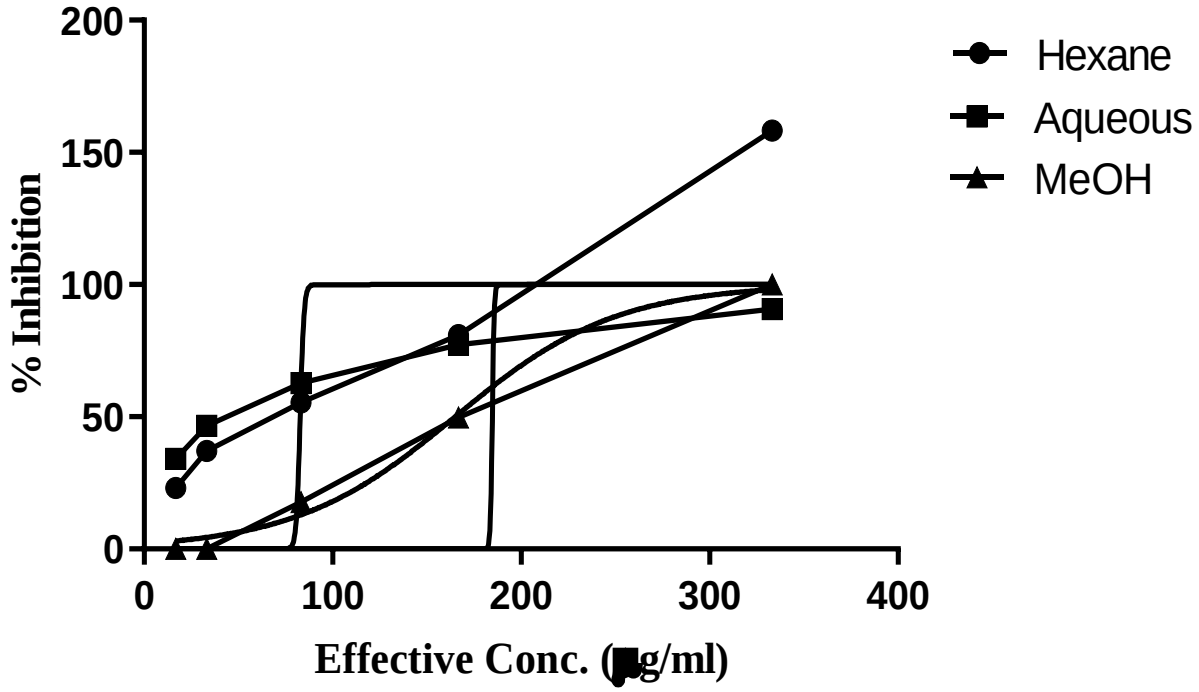


Figure 3.3: Antityrosinase activities of extracts

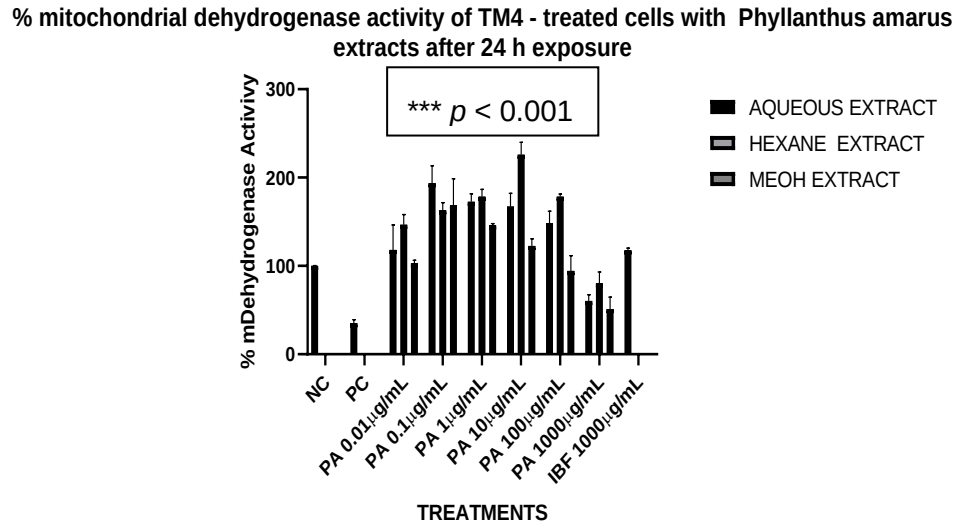


Figure 3.4: Mitochondrial dehydrogenase activity (%) of TM4 – treated cells,

with significant proliferation at 0.1 and 10 µg/mL of Phyllanthus amarus aqueous extract (***$p < 0.001$)

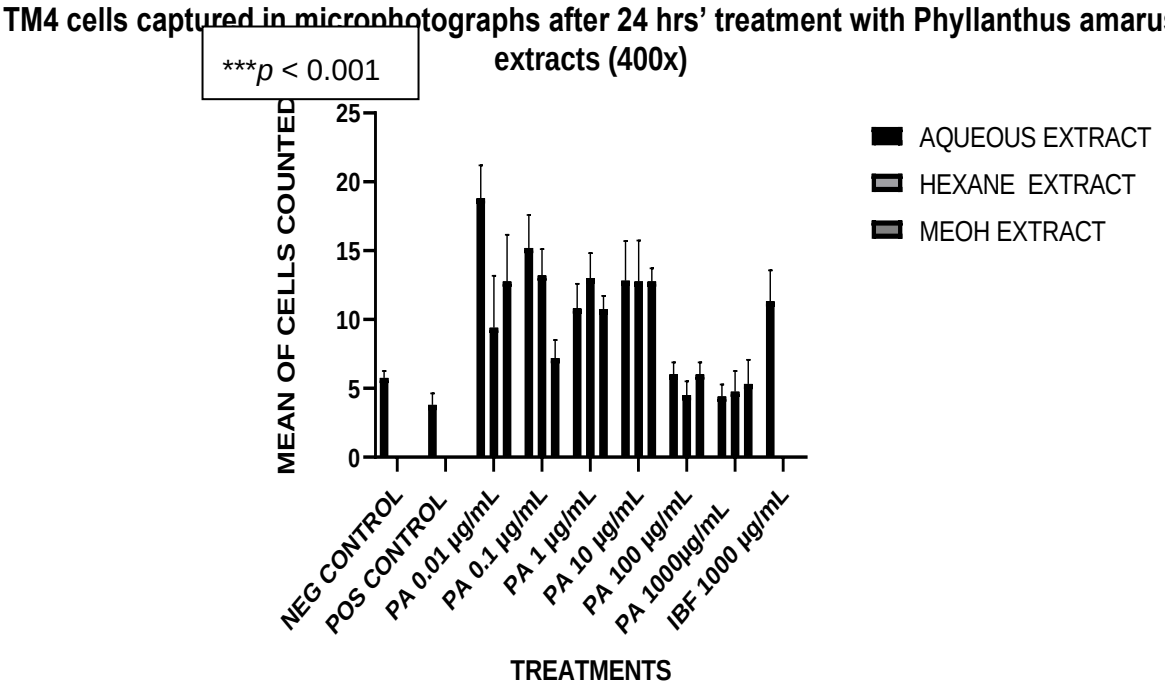


Figure 3.5: TM4 cell numbers counted in microphotographs after 24hrs exposure (400x), showing significant proliferation at lower concentrations (***$p < 0.001$)

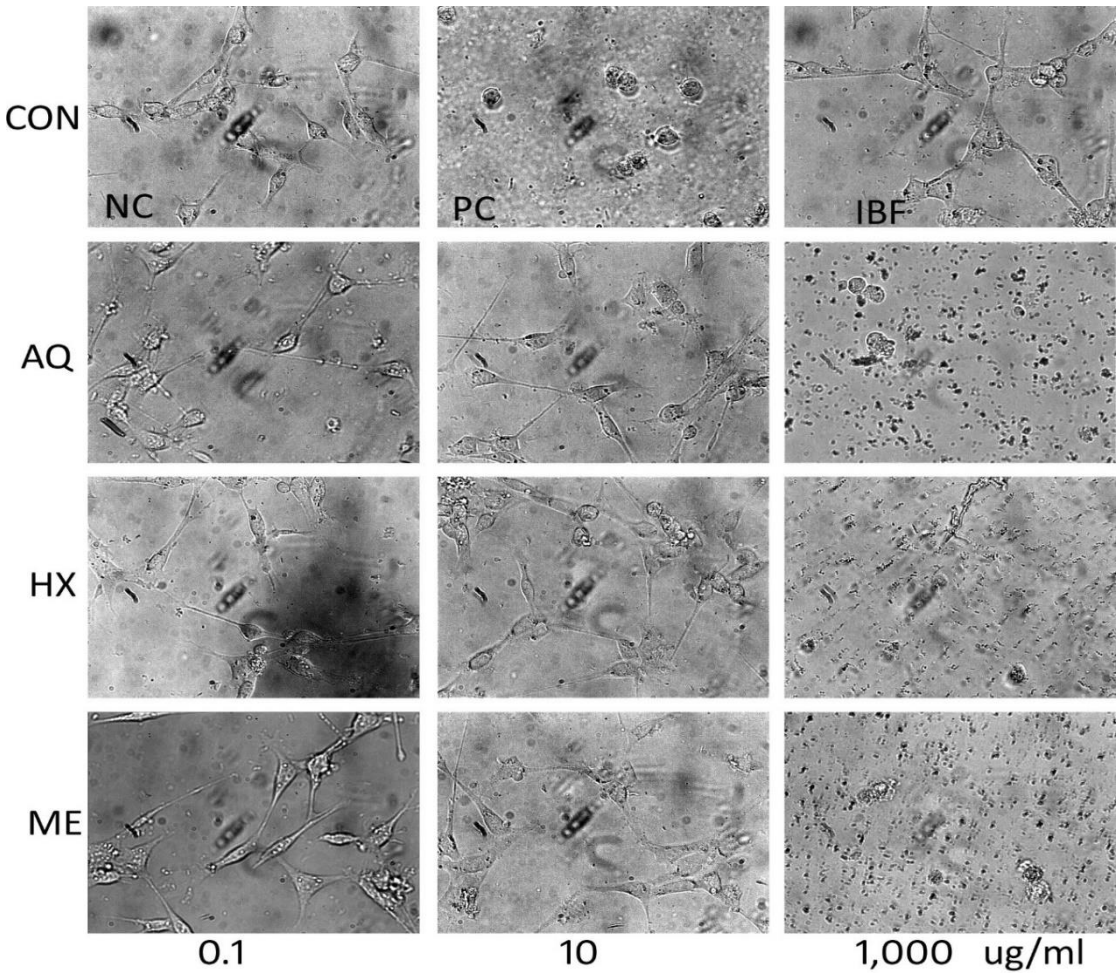


Figure 3.6: Microphotographs showing morphological changes in Phyllanthus amarus–treated mouse Sertoli (TM4) cells after 24 hours of exposure (400x).

CON, Controls; AQ, Aqueous extract; HX, Hexane extract; ME, Methanol extract; IBF, Ibuprofen.

3.4. Discussion

Scientific research has found an association between herbal teas, their polyphenols and cardiovascular disease (CVD), diabetes, cancer, and cognition, and it is opined that tea consumption greatly contributes to human health [33]. *Phyllanthus amarus,* a medicinal plant, used in this study is also consumed as a herbal tea, and the results from our study show that it has high polyphenols, which are regarded as naturally occurring antioxidants. They possess the potentials to scavenge free radicals due to their hydroxyl group [34]. They have been shown to contribute to high antioxidant activity observed in some extracts through different mechanisms exerted by different phenolic compounds or through synergistic effects with other non-phenolic

compounds [35]. The results presented in this study revealed the antioxidant capacity of the extracts with the use of different solvents for extraction. This is because not all extracts exhibited the same pattern due to their different antioxidant mechanisms [6]. Besides, the reaction mechanisms of the antioxidant assays differ [36]. These results show that higher antioxidant activity was displayed by the aqueous fraction (figure 3.1), water, being a more polar solvent. This is consistent with the findings, as reported by Mazlan et al. (2013) in a similar study. Also, as shown, higher levels of FRAP and TEAC were observed in the aqueous extract, whereas the methanol extract shows higher ORAC results than the aqueous extract (figure 3.2).

In the present study, the correlation between total antioxidant capacities and the contents of total polyphenol and flavonoid components of *P. amarus* was evaluated. The results revealed that there was no linear correlation between antioxidant properties and total polyphenol or flavonoids contents of the examined extracts. The lack of a linear correlation between these assays of the extracts might result from the existence of other compounds synergistically responsible for the antioxidant properties present in *P. amarus* extracts.

Moreover, it has been reported that samples with high antioxidant activity can be associated with significant antityrosinase activity, as both play essential roles in preventing free radical-related skin damage [9]. In the present study, the aqueous extract was the most active with the least inhibitory concentration at 50% inhibition (IC_{50} = 129.256 µg/mL), followed by hexane fraction (IC_{50} = 116.089 µg/mL) (figure 3.3) and they both exhibited significant tyrosinase inhibitory activity ($p < 0.05$) while its methanol extract produces no significant finding when compared with the kojic acid standard. In the same vein, the antioxidant potential was exhibited by the aqueous extract of this plant as evident in the FRAP and TEAC activities, suggesting this plant as a candidate for a natural alternative agent in food processing, cosmetic and health industries.

Sertoli cells in the testes are known to play a critical role in spermatogenesis [32]. Giving aqueous *Phyllanthus amarus* extract to male rats caused a severe disruption of sperm production with a marked degeneration and disorganization of seminiferous tubules [22]. Our study supports these findings as MTT assay (Figure 3.4) and morphology analysis (Figure 3.6) showed that all *Phyllanthus amarus* extracts tested were cytotoxic to TM4 Sertoli cells at a higher concentration of 100 or 1000 µg/mL. Mitochondrial dehydrogenase activities were lower in the higher concentrations of the extracts when compared with the negative control, and the cells were dead (1000 µg/mL) or showed some rounding and shrinkage at a concentration of 100 µg/mL. Cytotoxicity seems to vary within the three extracts, with MeOH extract being the most cytotoxic. This could be due to differences in the contents of the extracts. The results here

demonstrated decreased mitochondrial dehydrogenase activity as well as lower cell numbers and cell death at a concentration above 100 µg/mL for all the three extracts. This suggests compromised Sertoli cells viability, which eventually, may lead to impaired spermatogenesis. Also, it is worthy of note that ibuprofen was not cytotoxic at 1000 µg/mL and did not show any effect on cell morphology.

In contrast, in the lower concentration range of *Phyllanthus amarus* extracts, TM4 cells appeared to be well spread and healthy, but the mitochondrial dehydrogenase activities showed a significant concentration-dependent increase for all extracts tested. As the cell morphology indicates vital cells, this increased mitochondrial dehydrogenase activity is indicative of a marked increase in cell numbers rather than elevated cellular stress. This view is supported by cell counts from the microphotographs (Figure 3.5). At concentration ranging from 0.01 to 10 µg/mL extract, TM4 cell numbers were significantly higher compared to the untreated control. This significant stimulation of Sertoli cell proliferation could be due to estrogenic properties of the *Phyllanthus amarus* extracts. It is known that some of its components (e.g. Phyllanthin), are active phytoestrogens [37; 38]. On the other hand, TM4 Sertoli cells are isolated from immature rats; at this age, Sertoli cells express oestrogen receptors and cell proliferation can be activated by oestrogen exposure [39].

A report shows that bioflavonoids, a group of natural antioxidants which are widely distributed in fruits and vegetables, have a protective effect on the DNA damage induced by the hydroxyl radicals [40]. The observations in the present study could be due also to antioxidant substances present in the extracts that are known to display biphasic properties, being antioxidant at low concentrations and pro-oxidant at high concentrations. These properties may confer protection against oxidative stress and lipid peroxidation, in a way, that is promoting cell proliferation, especially at the lower concentrations [41; 42]. Therefore, determining the activities of superoxide dismutase and catalase in an in-vivo study with this plant extract would lend credence to these reports and may confirm testicular oxidative stress.

3.5. Conclusion

Results from this study showed that the aqueous extract demonstrated antioxidant capacity as evident in the FRAP and TEAC measurements. While the hexane and aqueous extracts possess good antityrosinase action, suggesting this plant as a candidate for a natural, alternative agent in food processing, cosmetic and health industries. Lower extract

concentrations stimulate Sertoli cell proliferation which might be due to phytoestrogenic activities of *Phyllanthus amarus* conferred by its active components such as phyllanthin and hypophyllanthin. In contrast, higher concentrations are cytotoxic. Further studies might be required to isolate and characterize the active principles in the extracts.

3.6. Ethical Approval

Institutional ethics approval was granted by the CPUT Health and Wellness Research Ethics Committee (Ref No: CPUT/HW-REC 2016/A1)

3.7. Human and Animal rights

Not applicable.

3.8. Consent for Publication

Not Applicable

3.9. Funding

Funding was received from the Cape Peninsula University of Technology (CPUT), and the National Research Foundation (NRF) granted to Prof OO Oguntibeju, South Africa with Grant numbers: RJ24 and RO22, respectively.

3.10. Conflict of interest

No conflict of interest.

3.11. List of abbreviations

ABTS	2,2'–azino-di-3-ethylbenzthialozine sulphonate
AAPH	2,2'-azobis-2-methyl-propanimidamide
AQ	Aqueous
Conc.	Concentration
CVD	Cardiovascular disease
DMACA	4-dimethylaminocinnamaldehyde

DMSO	Dimethyl sulfoxide
DMEM F-12	Dulbecco's Modified Eagle medium (HAM) [+] L-Glutamine
FRAP	Ferric reducing antioxidant power
FHI	Forestry herbarium Ibadan
IBF	Ibuprofen
KAE	Kojic acid equivalent
MEOH	Methanolic
ORAC	Oxygen reducing antioxidant capacity
ROS	Reactive Oxygen Species
SD	Standard deviation
TEAC	Trolox equivalent antioxidant capacity
TPTZ	Tripyridyl triazine

3.12. Acknowledgements

The financial support from the National Research Foundation NRF in the form of a grant awarded to Professor OO Oguntibeju (R022) and the University Research Fund (URF) of the Cape Peninsula University of Technology, South Africa is hereby acknowledged and appreciated. Mr Fanie Rautenbach is also thanked for his technical assistance.

References

[1] Olabiyi F.A., Aboua, YG. & Oguntibeju, O.O. 2018. Bioactive compounds of medicinal plants-properties and potential for human health. In: Innovations in plant science for better health: From soil to fork. Megh R. Goyal and Ademola O. Ayeleso (Eds). 187-208. Apple Academic Press (NY).

[2] Tremellen, K. 2008. Oxidative stress and male infertility: a clinical perspective. Human Reproduction Update, **14**(3), 243-258.

[3] Chompo, J., Upadhyay, A., Fukuta, M. & Tawata, S. 2012. Effect of *Alpinia zerumbet* components on antioxidant and skin disease-related enzymes. *BMC Complementary and Alternative Medicine*, **12**, 106-114.

[4] García-Lafuente, A., Guillamón, E., Villares, A., Rostagno, M.A. & Martinez, J.A. 2009. Flavonoids as anti-inflammatory agents: implications in cancer and cardiovascular disease. *Inflammatory Research,* **58**(9), 537-552.

[5] Pham-Huy, L.A., He, H. & Pham-Huy, C. 2008. Free radicals, antioxidants in disease and health. *International Journal of Biomedical Science,* **4**(2), 89.

[6] Mazlan, N.A., Mediani, A., Abas, F., Ahmad, S., Shaari, K., Khamis, S. & Lajis, N.H. 2013. Antioxidant, antityrosinase, anticholinesterase, and nitric oxide inhibition activities of three Malaysian Macaranga species. *The Scientific World Journal*, volume *2013*. Article ID 312741. 8 pages.

[7] Kim, Y.J. & Uyama, H. 2005. Tyrosinase inhibitors from natural and synthetic sources: structure, inhibition, mechanism, and perspective for the future. *Cellular and Molecular Life Sciences,* **62** (15), 1707-1723.

[8] Chang, T.S. 2009. An updated review of tyrosinase inhibitors. *International Journal of Molecular Sciences*, **10**(6), 2440-2475.

[9] Ko, H.H., Chang, W.L. & Lu, T.M. 2008. Antityrosinase and antioxidant effects of ent-kaurane diterpenes from leaves of Broussonetia papyrifera. *Journal of Natural products*, **71**(11), 1930-1933.

[10] Karuna, R., Bharathi, V.G., Reddy, S.S., Ramesh, B. & Saralakumari, D. 2011. Protective effects of Phyllanthus amarus aqueous extract against renal oxidative stress in Streptozotocin-induced diabetic rats. *Indian Journal of Pharmacology,* **43**(4), 414.

[11] Tahseen, M. & Mishra, G. 2013. Ethnobotany and Diuretic activity of some selected Indian medicinal plants. *The Pharma Innovation*. **2**, 112.

[12] Adeneye, A.A. 2012. The leaf and seed aqueous extract of *Phyllanthus amarus* improves insulin resistance diabetes in experimental animal studies. *Journal of Ethnopharmacology,* **144**(3), 705-11.

[13] Verma, S., Sharma, H. & Garg, M. 2014. *Phyllanthus amarus*: A Review. *Journal of Pharmacognosy & Phytochem*istry, **3**(2),18-22.

[14] Maciel, M.A.M., Cunha, A.F., Dantas, N.T.C. & Kaiser, C.R. 2007. NMR characterization of bioactive lignans from Phyllanthus amarus Schum and thonn. *Journal of Magnetic Resonance Imaging*, **6**(3), 76-82.

[15] Foo, L.Y. & Wong, H. 1992. Phyllanthusiin D, an unusual hydrolysable tannin from *Phyllanthus amarus*. *Phytochemistry,* **31**(2), 711-713.

[16] Foo, L.Y. 1993. Amariin, a di-dehydrohexahydroxydiphenoyl hydrolysable tannin from *Phyllanthus amarus*. *Phytochemistry,* **33**(2), 487-491.

[17] Kassuya, C.A., Silvestre, A., Menezes-de-Lima, O., Marotta, D.M., Rehder, V.L.G. & Calixto, J.B. 2006. Anti-inflammatory and antiallodynic actions of the lignan niranthin isolated from Phyllanthus amarus: evidence for interaction with platelet-activating factor receptor. *European Journal of Pharmacol*ogy, **546**(1), 182-188.

[18] Leite, D.F., Kassuya, C.A., Mazzuco, T.L., Silvestre, A., de Melo, L.V., Rehder, VL., Rumjanek, V.M. & Calixto, J.B. 2006. The cytotoxic effect and the multidrug resistance reversing action of lignans from Phyllanthus amarus. *Planta Medica*, **72**(15), 1353-1358.

[19] Houghton, P.J., Woldemariam, T.Z., O'Shea, S. & Thyagarajan, SP. 1996. Two securinega-type alkaloids from Phyllanthus amarus. *Phytochemistry*, **43**(3), 715-717.

[20] Keimer, A.K., Hartung, T., Huber, C. & Vollmar, A.M. 2003. *Phyllanthus amarus* has anti-inflammatory potential by inhibition of iNOS, COX-2 and cytokines via the NF-kB pathway. *Journal of Hepatology*, **38**, 289-297.

[21] Patel, J.R., Tripathi, P., Sharma, V. & Chauhan., N.S. 2011. uses, phytochemistry and pharmacology: a review. *Journal of Ethnopharmacology,* **138**(2), 286-313.

[22] Adedapo, A.A., Adegbayibi, A.Y. & Emikpe, B.O. 2005. Some clinico-pathological changes associated with the aqueous extract of the leaves of Phyllanthus amarus in

rats. *Phytotherapy Research: An International Journal Devoted to Pharmacological and Toxicological Evaluation of Natural Product Derivatives*, **19**(11), 971-976.

[23] Waterhouse, A. 2005. Folin-Ciocalteau method for total phenol in wine. Department of Viticulture and Enology. University of California, Davis, USA. Watt, J.M, Breyer-Brandwijk, M.G (1962). The medicinal and poisonous plants of southern and eastern Africa, **2**, 32.

[24] Zhishen, J., Mengcheng, T. & Jianming, W. 1999. The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. *Food Chemistry*, **64**(4), 555-559.

[25] Delcour, J. A. & Varebeke, D. J. D. 1985. A new colorimetric assay for flavanoids in pilsner beers. *Journal of the Institute of Brewing*, **91**(1), 37-40.

[26] Benzie, I. F. & Strain, J. J. 1996. The ferric reducing ability of plasma (FRAP) as a measure of “antioxidant power”: the FRAP assay. *Analytical Biochemistry*, **239**(1), 70-76.

[27] Prior, R. L., Hoang, H. A., Gu, L., Wu, X., Bacchiocca, M., Howard, L., ... & Jacob, R. 2003. Assays for hydrophilic and lipophilic antioxidant capacity (oxygen radical absorbance capacity (ORACFL)) of plasma and other biological and food samples. *Journal of Agricultural and Food Chemistry*, **51**(11), 3273-3279.

[28] Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M. & Rice-Evans, C. 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology and Medicine*, **26**(9-10), 1231-1237.

[29] Curto, E.V., Kwong, C., Hermersdörfer, H., Glatt, H., Santis, C., Virador, V., Hearing, V.J. Jr. & Dooley, T.P. 1999. Inhibitors of mammalian melanocyte tyrosinase: in vitro comparisons of alkyl esters of gentisic acid with other putative inhibitors. *Biochemistry and Pharmacology,* **57**(6), 663-672.

[30] Mosmann, T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of Immunological Methods*, **65**(1-2):55-63.

[31] Lappalainen, K., Jääskeläinen, I., Syrjänen, K., Urtti, A., Syrjänen. S. 1994. Comparison of cell proliferation and toxicity assays using two cationic liposomes. *Pharmaceutical Research*, 11(8), 1127-31.

[32] Monsees, T.K., Franz, M., Gebhardt, S., Winterstein, U., Schill, W.B., & Hayatpour, J. 2000. Sertoli cells as a target for reproductive hazards. *Andrologia*, **32**(4-5), 239-246.

[33] Howes, M.J.R. & Simmonds, M.S. 2014. The role of phytochemicals as micronutrients in health and disease. *Current Opinion in Clinical Nutrition & Metabolic Care*, **17**(6), 558-566.

[34] Hosseinimehr, S.J., Pourmorad, F., Shahabimajd, N., Shahrbandy, K. & Hosseinzadeh, R. 2007. In vitro antioxidant activity of *Polygonium hyrcanicum, Centaurea depressa, Sambucus ebulus, Mentha spicata and Phytolacca Americana. Pakistan Journal of Biological Sciences*, **10**(4), 637-640.

[35] Abbasi, M.A., Zafar, A., Riaz, T., Aziz-ur-Rehman., Arshad, S., Shahwar, D., Jahangir, M., Siddiqui, S.Z., *et al.,* 2010. Evaluation of the comparative antioxidant potential of aqueous and organic fractions of Ipomoea carnea. *Journal of Medicinal Plant Res*earch, **4**(18), 1883-1887.

[36] Nimse, S.B. & Pal, D. 2015. Free radicals, natural antioxidants, and their reaction mechanisms. *Rsc Advances*. **5**(35):27986-28006

[37] Islam, A., Naskar, S., Mazumder, U.K., Gupta, M. & Ghosal, S. 2008. Estrogenic properties of phyllanthin and hypophyllanthin from *Phyllanthus amarus* against carbofuran induced toxicity in female rats. *Pharmacology online*, **3**, 1006-1016.

[38] Iranloye, B., Oyeusi, K. & Alada, A. 2010. Effect of Aqueous Extract of Phyllanthus amarus Leaves on Implantation and Pregnancy in Rats. *Nigerian Journal of Physiological Sciences*, **25**(1), 63-66.

[39] Lucas, T.F., Pimenta, M.T., Pisolato, R., Lazari, M.F.M. & Porto, C.S. 2011. 17β-estradiol signalling and regulation of Sertoli cell function. *Spermatogenesis*, **1**(4): 318-324.

[40] Russo, A., Acquaviva, R., Campisi, A., Sorrenti, V., Di Giacomo, C., Virgata, G., Barcellona, M.L. & Vanella, A. 2000. Bioflavonoids as antiradicals, antioxidants, and DNA cleavage protectors. *Cell Biology and Toxicol*ogy, **16**(2), 91.

[41] Catala A. 2007. The ability of melatonin to counteract lipid peroxidation in biological membranes. *Current Molecular Medicine,* 638-649.

[42] Young, J., Whale, K.W. & Boyle, S.P. 2007. Cytoprotective effects of phenolic antioxidants and essential fatty acids in human blood monocyte and neuroblastoma cell lines: surrogates for neurological damage *in vivo. Prostaglandins, Leukotrienes and Essential Fatty Acid*, **78** (1),45-59.

CHAPTER FOUR: *Phyllanthus amarus* inhibits the activity of α-glucosidase and modulates glycaemic indices, and pancreatic oxidative stressed streptozotocin-diabetic male Wistar rats

FA Olabiyi[a,g], AA Oyagbemi[b], MO Tijani[c], OT Omobowale[d], YG Aboua[e], AA Adedapo[f], OO Oguntibeju[a*]

[a]Phytomedicine and Phytochemistry Group, Oxidative Stress Research Centre, Department of Biomedical Sciences, Cape Peninsula University of Technology, P.O Box 1906, Bellville 7535, South Africa.

[b]Department of Veterinary Physiology and Biochemistry, University of Ibadan, Nigeria.

[c]Department of Veterinary Pathology, University of Ibadan, Nigeria

[d]Department of Veterinary Physiology and Medicine, University of Ibadan, Nigeria.

[e]Department of Health Sciences, Namibia University of Science and Technology, Private Bag 13388, Windhoek, Namibia.

[f]Department of Veterinary Toxicology and Pharmacology, University of Ibadan, Nigeria.

[g]Department of Medical Laboratory Science, Afe Babalola University, P.M.B 5454 Ado-Ekiti, Nigeria.

*Corresponding author: Oluwafemi Omoniyi Oguntibeju

Email address: oguntibejuo@cput.ac.za Fax: +27(0)219538490

NB: Preparation of the manuscript for submission from this chapter is according to the Life Sciences (ELSEVIER, INC.) journal author's guidelines.

ABSTRACT

Aims: The antidiabetic activity of *Phyllanthus amarus* (PA) extracts through the evaluation of α-glucosidase as well as α-amylase activities, glycaemic indices, and pancreatic oxidative stressed diabetic rats were investigated.

Method: *In-vitro* antidiabetic action of PA aqueous together with the methanol extracts was examined using Enzyme-linked Immunosorbent assay techniques. *In vivo* evaluation was carried out in six weeks old, male Wistar rats. The rats were randomly divided into 5 groups (n = 10 rats per group); namely, i) Normal control, ii) Diabetic control, iii) Diabetes + PA 200mg/kgbwt., iv) Diabetes + PA 400mg/kgbwt., v) Diabetes + glibenclamide 0.2mg/kgbwt. T2D was induced by giving the animals 10% fructose to drink for 14 days, followed by an intraperitoneal injection of 55 mg/kg streptozotocin. Bodyweight and blood glucose measurements were performed. Serum insulin, Nitric oxide (NO), myeloperoxidase (MPO), glutathione-S-transferase (GST), glutathione peroxidase (GPx) and reduced glutathione (GSH) were evaluated in healthy as well as diabetic rats. Besides, histopathology of the pancreas was examined.

Key Findings: The extracts of PA caused a significant inhibition in the activity of α-glucosidase but produced no significant inhibition in the α-amylase activity. Diabetic rats treated with 400mg/kgbwt of PA aqueous extract reduced postprandial blood glucose, compared with diabetic rats. Also, 400mg/kgbwt of PA raised serum insulin levels, just like glibenclamide when compared with diabetic and healthy control rats. Both 200mg/kgbwt and 400mg/kgbwt of PA increased both pancreas GST and GPx activities. However, only 400mg/kgbwt of PA extract gave a significant increase in GPx activity.

Significance: PA extracts possess a significant α-glucosidase inhibition, improve serum insulin levels and antioxidant enzymes activities, thereby modulating oxidative stress precipitated by diabetes. Therefore, PA could be a natural alternative to antidiabetic drugs.

Keywords: *Phyllanthus amarus*, antidiabetic activity, α-glucosidase, streptozotocin, oxidative stress, Wistar rats

4.1. Introduction

Diabetes mellitus is an endocrine disease, typified by high blood glucose levels (hyperglycaemia). It is due to defects in insulin production or insulin action [1], thus impaired carbohydrate, lipid and protein metabolism, leading to protracted health implications [2,3]. In diabetic patients, prolonged damage, dysfunction, and organs failure as in the eyes, kidneys, nerves, heart, and blood vessels [4] all have to do with uncontrolled hyperglycaemia [5,6]. Also, in people with diabetes, hyperglycemia induces free radicals, particularly the reactive oxygen species (ROS), thus, impairing endogenous antioxidant defence system, besides antioxidant enzymes and non-enzymatic antioxidants [7]. Overproduction of ROS leads to more oxidation of lipids, proteins, and glucose auto-oxidation, which in turn weakens the capacities of the body's antioxidant defence system[8], leading to oxidative stress [9]. Thus, oxidative stress impairs the activity of antioxidant enzymes [8,10]. Increased lipid peroxidation and reduced antioxidant enzymes activity are found to be responsible for the progression of diabetes and its complications [11]. Both endogenous, together with exogenous antioxidants, help to mitigate the effect of ROS, leading to reduced oxidative stress [12]. Consequently, reduction of oxidative stress levels in diabetes counterbalanced hyperglycaemia [13] and eventually attenuated diabetic complications [14].

Oral antidiabetic drugs are useful in the clinical management of diabetes. These conventional antidiabetic agents act by various mechanisms including the stimulation of the pancreatic β-cells to produce more insulin, increasing the sensitivity of muscles and other tissues to insulin, decreasing gluconeogenesis by the liver and delaying the absorption of carbohydrates from the gastrointestinal tract [15]. These oral antidiabetic drugs with different mechanisms are quite useful in their actions. However, due to their increasing undesirable side effects and high cost, natural plant products are explored nowadays and considered as an alternative therapy.

Phyllanthus amarus (PA) is a herb in the Euphorbiaceae family widely found in the tropical and sub-tropical areas around the world. Its ethnobotanical uses include local management of liver disorders, diabetes, obesity, hyperlipidaemia, and topical application to treat skin lesions, among others. The phytochemical evaluation revealed that the herb has lots of constituents like phenolic acids with tetracyclic triterpenoids [16], flavonoids [17], tannins [18,19], lignans [16,20,21] as well as alkaloids [22]. Other bioactive compounds isolated from PA demonstrate an array of pharmacological functions. Examples of such features include its activities to treat viral and bacterial infections, diabetes, inflammation, pain, diuresis, hyperlipidaemia. It also possesses antioxidant, hepatoprotective and nephroprotective properties [23,6].

A previous study reported that PA aqueous extract exhibited antidiabetic activity in graded doses of 200mg/kgbwt and 400mg/kgbwt./day in alloxan-diabetic rats [24], however, the mechanism of its antidiabetic action is unclear; besides, its potential to modulate the complex responses associated with oxidative stress needs more scientific elucidation. Also, our previous report revealed that the PA aqueous extract possesses the highest antioxidant capacity [25]. Therefore, the current study examined the effect of PA extracts on α-glucosidase and α-amylase activities [26], the glycaemic indices and pancreatic oxidative stress in streptozotocin-diabetic rats [12].

4.2. Methodology

4.2.1. Reagents and Chemicals

The following enzymes and chemicals; α-glucosidase (Saccharomyces cerevisiae), α-amylase (procaine pancreas), 3,5, di-nitro salicylic acid (DNS), P-nitro-phenyl- α-D-glucopyranoside (p-NPG) and streptozotocin (18883-66-4) were procured from Merck Group-Sigma-Aldrich Chemical Company, South Africa. Rat insulin ELISA kit bought from Thermofisher Scientific Inc. South Africa. D-Fructose was purchased from Loba Chemie PVT. LTD, Mumbai, India. Glibenclamide was purchased from a local pharmacy.

4.2.2. Plant Materials

Phyllanthus amarus whole plant was harvested from the south-west, Nigeria. A botanist assisted in identifying and authenticating the plant. A specimen token was deposited at the FRIN Ibadan, Nigeria (herbarium FHI NO: 110242).

4.2.2.1. Extracts Preparation

Fresh *Phyllanthus amarus* leaves were shade-dried, then ground to powder using a mechanical mill (Mincer 9FQ-28, China). 100-gram powder of aqueous extract was soaked in 1 litre of distilled water, stirred, then left for 24h at room temperature. The homogenate was filtered using Whatman no-1 filter paper; this was followed by evaporating the resultant filtrate to dryness at reduced pressure with a rotary evaporator (Buchi Rotavapor II, Buchi Germany) at 40^{o}C. The

product was lyophilised in a freeze drier (VirTis Genesis 25EL, SP Scientific, NY, USA) for sixteen hours; the dried extract was kept at 4^{o}C till it is required for use [25].

4.2.2.2. Experimental Animals

Fifty (50) healthy, male Wistar rats of about six weeks old, weighing between 190±10gm were purchased and kept at the animal facility, the Vet Physiology and Biochemistry Department, University of Ibadan, Nigeria [27]. They were kept in rats' cages at controlled, standard, laboratory conditions; humidity between 45% to 55%, an ambient temperature between 22^{o}C to 26^{o}C and regular photoperiod (12h dark: 12h light). They were fed twice daily (09:00 am and 4:00 pm) with rats' chow, a pelleted feed produced by Ladokun and Sons Livestock Feed, Nigeria Limited. They were always allowed access to clean water in clean bowls.

4.2.2.3. Ethical Statement

Ethics clearance was obtained from the Health and Wellness Sciences Faculty Research Ethics Committee of CPUT, South Africa (CPUT/HW-REC 2016/A1). Besides, the protocol and the handling of the animals follow the guidelines set by the Animal Ethics Committee, the University of Ibadan, Nigeria, where the animal experiment was done (approval no: UI-ACUREC/19/0017).

4.2.2.4. Type 2 Diabetes Mellitus induction in the Experimental Animals

Animals were allowed one week after purchase, to acclimatize to their new environment. The animals were then administered 10% fructose *ad libitum* for 2 wks, after that, a single, intraperitoneal injection (i.p) of streptozotocin (55mg/kgbwt) in 0.1M cold citrate buffer (pH 4.5) was given. Non-diabetic models received citrate buffer which served as the vehicle. Hyperglycaemia using Accu-chek® glucometer (Roche, Germany) was confirmed four days post-STZ administration, after which animals with blood glucose ≥11.1mmol/L were considered diabetic. Treatments with the extract and standard antidiabetic drug, glibenclamide commenced immediately.

4.2.2.5. Experimental Design

The rats were randomly grouped into five, with ten animals each (n=10). **NC**, the healthy, non-diabetic control rats' group, received standard rat chow and vehicles only (citrate buffer and distilled water); group **DC** was the diabetic untreated control rats, are fed with rat chow and water only. The PA 200mg group were rats fed with 200mg/kgbwt/day PA extract for four wks. The PA 400mg group comprised of rats that were given 400mg/kgbwt/day PA extract, also for four wks. GLIBEN 0.2mg-diabetic rats received 0.2mg of glibenclamide per kg body weight for four wks. Before termination and sacrifice, rats were eight hours fasted; after that, an oral glucose tolerance test (OGTT) was carried out on the rats. Fasting blood samples (0 min) were taken first; then the rats received daily treatments. Thirty minutes later, a single dose of D-glucose (50%, $^{w}/_{v}$) solution (2g/kgbwt) was orally administered to each animal, followed by the measurement of blood glucose levels at 30, 60, 90 and 180 minutes. At the termination of the experiment, animals in all groups were euthanised; blood sample, pancreas, along with other organs were collected for biochemical evaluations. Serum insulin, glycosylated haemoglobin and histopathological studies of the pancreas were performed.

4.2.2.6. Preparation of Serum and Tissues for Biochemical Assays

The rats were anaesthetised and euthanised using i.p sodium pentobarbital, 60mg/kgbwt followed by cervical dislocation, and blood samples collected as previously described [28] using a clean heparinized capillary tube into labelled plain, lithium heparin and EDTA bottles. The heparinized blood samples were then centrifuged at 3,500 revolutions per minute (rpm) for 20 minutes. The supernatant was removed, labelled appropriately, and stored in a deep freezer [29] at -80^{o}C for further analysis while the whole blood in EDTA bottles was used for HbA1c assays.

4.2.2.7. Homogenization

Pancreatic tissue was immediately excised and washed in ice-cold buffered saline (PBS) solution and weighed. The tissue samples were homogenized in the buffer (1gm/2mL) in a homogenizer (Yellow^{line}D1 25 basic, IKA® -WERKE GMBH & CO, Germany); then briefly centrifuged for 20mins. This supernatant was collected and used for the endogenous antioxidant and biochemical evaluations [30].

4.2.2.8. Body Weights Change

Body weights were monitored weekly with the aid of a portable, battery-operated weighing scale (KERN TCB 200-1, Kern & Sohn, South Africa). The body weight change and the percentage of body weight change during the study period was calculated, using the formulae, as shown below:

$$\boldsymbol{Body\,weight\,change\,(g) = Final\,body\,weight\,(g) - Initial\,body\,weight\,(g)}$$

$$\%\,\boldsymbol{Bodyweight\,change} = \boldsymbol{(Bodyweight\,change\,(g) \div Initial\,bodyweight\,(g)\,X\,100}$$

4.2.3. *In-vitro* Anti-diabetic Evaluation

4.2.3.1. α-amylase inhibitory activity

Alpha-amylase inhibitory activity of PA methanol and aqueous extracts were performed following a modified method [31], as described by (Telagari and Hullatti [32]. Briefly, inside 96–well plate, reaction mixture containing 50µL phosphate buffer (100mM, pH = 6.80), 20µL α-amylase (2 U/mL), and 20µL of differing extracts concentrations (3.9625µg/mL, 7.8125µg/mL, 15.625µg/mL, 31.25µg/mL, 62.5µg/mL, 125µg/mL) was incubated at 37^{o}C for 20 min. Then, 20µL of 1% soluble starch (100mM phosphate buffer, p^{H} = 6.80) was added as a substrate and incubated further at 37^{o}C for 30 min; 50µL of the DNS colour reagent was then added and boiled for 30 min. The absorbance of the reactants was measured at 540nm using microplate ELISA reader (Multiskan Spectrum by Thermo Electron Corporation, MA, USA. Each experiment was performed three times.

The percentage inhibition was calculated as follows:

$$\%\,\boldsymbol{inhibition} = \left\{\frac{A_{control} - A_{sample}}{A_{control}}\right\} x100$$

Where, A_{sample} is the absorbance of test samples and $A_{control}$ is the absorbance of control.

4.2.3.2. α-glucosidase inhibitory activity

Alpha-glucosidase inhibitory activity of PA methanol and aqueous extracts was performed by following a modified method [33] as described by (Telagari and Hullatti [32], using p-NPG as a substrate. Briefly, inside 96–well plate, reaction mixture containing 50µL phosphate buffer (100mM, pH =6.80), 10µL alpha-glucosidase (1U/mL), and 20µL of varying extracts

concentrations (1.953µg/mL, 3.90625µg/mL, 7.8125µg/mL, 15.625µg/mL, 31.2500µg/mL and 62.500µg/mL) was added. Then, 20µL p-NPG (5mM) was added as a substrate and incubated for 30 min at 37^{o}C. The reaction was halted by adding 50µL Na_2CO_3 (0.1M) while absorbance of the released p-nitrophenol was measured at 405nm using microplate ELISA reader (Multiskan Spectrum by Thermo Electron Corporation, MA, USA).

Each experiment was performed three times, and the percentage inhibition calculated as follows:

$$\%\, inhibition = \left\{\frac{A_{control} - A_{sample}}{A_{control}}\right\} x100$$

Where, A_{sample} is the absorbance of test samples and $A_{control}$ is the absorbance of control.

Their IC_{50} was determined using GraphPad software.

4.2.4. Glycaemic indices: Fasting blood glucose, OGTT, Glycosylated Haemoglobin and Serum Insulin

4.2.4.1. Fasting Blood Glucose (FBG)

Fasting blood glucose was measured based on the glucose dehydrogenase method [34], using a portable Accu-Chek® Active glucose meter (Roche, Germany). On a test strip that was slid in the measurement window, one drop (10µL) from the rat's tail prick was applied on the test field of the test strip, the meter then displays the concentration of glucose in mmol/L.

4.2.4.2. Glycosylated Haemoglobin

Glycosylated haemoglobin (HbA1c) mmol/mol, was quantitatively evaluated in blood samples by Tina-quant Haemoglobin A1c Gen.3-Hemolysate and whole blood on Roche/Hitachi Cobas c311 auto-analyser systems. The HbA1c determination depends on the turbidimetric inhibition of immunoassay (TINIA) for haemolyzed whole blood. Thus, expressing results as mmol/mol HbA1c, values were calculated from the HbA1c/Hb ratio:

Representing mmol/mol HbA1c acc. to IFCC,

$$HbA1c\left(\frac{mmol}{mol}\right) = \left(\frac{HbA1c}{Hb}\right) x1000$$

4.2.4.3. Serum Insulin

Serum insulin was measured using Thermoscientific™ Pierce™ Rat insulin, ELISA Kit [35], following the manufacturer's instruction. The concentration of insulin (µU/mL) in each sample was determined by interpolating the Insulin concentration to the absorbance value from a prepared standard curve. Expressing the results in SI unit, 1µU/mL = 6.945ρmol/L.

4.2.5. Assessment of Antioxidant Enzymes activity, Reduced glutathione (GSH) level and Tissue Lipid peroxidation

4.2.5.1. Superoxide Dismutase

The superoxide dismutase (SOD) activity in pancreatic tissue homogenate was evaluated by a modified Misra and Fridovich method [36]. Briefly, 50mg of epinephrine was dissolved in 100mL distilled water and acidified with 0.5mL of concentrated hydrochloric acid. 30µL homogenate was added to 2.5 mL 0.05 M carbonate buffer (p^H 10.2) followed by the addition of 300µL of 0.03mM adrenaline. The increase in absorbance at 480nm was monitored every second for 150 seconds.

CALCULATION

$$Increase\ in\ absorbance\ per\ minute = \frac{A_3 - A_0}{2.5}$$

Where, A_0 = absorbance at 0 seconds

A_3 = absorbance 150 seconds

$$\%\ inhibition = \frac{increase\ in\ absorbance\ for\ substrate}{increase\ in\ absorbance\ of\ blank}\ x\ 100$$

1unit of SOD activity was given as the amount of SOD necessary to produce half-maximal oxidation of adrenaline to adrenochrome for 1 minute [37].

4.2.5.2. Determination of Glutathione S-transferase activity in the Tissue sample

The glutathione-S-transferase activity was determined by following a modified method of Prohaska and Ganther [38]. The assay measures total GST activity (cytosolic and microsomal) at 340nm by measuring the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) with reduced glutathione.

Calculation:

$$GST\ activity, \left\{\frac{nmol}{min}\right\}/mL = \left(\frac{\frac{\Delta A_{340}}{min}}{0.00503\mu M^{-1}}\right) X \left(\frac{0.2mL}{0.02mL}\right) X\ sample\ dilution$$

4.2.5.3. Glutathione Peroxidase

The glutathione peroxidase activity was estimated following a method of Beutler et al. [39]. Briefly, 0.5mL phosphate buffer, 0.1mL $NaNO_3$, 0.2mL GSH, 0.1mL H_2O_2, 0.5mL sample and 0.6mL of distilled water was placed into a test tube. This mixture was incubated at 37^0C for 5 minutes; 0.5mL of TCA was added and centrifuged at 3, 000 rpm for 5 minutes. To 1mL of supernatant, 2 mL of K_2HPO_4 and 1mL of DTNB was added. The absorbance was read at 412nm against the blank.

One unit of glutathione peroxidase activity is defined as the amount of enzyme required to utilize 1nmol of NADPH/minute at 25^0C.

4.2.5.4. Estimation of Reduced Glutathione (GSH) level

The method of Beutler et al. [39] was followed in estimating the level of reduced glutathione (GSH). Briefly, 0.25mL of the sample was placed into the tubes and, 0.25mL of the precipitating solution was mixed with the sample. The mixture was centrifuged at 1700 x ***g*** for 5 minutes. 20µL of the supernatant was put in another test tube, followed by 4.5mL of Ellman's reagent. The solution was read at 412nm against distilled water as blank. GSH was proportional to the absorbance, and the concentration was extrapolated from a prepared GSH calibration curve.

4.2.5.5. Estimation of Malondialdehyde (MDA) content

Malondialdehyde (MDA) content was measured using the thiobarbiturate (TBA) test in the pancreatic tissues according to a method as previously described by Varshney and Kale [40]. To 1.6mL of Tris-KCl, 0.5mL of 30% TCA, 0.4mL of sample and 0.5mL of 0.75% TBA prepared in 0.2M HCl were added. The reactants were boiled in a water bath [41] at 80^0C for 45 minutes, ice-cooled and centrifuged at 1700 x ***g*** for 20 minutes [42]. After that, the absorbance of the

coloured solution was read at 532nm in an ELISA reader (Multiskan Spectrum by Thermo Electron Corporation, MA, USA). MDA (nMol) as a function of lipid peroxidation was calculated by multiplying the absorbances by a molar extinction coefficient of 1.56×10^5/M/cm.

.

4.2.5.6. Estimation of Serum Myeloperoxidase

The activity of myeloperoxidase (MPO) was determined according to the method of Xia and Zweier [43]. Briefly, 200µL of O-dianisidine and H_2O_2 mixture was pipetted in the cuvette followed by 10µL serum sample. The reactant mixture was read every 30 seconds at 460nm wavelengths.

The amount of MPO generated was calculated from the formula below:

$$MPO\ generated = \frac{change\ in\ activity}{mg\ protein}\ x\ 10$$

T_1=30-0 and T_2 = 60 seconds.

4.2.5.7. Estimation of Serum Nitric Oxide

A combined modified method, as described by Maciel et al. [44] and Green et al. [45], was followed to measure serum nitric oxide (NO) levels. In this method, serum NO levels were determined by the measurement of NO_X (nitrite and nitrate) after enzymatic nitrate to nitrite conversion by nitrate reductase [46]. Briefly, 50µL of undiluted serum was incubated with the same volume of reductase buffer (0.1M potassium phosphate, p^H 7.5, containing 1mM nicotinamide adenine dinucleotide phosphate, 10mM flavin adenine dinucleotide and 4units of nitrate reductase/mL for 20 hours at 37^{o}C.

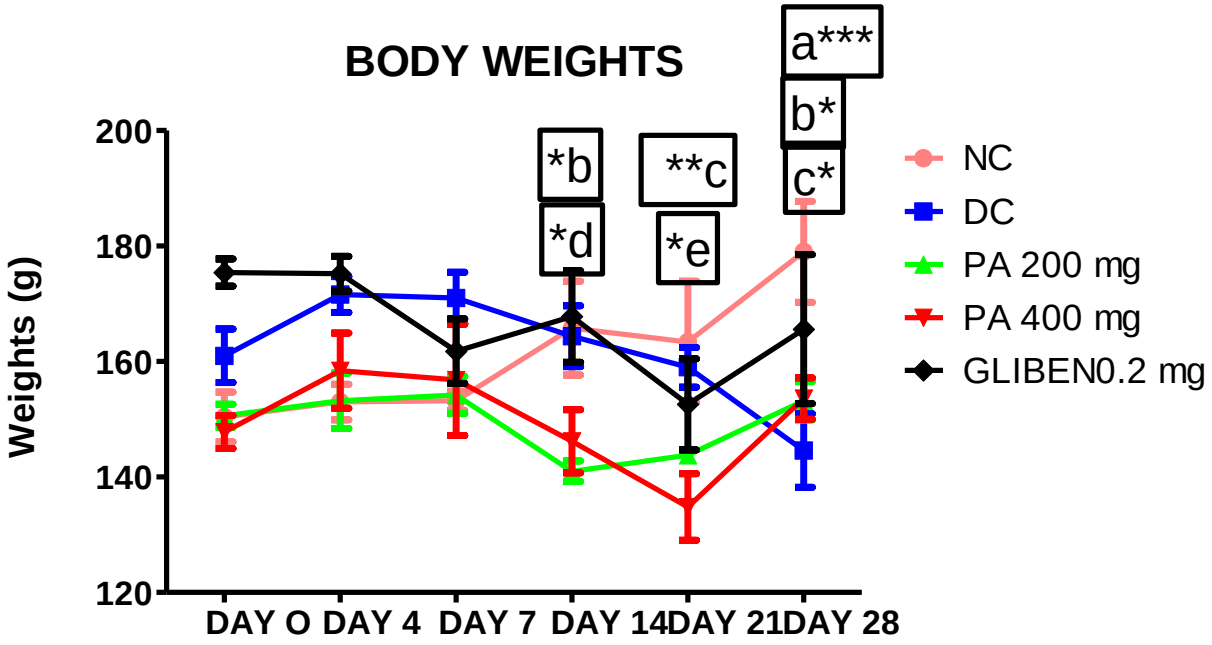
4.2.6. Histopathology

The pancreas was collected into 10% formol-saline for proper fixation. This tissue was processed and embedded in paraffin wax. Sections of 5µm thick were made and stained with haematoxylin and eosin (H&E) for histopathological examination [47]. Histopathology analysis was carried out a qualified veterinary pathologist with no prior knowledge of the animal groupings and their treatments.

4.2.7. Statistical Analysis

GraphPad Prism software, version 5.0 (GraphPad Software, San Diego, California, USA), was used for all graphical evaluations and representations. Data were expressed as mean ± SEM. A one-way ANOVA test with Bonferroni's multiple comparison test was used. Data were taken as significant if $p<0.05$.

4.3.



4.4.

4.5. Results

Results show that at 0.78, 1.56, 3.13, 6.25 and 12.5µg/mL, aqueous extract caused 23%, 23%, 41%, 76% and 91% reduction in the activity of α-glucosidase, in that order. Conversely, the methanol extract inhibited the activity of the enzyme by 27%, 27%, 44%, 32% and 79% respectively (Table 4.1). Their IC_{50} were 3.9 and 5.5µg/mL, and log of half-maximal inhibition ($LogIC_{50}$) are 0.5922 and 0.7398, respectively (Figure 4.1). However, the aqueous extract did not produce significant inhibition on α-amylase (Table 4.2).

Weight measurements were performed at days 0, 4, 7, 14, 21 and 28 after streptozotocin administration. The results show there was persistent weight loss among the diabetic control rats

as well as among all the diabetic treated groups up till day 21 (Figure 4.2). From the figure, there was significant weight loss in diabetic controls versus healthy controls till day 28 (p<0.001), in the PA 200mg/kgbwt versus healthy controls at days 14 and 28 (p<0.05). Nevertheless, there was significant weight loss in the PA 400mg/kgbwt versus healthy control animals at day 21 (p<0.01) and at day 28 (p<0.05). There was also significant weight loss in diabetic controls vs diabetic treated with PA 200mg/kgbwt at day 14 (p<0.05), and versus PA 400mg/kgbwt at day 21 (p<0.05). Moreover, significant weight loss among the diabetic treated rats with GLIBEN 0.2mg/kgbwt versus PA 200mg/kgbwt at day 14 (p<0.01) was noted.

Diabetic rats treated with PA aqueous extract at 400mg/kgbwt for the four weeks showed a 27.6% cut in postprandial blood glucose when compared to untreated diabetic animals (Figure 4.3A). There was a 36.6% increase in the postprandial blood glucose levels of rats treated with glibenclamide when compared with untreated diabetic control rats which remains persistent.

Serum insulin in the animals treated with PA 400mg/kgbwt and glibenclamide versus the diabetic non-treated control rats and healthy control rats produce no significant difference (p>0.05) (Figure 4.3C). The HbA1c in diabetic animals and the experimental groups were significantly increased (p<0.0001) when compared with the healthy controls (Figure 4.3B).

As shown in figure 4.4 below, serum myeloperoxidase (MPO) activity, an inflammatory biomarker, remain significantly raised in the diabetic group DC (p<0.0001) compared to the healthy controls NC, while the extracts PA 200mg/kgbwt, as well as the PA 400mg/kgbwt, brought serum MPO down to normal levels compared to the diabetic animals (p<0.0001) in a similar manner to glibenclamide. Besides, Serum Nitric oxide (NO), another inflammatory biomarker was significantly reduced by GLIBEN 0.2mg/kgbwt compared to healthy control rats***NC (p<0.001); and by PA 200mg/kgbwt. compared to diabetic control **(p<0.001). As depicted in table 4.5, PA 400mg/kgbwt increased glutathione (GSH) in the pancreatic tissue by 38.41% when compared with normal controls (p>0.05). The glutathione peroxidase (GPx) activity of PA 200mg/kgbwt. the group was increased by 23% when compared with the healthy controls. More so, both 200mg/kgbwt. with 400 mg/kgbwt. caused a significant increase in GPx activity (p<0.0001) like glibenclamide (p<0.001) when compared with diabetic untreated control (Figure 4.5D). Both PA 200mg/kgbwt. and PA 400mg/kgbwt. raised glutathione-S-transferase (GST) activity by 20.3% and 17.1%, respectively (Figure 4.5E and Table 4.5), but these increases are not significant (p>0.05). Moreover, the PA 200mg/kgbwt together with PA 400mg/kgbwt extracts tend to bring the malondialdehyde (MDA) to normal levels in a similar

manner to glibenclamide versus diabetic untreated rats (Figure 4.5C). There was no significant difference in pancreatic H_2O_2 content among all the experimental groups.

Histologically, all the treatment groups showed varying degrees of damage to the islets of Langerhans. The PA 200mg group showed severely depleted islets of Langerhans, but there was no necrosis. The islets of Langerhans were also similarly depleted and severely shrunken in the PA 400mg group with no necrosis. The GLIBEN 0.2mg group showed total depletion of the islets of Langerhans as well as atrophy of exocrine pancreatic tissue, severe interstitial fibrosis, and infiltration by inflammatory cells (as shown in Figure 4.6).

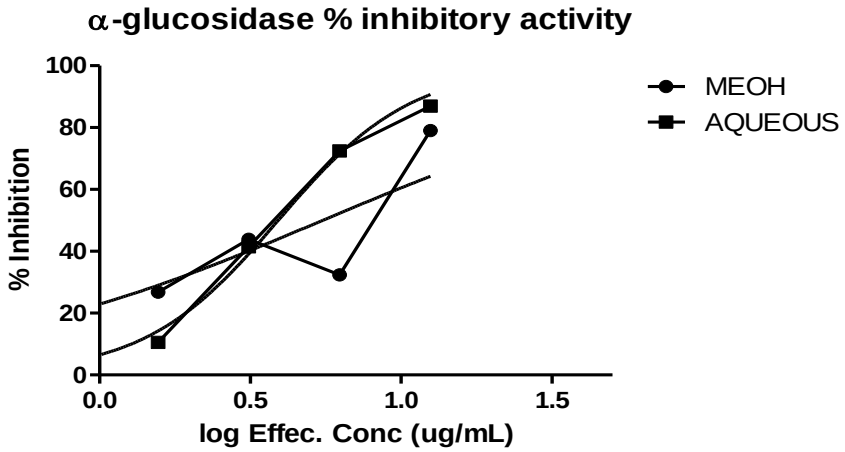


Figure 4.1: PA extracts' inhibition on alpha-glucosidase

Table 4.1: PA extracts' inhibition on alpha-glucosidase activity

Concentration (µg/mL)	**% Inhibition**	
	Methanol Extract	**Aqueous Extract**
12.5	79	91

6.25	32	76
3.125	44	41
1.5625	27	23
0.78125	27	23

Table 4.2: α-amylase inhibitory activity exhibited by varying concentrations of aqueous PA extracts

Effective Concentrations (µg/mL)	**% inhibition**
600	33.20
300	29.70
150	24.16
75	0.91
37.5	-1.20

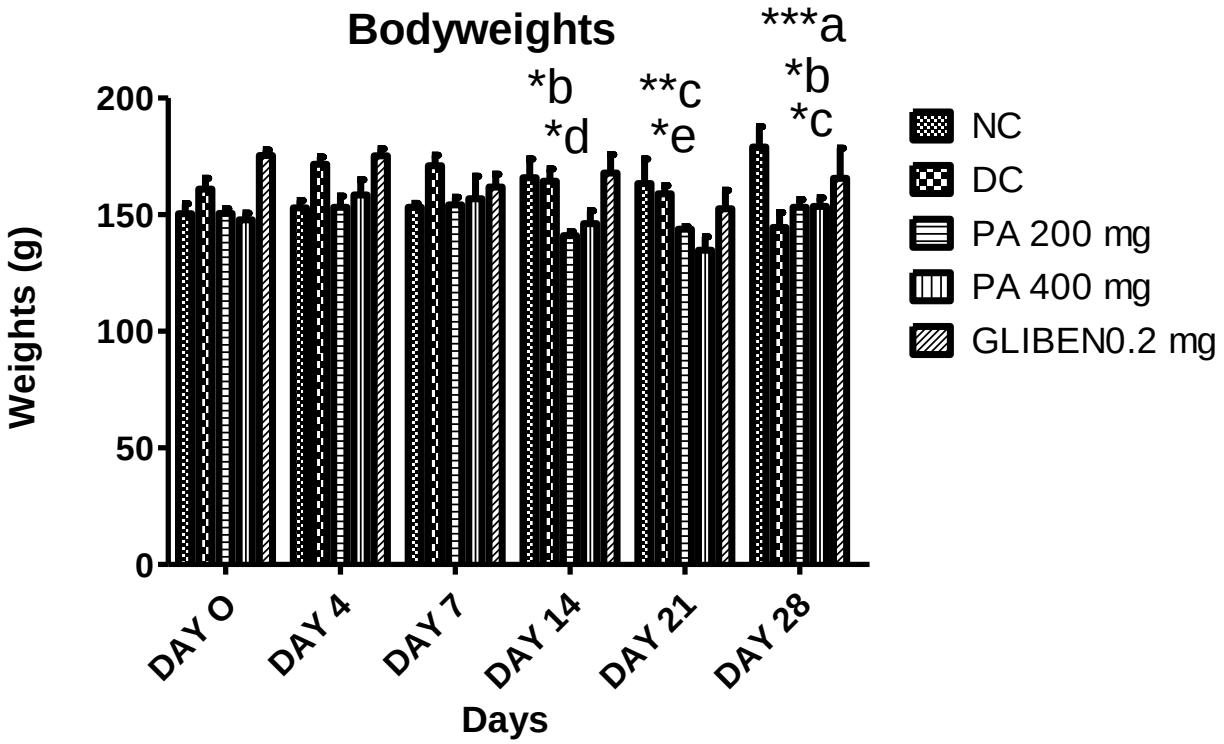


Figure 4.2: Progressive weights of the animals during the study period

$^{***a}p < 0.001$ (NC vs DC at day 28), $^{*b}p < 0.05$ (NC vs PA 200 mg at days 14 and 28);

$^{**c}p < 0.01$ (NC vs PA 400 mg at 21), $^{*c}p < 0.05$ (NC vs PA 400 mg at 28);

$^{*d}p < 0.05$ (DC vs PA 200 mg at day 14); $^{*e}p < 0.05$ (DC vs PA 400 mg at day 21;

$^{**f}p < 0.01$ (PA 200 mg vs GLIBEN 0.2 mg at day 14). Values are mean ±SD (Two ways ANOVA followed by Bonferroni multiple comparison test).

Table 4.3: Mean± (SD) progressive body weights and % weight change

Groups	Initial body weights (g)	Final body weights (g)	% Bodyweight changes
NC	153±6.2	179±17.5	+17.0%
DC	171.6±10.0	144.6±14.3	-15.8% ($p < 0.05$)
PA 200mg	153.2±9.6	153.2±6.6	0%
PA 400mg	158.4±14.6	153.6±8.0	-2.5% ($p < 0.05$)
GLIBEN. 0.2 mg	175.2±6.0	165.6±25.8	-5.5% ($p < 0.05$)

–

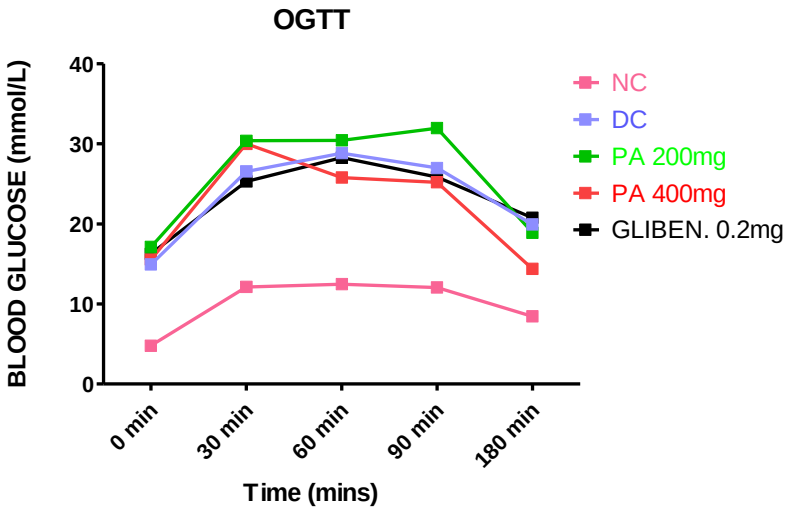
As shown above, there was a significant loss of weight among the experimental animals compared to the healthy controls. The PA 200mg/kgbwt group shows no mean weight change.

Table 4.4: Mean ± (SEM) Fasting Blood Glucose levels of experimental and control rats

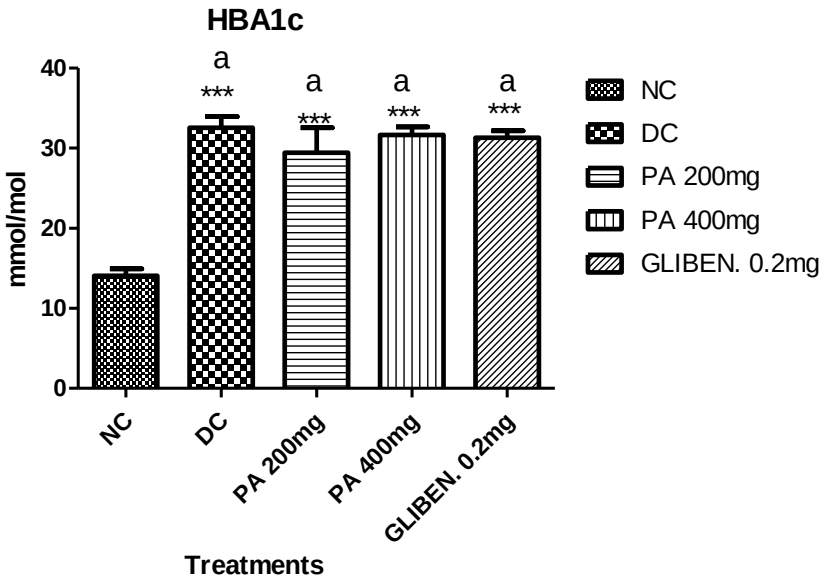
Groups	Initial FBG (mmo/L)	Final FBG (mmo/L)	Change in FBG (mmol/L)
NC	3.8 ± 0.5	4.7 ± 0.9	+0.9 (23.6%)
DC	10.3 ± 4.4	13.3 ± 7.0	+3.0 (29.1%)
PA 200mg	15.1 ± 1.1	20.9 ± 2.2	+5.8 (38.4%)
PA 400mg	11.1 ± 1.3	21.2 ± 0.7	+10.1 (91.0%)
GLIBEN. 0.2mg	14.1 ± 4.6	19.2 ± 1.8	+5.1(36.2%)

The result shows persistently high FBG levels in the experimental groups.

A


OGTT
BLOOD GLUCOSE (mmol/L)
40
30
20
10
0
0 min
30 min
60 min
90 min
180 min
Time (mins)
NC
DC
PA 200mg
PA 400mg
GLIBEN. 0.2mg

B


HBA1c
mmol/mol
40
30
20
10
0
a
***
a
***
a
***
a
***
NC
DC
PA 200mg
PA 400mg
GLIBEN. 0.2mg
Treatments

C

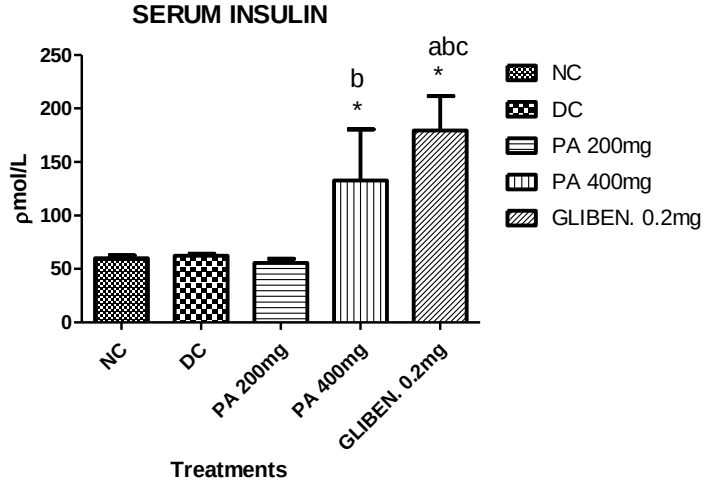

SERUM INSULIN
ρmol/L
250
200
150
100
50
0
b
*
abc
*
NC
DC
PA 200mg
PA 400mg
GLIBEN. 0.2mg
Treatments

Figure 4.3: Effect of PA on OGTT, HbA1c and serum insulin levels of experimental and control rats

A. 3h OGTT; B. Glycosylated haemoglobin (HbA1c NC compared to DC; NC compared to PA 200mg; NC compared to PA 400mg; NC compared to GLIBEN 0.2mg ($^{***}p < 0.0001$) and, C. Serum insulin levels of experimental animals and controls over the study period *NC compared to PA 400mg; DC compared to GLIBEN 0.2mg ($^{*}p<0.05$).

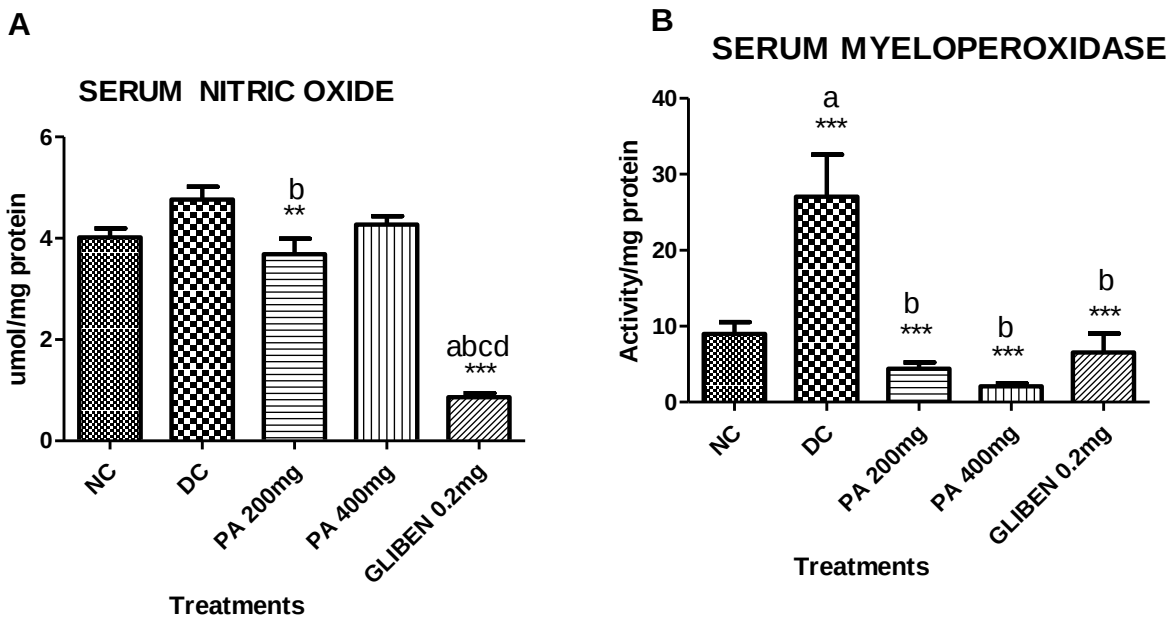


Figure 4.4: Effects of PA on inflammatory biomarkers of experimental and control rats

A. Serum Nitric oxide (NO) was brought down by GLIBEN 0.2mg compared to normal rats***NC ($p<0.001$); and *PA 200mg compared to diabetic control ** ($p<0.001$). B. Showing serum myeloperoxidase (MPO) activity being reduced by PA 200mg/kgbwt, PA 400mg/kgbwt and GLIBEN 0.2mg/kgbwt; corresponding to diabetic control animals DC ($^{***}p<0.001$).

Table 4.5: Mean ± (SEM) Serum Myeloperoxidase, Pancreatic tissue MDA, GPx, GSH, GST and H_2O_2 in the experimental and control rats

	NC	DC	PA 200mg/kgbwt	PA 400mg/kgbwt	GLIBEN 0.2mg/kgbwt
MPO	8.73±1.59	27.04±5.53	4.43±0.83	2.07±0.42	6.54±2.52
MDA	5.29±0.52	7.97±1.21	7.63±0.32	7.25±0.69	6.78±0.54
GPx	271.8±17.07	204±8.59	334±7.27	315.5±16.34 (23%)	276.8±16.46
GSH	18.59±0.71	19.65±0.53	20.03±1.47	21.13±0.37 (38.41%)	20.29±0.69
GST	29.56±1.54	23.14±1.72	35.57±1.32 (20.3%)	34.61±3.12 (17.1%)	35.56±2.72
H_2O_2	91.30±4.10	87.23±3.04	90.56±4.30	89.12±3.29	89.50±4.02

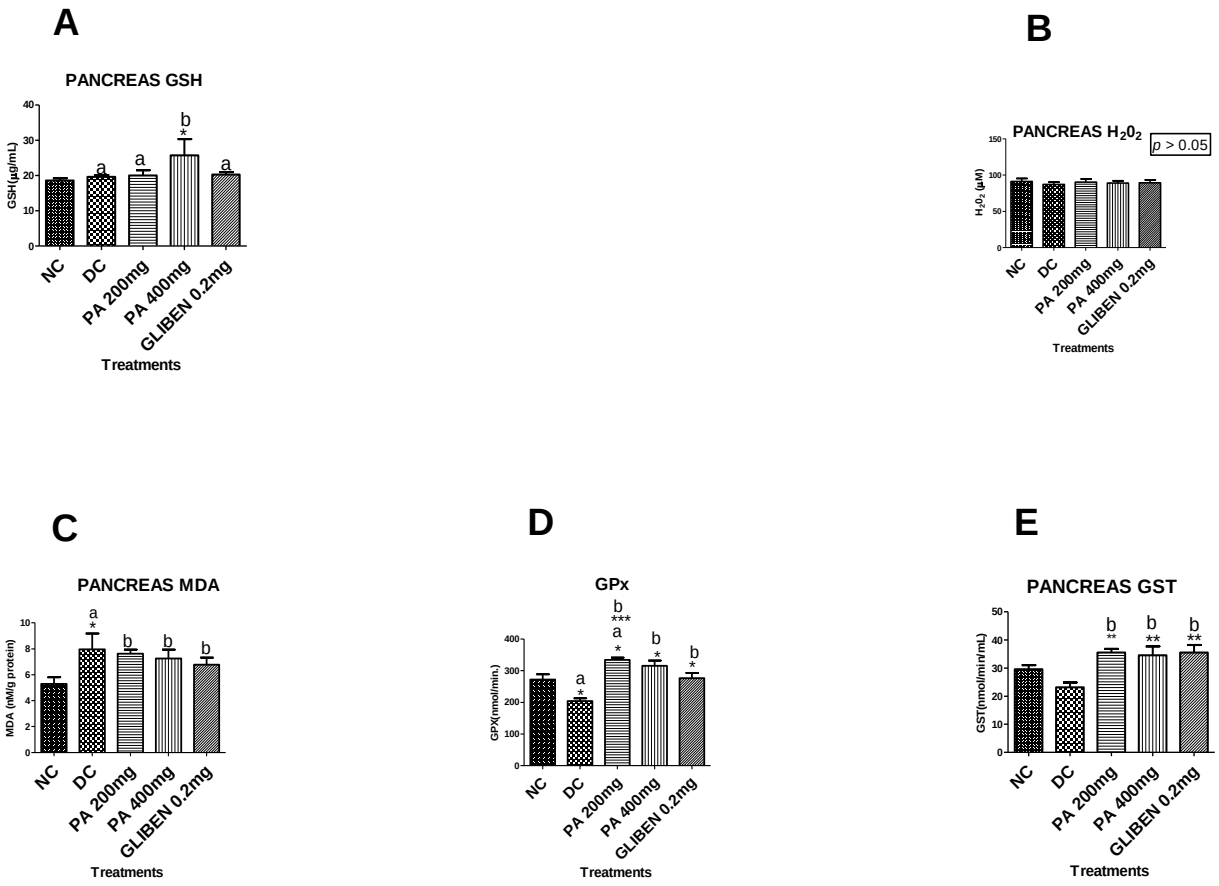


Figure 4.5: Effect of pancreatic oxidative stress biomarkers of experimental and control rats

A. Glutathione (GSH) in pancreas tissue of experimental animals and controls; B. pancreas H_2O_2 of experimental animals and controls; C. pancreas malondialdehyde (MDA) of experimental animals and controls; D. Glutathione peroxidase activity in pancreas tissue of experimental animals and controls *NC compared to DC (p<0.05); *NC compared to PA 200mg (+23%); ***DC compared to PA 200mg; ***DC compared to PA 400mg; **DC compared to GLIBEN 0.2mg. (***p<0.0001); (**p<0.001); (*p<0.05); and **E**. Glutathione-S-transferase activity in pancreas tissue of experimental animals and controls **DC compared to PA 200mg; **DC compared to 400mg; **DC compared to GLIBEN 0.2mg (**p<0.001); NC compared to PA 200mg (+20.3%); NC compared to PA 400mg (+17.1%).

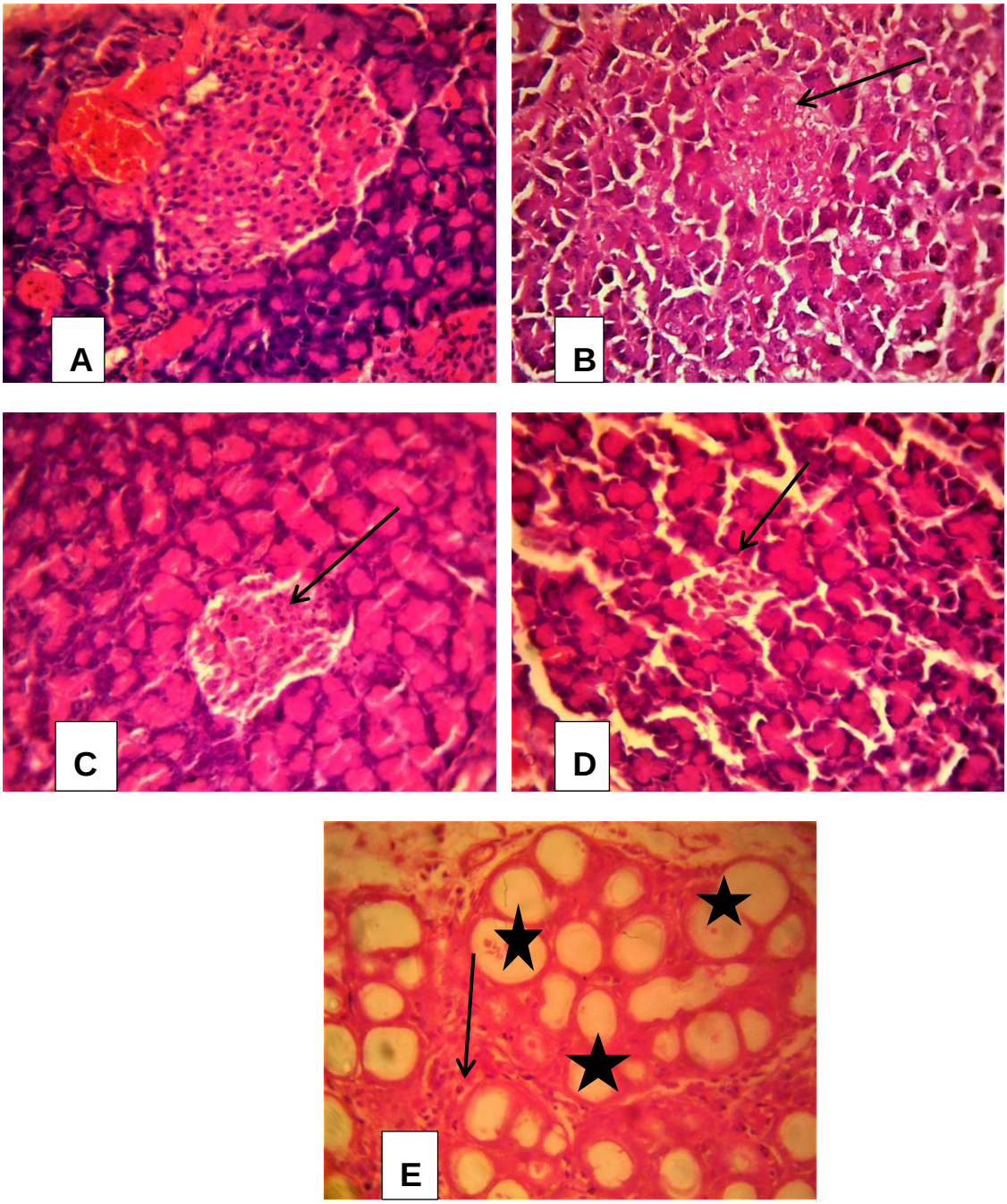


Figure 4.6: Histology of the pancreatic tissues

A. Healthy control: H&E, x600 showing No visible lesion; B. Diabetic Control: H&E, x600 with Severely shrunken, depleted and necrotic Islet of Langerhans (arrow); C. PA 200mg: H&E, x600 depicting Severe depletion of cells in the Islets of Langerhans (arrow) with no necrosis; **D**. PA 400mg: H&E, x600 Severely shrunken and depleted islet of Langerhans (arrow) with no necrosis; **E**. GLIBEN 0.2mg: H&E, x600. Total depletion of pancreatic acinar cells (stars), absence of Islets of Langerhans with severe interstitial fibrosis and infiltration by inflammatory cells (arrow).

4.6. Discussion

Hyperglycaemia which increases ROS is reported to be the underlining mechanism of oxidative stress in diabetes, hence, its role in diabetic complications [10]. Consequently, uncontrolled diabetes mellitus results in extensive damage, dysfunction, and failure of different organs. Therefore, intensive glycaemic control is very vital to mitigate the risks of microvascular complications in T1D and T2D. Our *In-vitro* analysis in this study revealed that PA aqueous and methanol extracts caused a significant inhibition in the activity of α-glucosidase, dose-dependently (figure 4.1).

Furthermore, it is known that Inhibitors of α-glucosidase breakdown starch and disaccharides to glucose; therefore, they may reduce the postprandial glucose levels by delaying the carbohydrate digestion and absorption [48]. In the present study, diabetic rats treated with PA aqueous extract at 400mg/kg body weight showed a reduction in postprandial blood glucose (as in figure 4.3A) when compared with the untreated diabetic rats. Our findings clearly showed that PA extract acted by inhibition of α-glucosidase, thereby delaying the absorption of glucose from the intestinal tract, hence, its antihyperglycemic effect. This study corroborates the previous investigators that showed that PA extracts at various doses tested, significantly reduced blood glucose levels [49,50]. Besides, in the study, we demonstrated that the antidiabetic effect exhibited by the PA extract is antihyperglycemic (as depicted in the OGTT results in figure 4.3A) and not hypoglycaemic as earlier reported [51] thus corroborating the work of Moshi et al. [52]. We observed persistent weight loss in the diabetic control animals as well as in all the diabetic treated groups up till day 21, after which there is a probable sign of weight gain after day 28 (figure 4.2). The reduction in weight supports one of the typical symptoms of diabetes mellitus, which is unexplained weight loss due to excessive hunger and urination. Although an earlier report showed that there was weight gain in diabetic animals after treatment with PA extract [53]. This claim could not be established in the present study. More so, scientific reports showed that tannin is abundantly present in *Phyllanthus amarus* [18,19]. Research showed that tannins have an antinutritional property that causes weight loss in rodents by forming complexes with proteins in the intestinal lumen [54,55] or by causing loss of appetite in the animals [56]. As a result, the abundant presence of tannin in this plant extract and the strain of the rats used in the present study could be possible reasons for the failure of the animals to experience weight gain following treatment with PA.

Furthermore, Adeneye [53] demonstrated that PA control glucose level in diabetes mellitus mediated by improving insulin resistance, hence supporting its ethnomedicinal use in the

traditional management of diabetes mellitus. Similarly, our study revealed that PA 400 mg/kg body weight increased serum insulin levels similarly to glibenclamide (a standard antidiabetic drug used in this study as depicted in Figure 4.3C) compared to the non-treated diabetic control rats as well as the healthy control animals (p>0.05). The finding agrees with a previous report that showed 400mg/kgbwt effectively controlled blood glucose in rats [24]).

Glycaemic control is very critical in the management of diabetes mellitus. However, glycaemic control is just one aspect of an overall management plan that encompasses the effective treatment of hypertension and dyslipidaemia [57,58], as both conditions are commonly encountered in patients with T2D. Nevertheless, results from randomized controlled trials have conclusively demonstrated that the risk of microvascular complications can be reduced by intensive glycaemic control in patients with type 1 and type 2 diabetes mellitus [59,60]. These trials led to the American Diabetes Association's glycosylated haemoglobin (HbA1c) goal of 7% after 60% and 25% reduction in diabetic retinopathy, nephropathy, and neuropathy. Besides, HbA1c reflects average plasma glucose over the previous eight to 12 weeks [61] and thereby giving a clear picture of glycaemic control [62]. Our study shows that HbA1c in diabetic untreated animals and the diabetic treated rats were raised (p<0.0001) versus the healthy controls (Figure 4.3B). The failure of this study to achieve a reduction in the HbA1c could also be due to the short study period, as assessing response to antidiabetic therapy involves periodic - generally 3 - to 6 – monthly- measurement of HbA1c [48].

Nitric oxide (NO) is increased in the course of vascular diseases such as diabetes and hypertension [63]. Higher glucose levels in serum were suggested to be responsible for the activation of endothelial cells to enhance NO levels. Besides, NO plays a vital role in the vascular flow of blood to the skeletal tissue, thereby increasing delivery and uptake of glucose by myocytes [64]. Tessari et al. [65] suggested that insulin resistance might be responsible for the reduced urinary excretion of NO in type 2 diabetes since the stimulation of NOS activity is a downstream effect of Akt activation by insulin. In the present study, PA 200mg/kgbwt significantly reduced serum NO levels compared with the diabetes untreated rats (Figure 4.4A). The observed low levels of NO are deemed to be beneficial for several physiological and cellular functions, while high levels of NO could be detrimental to the cells. The negative impact of nitric oxide is because its high levels may react with superoxide anion to generate peroxynitrite radical, which binds to proteins and thus affects their function [66]. This finding agrees with Adela et al. [67], who observed a significant increase in serum NO levels in hyperglycemic rats compared to healthy controls. Besides, the activity of serum myeloperoxidase (MPO), a

biomarker of oxidative stress and inflammation, remains high in the diabetic untreated rats (p<0.0001)versus the healthy controls while the extracts PA 200mg/kgbwt and PA 400mg/kgbwt brought down serum MPO compared with the diabetic rats (p<0.0001) in a similar manner to the glibenclamide (Figure 4.4B). The ability of the extract to bring down the serum level of myeloperoxidase activity in this report validates the plant extract's use in the local management of inflammation and diabetes [6,23,46,53].

There is a crosslink between hyperglycaemia, oxidative stress and diabetic complications [68,69]. Study shows that hyperglycaemia elicits oxidative stress by releasing free radicals [70]. During normal physiological conditions, networks of antioxidant defences protect the body from the harmful effects of free radicals [12]. However, these defences become weakened during diabetes, exacerbated by chronic hyperglycaemia to produce reactive oxygen species [71]. The abnormally high levels of free radicals and a concomitant decline in these antioxidant defences [72] may result in damage to cellular organelles and enzymes, which further results in increased lipid peroxidation and eventual insulin resistance [73]. In the present study, PA 400 mg/kgbwt increased glutathione (GSH) in the pancreatic tissue by +38.41% compared with the normal controls (p>0.05) (Figure 4.5A) while glutathione peroxidase (GPx) activity in PA 200mg group increased by 23% compared with the normal control (Figure 4.5D). PA 200mg/kgbwt and PA 400mg/kgbwt caused an insignificant increase (p>0.05) in glutathione-S-transferase (GST) activity by +20.3% and +17.1% respectively (Figure 4.5E). The two doses of the plant extract tend to bring the malondialdehyde (MDA) to normal levels similarly to the glibenclamide compared with the diabetic control rats (Figure 4.5C). These findings indicate that the plant extract has the potential to mitigate oxidative stress precipitated by diabetes.

Histologically, the most dramatic changes were observed in the GLIBEN 0.2mg/kg group. Here, the pancreatic islets were depleted. This depletion could indicate that glibenclamide treatment did not improve the progression of diabetes in this instance. The PA 200mg and PA 400mg groups showed depletion of the pancreatic islets. Besides, necrosis of the pancreatic islet cells was not a feature of these treatment groups compared to the diabetic controls. The absence of necrosis suggests that PA at 200mg/kgbwt and PA 400mg/kgbwt potentially put a halt to the progressive destruction of the islets of Langerhans. It is highly probable that the small-sized islets observed in PA 200mg and 400mg groups are signs of the ongoing regeneration of pancreatic islets that were hitherto destroyed during diabetes. Based on these findings, the possible ability of PA to stimulate endogenous β-cell proliferation needs further investigation. It has been reported that the proliferative rate of β-cells in one-month-old rats and seven-month-

old rats were approximately 4% and 0.5% per day, respectively [74]. Since the rats investigated in this study were six weeks old and well within the age range that pancreatic β-cell regeneration has been reported, it is highly probable that β-cell recovery occurred in the pancreatic islets in the PA 200mg/kg and PA 400mg/kg groups.

4.7. Conclusion

Oxidative stress during diabetic condition disturbs glycaemic indices. The findings from our study show that PA aqueous extract possesses a significant inhibition on α-glucosidase improves serum insulin levels, together with the activities of antioxidant enzymes, thereby modulating oxidative stress caused by diabetes. Therefore, this plant extract should be explored further as it could aid the development of antidiabetic drugs.

4.8. CRediT author statement

OOO & YGA: Conceptualization, supervision, writing - review & editing. FAO & AAO: methodology, investigation, and data analysis. FAO, AAO, MOT: Data interpretation. FAO: Writing - first draft preparation. OOO: Funding acquisition. OOO.

4.9. Acknowledgements

We with this thank Mr Fanie Rautenbach and Dr Jelili Badmus of OSRC, CPUT, Bellville Campus, South Africa for their technical input.

4.10. Declarations of interest

None to declare.

4.11. Funding Source Declaration

This research work was funded by the Cape Peninsula University of Technology's University Research Fund (RJ 24), and the National Research Foundation Grant (R022) awarded to Professor OO Oguntibeju. The funders took no part in the experimental formulation, data acquisition, analysis, and interpretation, writing of the reports and the decision as to where to submit the article for publication

References

[1]. Oliveira, J.S., Silva, A.A.N. and Silva Junior, V.A., 2017. Phytotherapy in reducing glycaemic index and testicular oxidative stress resulting from induced diabetes: a review. *Brazilian Journal of Biology*, *77*(1), pp.68-78

[2]. Gezginci-Oktayoglu, S., Basaraner, H., Yanardag, R. and Bolkent, S. The effects of combined treatment of antioxidants on the liver injury in STZ diabetic rats. Dig Dis Sci. 2009, 21;54(3):538–46.

[3]. American Diabetes Association. Diagnosis and classification of diabetes mellitus. Diabetes Care. 2013;36(1):67–74.

[4]. Ayeleso, A.O., Matumba, M.G., Ntambi, J.M. and Mukwevho, E. 2020. Insights into the metabolism of lipids in obesity and diabetes. In *Lipid Signaling and Metabolism* (pp. 345-357). Academic Press.

[5]. Tesfaye, S. and Gill, G. Chronic diabetic complications in Africa. African J. Diabetes Med. 2011;19(1):4–8.

[6]. Patel, J.R., Tripathi, P., Sharma, V., Chauhan, N.S. and Dixit, V.K. Phyllanthus amarus: Ethnomedicinal uses, phytochemistry and pharmacology: A review. J Ethnopharmacol 2011;138(2):286–313.

[7]. Matough, F.A., Budin, S.B., Hamid, Z.A., Alwahaibi, N. and Mohamed, J. The role of oxidative stress and antioxidants in diabetic complications. Sultan Qaboos Univ Med J. 2012;12(1):5–18.

[8]. Alabi, T.D., Olabiyi, F.A. and Oguntibeju, O.O., 2020. Palm oil: its antioxidant potential in diabetes mellitus. In *Diabetes* (pp. 285-291). Academic Press.

[9]. Adly, A.A.M. Oxidative stress and disease: An updated review. Vol. 3, Research Journal of Immunology. 2010. p. 129–45.

[10]. Wiernsperger, N. Oxidative stress as a therapeutic target in diabetes: revisiting the controversy. Diabetes Metab. 2003; 29(6):579–85.

[11]. Rao, Y.M, Lakshmi, I.A, Bhargavi, C. and Umavenkatesh, S. Effect of captopril and allylmercaptocaptopril on antioxidant status in Streptozotocin-induced diabetic rats. Int J PharmTech Res. 2010;4(2):2251–5.

[12]. Bonini, M.G., Dull, R.O. and Minshall, R.D. 2014. Systems Biology of Free Radicals and

Antioxidants. *Berlin: Springer*, 1343-1363.

[13]. Gao, R., Wang, Y., Wu, Z, Ming, J. and Zhao, G. Interaction of barley β-glucan and tea polyphenols on glucose metabolism in streptozotocin-induced diabetic rats. J Food Sci. 2012;77(6):128–34.

[14]. Nathan, D.M. The diabetes control and complications trial/epidemiology of diabetes interventions and complications study at 30 years: Overview. Diabetes Care. 2014;37(1):9–16.

[15]. Al-Achi, A. Herbs that affect blood glucose levels. Women's Heal Prim Care. 2005;8(7):325–30.

[16]. Maciel, M.A.M., Cunha, A.F. and Dantas, TNC. NMR Characterization of Bioactive Lignans from Phyllanthus amarus Schum &. Ann Magn Reson. 2007;6(3):76–82.

[17]. Verma, S., Sharma, H. and Garg, M. Phyllanthus amarus: A review. J Pharmacogn Phytochem. 2014;3(2):18–22.

[18]. Foo, L. and Wong H. Phyllanthusiin D, an unusual hydrolysable tannin from Phyllanthus amarus. Phytochemistry. 1992;31(2):711–3.

[19]. Foo, L.Y. Amariin, a di-dehydrohexahydroxydiphenoyl hydrolysable tannin from Phyllanthus amarus. Phytochemistry. 1993;33(2):487–91.

[20]. Kassuya, C.A.L., Silvestre, A., Menezes-de-Lima, O., Marotta, D.M., Rehder, V.L.G. and Calixto, JB. Antiinflammatory and antiallodynic actions of the lignan niranthin isolated from Phyllanthus amarus. Evidence for interaction with platelet-activating factor receptor. Eur J Pharmacol. 2006;546(1–3):182–8.

[21]. Leite, D.F.P., Kassuya, C.A.L., Mazzuco, T.L., Silvestre, A., De Melo, L.V., Rehder, VLG, Rumjanek, V.M. and Calixto, J.B. The cytotoxic effect and the multidrug resistance reversing action of lignans from Phyllanthus amarus. Planta Med. 2006;72(15):1353–8.

[22]. Houghton, P.J., Woldemariam, T.Z. and O'Shea, S. Thyagarajan SP. Two securinega-type alkaloids from Phyllanthus amarus. Phytochemistry. 1996;43(3):715–7.

[23]. Kiemer, A.K., Hartung, T., Huber, C. and Vollmar, A.M. Phyllanthus amarus has anti-inflammatory potential by inhibition of iNOS, COX-2, and cytokines via the NF-kB pathway. J Hepatol. 2003;38:289–97.

[24]. Adedapo, A., Ofuegbe, S. and Oguntibeju, O. The antidiabetic activities of the aqueous

leaf extract of Phyllanthus amarus in some laboratory animals. In: Antioxidant-Antidiabetic Agents and Human Health. 2014. Rijeka. InTech. 115–37.

[25]. Olabiyi, F.A., Aboua, Y.G., Popoola, O.K., Monsees, T.K. and Oguntibeju, O.O. Evaluation of antioxidant, antityrosinase activities and cytotoxic effects of Phyllanthus amarus extracts. Nat Prod J. 2020;10(2):130–8.

[26]. Wang, R.Y., Huang, J.H., Hsu, G.S.W., Lin, W.T. and Lu, Y.F., 2017. Hypoglycaemic and antioxidative effects of Glossogyne tenuifolia on streptozotocin-nicotinamide-induced diabetic rats. *American Journal of Plant Sciences*, *8*(5), pp.1170-1181.

[27]. Ajiboye, T.O., 2018. Colistin sulphate induced neurotoxicity: Studies on cholinergic, monoaminergic, purinergic and oxidative stress biomarkers. *Biomedicine & Pharmacotherapy*, *103*, pp.1701-1707.

[28]. Akinyede, A., Ikumawoyi, V.O., Kujembola, O. and Nwaiwu, O. Toxicological evaluation of a polyherbal formulation on biochemical parameters in Sprague-Dawley rats. *Nigerian Quarterly Journal of Hospital Medicine*, 2016, *26*(3), pp.546-550.

[29]. Haberal, E.T., Ulu, İ., Özcan, C., Gök, G.A., Kıran, G. and Kucur, M. Is there a relationship between delivery mode and serum irisin levels in pregnant women? *Journal of Laboratory Medicine*, 2017, *41*(6), pp.317-323.

[30]. Uittamo, J., Nieminen, M.T., Kaihovaara, P., Bowyer, P., Salaspuro, M. and Rautemaa, R. Xylitol inhibits carcinogenic acetaldehyde production by Candida species. *International journal of cancer*, 2011, *129*(8), pp.2038-2041.

[31]. Ademiluyi, A.O. and Oboh, G. Soybean phenolic-rich extracts inhibit key-enzymes linked to type 2 diabetes (α-amylase and α-glucosidase) and hypertension (angiotensin I converting enzyme) in vitro. Exp Toxicol Pathol. 2013; 65(3):305–9.

[32]. Telagari, M. and Hullatti, V. In-vitro α-amylase and α-glucosidase inhibitory activity of Adiantum caudatum Linn. and Celosia argentea Linn. extracts and fractions. Indian J Pharmacol 47(4), 425. 2015;47(4):425.

[33]. Shai, L.J., Magano, S.R., Lebelo, S.L. and Mogale, A.M. Inhibitory effects of five medicinal plants on rat alpha-glucosidase: comparison with their effects on yeast alpha-glucosidase. J Med Plants Res. 2011;5(13):2863–7.

[34]. Owiredu, W.K.B., Amegatcher, G. and Amidu, N. Precision and accuracy of three blood glucose meters: Accu-chek advantage, one-touch horizon and sensocard. J Med Sci. 2009;9(4):185–93.

[35]. Al-Hindi, B., Yusoff, N.A., Atangwho, I.J., Ahmad, M., Asmawi, M.Z. and Yam, M.F., 2016. A soxhlet extract of Gongronema latifolium retains moderate blood glucose-lowering effect and produces structural recovery in the pancreas of STZ-induced diabetic rats. *Med. Sciences*, *4*(2), p.9

[36]. Misra, H. and Fridovich, I. The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. J Biol Chem. 1972;217:3170–5.

[37]. Pougoue, J.K., Fokunang, E.T., ngoupayo, J., beringyuy, E.B., njinkio., B.N., MBAH J.A., fonmboh, J.D., ngameni, B. and fokunan, C.N. Evaluation of antioxidant properties of secondary metabolites in aqueous extracts of Ficus: thonningii Blume tested on Wistar rats. J Anal Pharm Res. 2020;9(1):27?35. DOI: 10.15406/japlr.2020.09.00348.

[38]. Prohaska, J.R. and Ganther, H.E. Glutathione peroxidase activity of glutathione-S-transferases purified from rat liver. Biochem Biophys Res Commun. 1977;76(2):437–45.

[39]. Beutler, E., Duron, O. and Kelly, B. Improved method for the determination of blood glutathione. J Lab Clin Med. 1963;6:882–8.

[40]. Varshney, R. and Kale, R.K. Effects of calmodulin antagonists on radiation-induced lipid peroxidation in microsomes. Int J Radiat Biol. 1990;58(5):733–43.

[41]. Zahra, S., Ahmed, M., Qasim, M. *l,.* Gul, B., Zia, M., Mirza, B. and Haq, I.U., Polarity based characterization of biologically active extracts of *Ajuga bracteosa* Wall. Ex Benth and RP-HPLC analysis. *BMC Complement Altern Med.* 2017, **17,** 443. https://doi.org/10.1186/s12906-017-1951-5.

[42]. Hatami, M. and Ghorbanpour, M. Effect of nanosilver on physiological performance of pelargonium plants exposed to dark storage. *J. Horticul. Res.* 2013, *21*(1), pp.15-20.

[43]. Xia, Y. and Zweier, J.L. Measurement of myeloperoxidase in leukocyte-containing tissues. Anal Biochem. 1997; 245(1):93–6.

[44]. Maciel, E.A.P., Athanazio, D.A., Reis, E.A.G., Cunha, F.Q., Queiroz, A., Almeida, D., McBride, A.J., Ko, A.I. and Reis. High serum nitric oxide levels in patients with severe leptospirosis. Acta Trop. 2006;100(3):256–60.

[45]. Green, L.C., Ruiz de Luzuriaga, K., Wagner, D.A., Rand, W., Istfan, N., Young, V.R. and

Tannenbaum, S.R. Nitrate biosynthesis in man. Proc Natl Acad Sci U S A. 1981;78(12):7764–8.

[46]. Sarchielli, P., Pini, L.A., Coppola, F., Rossi, C., Baldi, A., Mancini, M.L. and Calabresi, P. Endocannabinoids in chronic migraine: CSF findings suggest a system failure. *Neuropsychopharmacology*, 2007, *32*(6), pp.1384-1390.

[47]. Drury, R. and Wellington, E. Carlton's histopathological techniques: Oxford University Press. London, New York. 1976.

[48]. Krentz, A.J. and Bailey, C.J., 2005. Oral antidiabetic agents. *Drugs*, *65*(3), 385-411.

[49]. Lawson-Evi P., Eklu-Gadeg, K., Agbonon, A., Aklikokou, K., Creppy, E. and Gbeassor, M. Antidiabetic activity of Phyllanthus amarus schum and Thonn (Euphorbiaceae) on alloxan-induced diabetes in male Wistar rats. J Appl Sci. 2011;11(16):2968–73.

[50]. Adedapo, A.A., Ofuegbe, S.O. and Adeyemi, A.A. The anti-diabetic activities of the methanol leaf extract of Phyllanthus amarus in some laboratory animals. Asian J Med Sci. 2013; 4(3):23–34.

[51]. Adeneye, A.A., Amole, O.O. and Adeneye, A.K. Hypoglycemic and hypocholesterolemic activities of the aqueous leaf and seed extract of Phyllanthus amarus in mice. Fitoterapia. 2006;77(7–8):511–4.

[52]. Moshi, M.J., Lutale, J.J.K., Rimoy, G.H., Abbas, Z.G., Josiah, R.M. and Swai, A.B.M. The effect of Phyllanthus amarus aqueous extract on blood glucose in non-insulin-dependent diabetic patients. Phytother Res. 2001;15(7):577–80.

[53]. Adeneye, A.A. The leaf and seed aqueous extract of Phyllanthus amarus improves insulin resistance diabetes in experimental animal studies. J Ethnopharmacol. 2012;144(3):705–

[54]. Butler, L.G. Antinutritional effects of condensed and hydrolyzable tannins. Vol. 59, Basic life Sci. 1992. 693–8.

[55]. Carbonaro, M., Grant, G. and Pusztai, A. Evaluation of polyphenol bioavailability in rat small intestine. Eur J Nutr. 2001;40(2):84–90.

[56]. Oliveira, A.C.P., Endringer, D.C., Amorim, L.A.S., Brandão, M.D.G.L. and Coelho, M.M. Effect of the extracts and fractions of Baccharis trimera and Syzygium cumini on glycaemia of diabetic and non-diabetic mice. J Ethnopharmacol. 2005;102(3):465–9.

[57]. Tuomilehto, J. Controlling glucose and blood pressure in type 2 diabetes: starting treatment earlier may reduce complications. Br Med J. 2000;321:394–6.

[58]. Adler, A. Cardiovascular risk reduction in diabetes underemphasised and overdue. Messages from major trials. Clin Med (Northfield Il). 2001;1(6):472–7.

[59]. The Diabetes Control Complications Trial Research Group. The effect of intensive treatment of diabetes on the development and progression of long -term complications of insulin-dependent diabetes mellitus. N Engl J Med. 1993;329(14):977–86.

[60]. UK Prospective Diabetes Study (UKPDS) Group. Intensive blood-glucose control with sulfonylureas or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes. Lancet. 1998;352(2):837–53.

[61]. Nathan, D.M., Turgeon, H. and Regan, S. Relationship between glycated haemoglobin levels and mean glucose levels over time. Diabetologia. 2007;50(11):2239–44.

[62]. World Health Organization. Use of glycated haemoglobin (HbA1c) in the diagnosis of diabetes mellitus. Abbreviated Report of a WHO Consultation. 2011.

[63]. Tikkanen, I. and Fyhrquist, F. Nitric oxide in hypertension and renal diseases. Ann Med. 1995;27(3):353–7.

[64]. Goboza, M. Modulatory and antidiabetic effects of vindoline and Catharanthus roseus in type 2 diabetes mellitus induced male Wistar rats and in RIN-5F cell line. Cape Peninsula University of Technology, 2019. 9.

[65]. Tessari, P., Cecchet, D., Cosma, A., Vettore, M., Coracina, A., Millioni, R, Iori, E., Puricelli, L., Avogaro, A. and Vedovato, M. Nitric oxide synthesis is reduced in subjects with type 2 diabetes and nephropathy. Diabetes. 2010; 59(9):2152–9.

[66]. Pacher, P., Beckman, J.S. and Liaudet, L. Nitric oxide and peroxynitrite in health and disease. Physiol Rev. 2007; 87(1):315–424.

[67]. Adela, R, Nethi, S.K., Bagul, P.K., Barui, A.K., Mattapally, S., Kuncha, M, Patra, C.R., Reddy, P.N.C. and Banerjee, S.K. Hyperglycaemia enhances nitric oxide production in diabetes: A study from South Indian patients. PLoS One. 2015;10(4):1–17.

[68]. Asmat, U., Abad, K. and Ismail, K. Diabetes mellitus and oxidative stress-A concise review. Saudi Pharm J. 2016;24(5):547–53.

[69]. Yaribeygi, H., Atkin, S.L. and Sahebkar, A. A review of the molecular mechanisms of

hyperglycemia-induced free radical generation leading to oxidative stress. J Cell Physiol. 2019;234(2):1300–12.

[70]. Ahmed, R.G. The physiological and biochemical effects of diabetes on the balance between oxidative stress and antioxidant defence system. Med J Islam World Acad Sci. 2005;15(1):31–42.

[71]. Erejuwa, O. Oxidative stress in diabetes mellitus: Is there a role for hypoglycemic drugs and/or antioxidants? In: Oxidative Stress and Diseases. 2012. p. 217–46.

[72]. Nwanna, E.E., Ibukun, E.O. and Oboh, G., 2013. Inhibitory effects of methanolic extracts of two eggplant species from South-western Nigeria on starch hydrolysing enzymes linked to type-2 diabetes. *Afri. J. Pharm. & Pharmacol. 7*(23), pp.1575-1584.

[73]. Maritim, A.C., Sanders, RA. and Watkins, J.B. Diabetes, oxidative stress, and antioxidants: A review. J Biochem Mol Toxicol. 2003;17(1):24–38.

[74]. Montanya, E., Nacher, V., Biarnes, M. and Soler, J. Linear correlation between β-cell mass and body weight throughout the lifespan in Lewis rats. Role of β-cell hyperplasia and hypertrophy. Diabetes. 2000;49(8):1341–6.

CHAPTER FIVE: *Phyllanthus amarus* attenuates hepatic damage and associated biochemical derangements in a diabetic animal model

Folorunso Adewale Olabiyi[1,6], Ademola Adetokunbo Oyagbemi[2], Monsuru Tijani[3], Yapo Guillaume Aboua[4], Alex Adeolu Adedapo[5] & Oluwafemi Omoniyi Oguntibeju[1]*,

[1]Phytomedicine and Phytochemistry Research Group, Department of Biomedical Sciences, Oxidative Stress Research Centre, Cape Peninsula University of Technology P.O. Box 1906, Bellville 7535, South Africa.

[2]Department of Veterinary Physiology and Biochemistry, University of Ibadan, Nigeria.

[3] Department of Veterinary Pathology, University of Ibadan, Nigeria.

[4]Department of Health Sciences, Namibia University of Science and Technology, Private Bag 13388, Windhoek, Namibia.

[5]Department of Veterinary Pharmacology and Toxicology, University of Ibadan, Nigeria,

[6]Department of Medical Laboratory Science, Afe Babalola University, P.M.B 5454 Ado-Ekiti, Nigeria

All correspondence to oguntibejuo@cput.ac.za; Tel.: +27-219538495

NB: Preparation of the manuscript for submission from this chapter is according to the BIOMEDICINES journal author's guidelines.

ABSTRACT

This research seeks to assess the inherent protective mechanisms of *Phyllanthus amarus* (PA) out of hepatic, renal and cardiac complications in a T2D diabetic animal model. T2D was created in male Wistar rats with fructose: streptozotocin model, followed by oral feeding with PA (200mg/kgBWT. and 400mg/kgBWT) or glibenclamide (0.2 mg/kgBWT) daily for four weeks. The effects of the treatments on the liver, kidney and cardiac functions of diabetic rats were assessed in blood and tissue samples. There was persistent emaciation in the animals, except among the healthy controls up to day 21 of the experiment and a significant difference in absolute and relative liver weights of diabetic untreated rats compared with the healthy control (p<0.0001). PA 200mg/kgBWT significantly reduced the triglycerides levels compared with diabetic non-treated animals (p<0.0001). PA 200mg/kgBWT and PA 400mg/kgBWT caused a rise in the activity of GPx together with reduced lipid peroxidation in the rats' liver, kidney as well as the heart when compared with glibenclamide and diabetic untreated rats. PA 200mg/kgBWT, as well as the PA 400mg/kgBWT, normalised the activities of ALP and AST respectively corresponding to the diabetic control rats.

In comparison, PA 200mg/kgBWT normalised the activity of GGT when corresponding with the diabetic control animals. Besides, there was notable hypoproteinemia in all the diabetic groups (p<0.0001) similarly to the healthy control rats. Conclusively, *Phyllanthus amarus* possesses an ameliorative effect on the hepatic damage precipitated by diabetes.

Keywords: *Phyllanthus amarus*, triglycerides, oxidative stress, liver, kidney, heart, Wistar rats

5.1. Introduction

Diabetes mellitus is a chronic, metabolic disorder of carbohydrate, protein, and lipid metabolism. It is either due to complete or partial insulin deficiency or insulin action [1;2]. This abnormality is typified by an excessive amount of free glucose in the bloodstream, a condition known as hyperglycaemia. The chronic hyperglycaemia of diabetes leads to the release of reactive oxygen species, resulting in oxidative stress. A recent study suggests that hyperglycaemia is the pathophysiological basis for oxidative stress [3], occurring in cases of an imbalance between the body's natural defence system and the reactive oxygen species. It is the resultant stress created on various cells and organs by hyperglycaemia that produces complications. As a result, in diabetic patients, prolonged damage, dysfunction, and organs failure, especially the eyes, kidneys, nerves, heart, testes and blood vessels are related to uncontrolled hyperglycaemia [2;4;5;6].

In the clinical setting, drugs are often prescribed to treat diabetes. These conventional diabetic treatments bring about side effects such as gastrointestinal disturbances, flatulence, abdominal discomfort, hypoglycaemia, weight gain, dizziness, bloating and diarrhoea. Adverse effects of the drugs, failure rates with their exorbitant cost make people hunt for alternative or complementary medicine because they are natural in origin with fewer side effects [7]. Hence, the current global awareness and consideration for natural products such as medicinal plants.

Phyllanthus amarus is a herb found in the tropics around the world. It possesses antidiabetic, hypolipidemic, anti-inflammatory potentials [8]. Many phytochemicals in the Phyllanthus species constituting their active principle and available in the plant are lignans, glycosides, flavonoids, alkaloids, ellagitannins and phenylpropanoids that are present in their leaves, stems, as well as the roots. Some lipids such as sterols and flavonols are also found in the plant [9]. *Phyllanthus amarus* is known to have two lignans, viz phyllanthin and hypophyllanthin, from the leaves of the plant [10;11]. Niranthin, nirtetralin, phyltetralin and lintetralin; the four-flavanone glycoside has also been reported to be from the leaves of *Phyllanthus amarus* [12;13]. This study is uniquely designed to investigate the mechanism of its antidiabetic, radical scavenging, and protective effect against complications on the liver, kidney, and cardiovascular system in a rat model of diabetes.

Therefore, this study evaluated potential protective mechanisms of *Phyllanthus amarus* against hepato-renal and cardiac complications in streptozotocin-induced diabetes male Wistar rats. Besides, we measured various biochemical markers in the liver, kidney, and heart tissues after different treatment protocols in a streptozotocin-induced diabetic male Wistar rat.

5.2. Materials and methods

5.2.1. Reagents

Streptozotocin (18883-66-4) was procured from Merck Group-Sigma-Aldrich Chemical Company, South Africa. D-Fructose was bought from Loba Chemie PVT. LTD, Mumbai, India. Cobas Elecsys® reagents, controls, and calibrators for use on Roche/Hitachi Cobas c 311 and Cobas e 411 analysers were purchased from Roche Diagnostics (Roche, Germany).

5.2.2. Bioactive Materials

Fresh whole *Phyllanthus amarus* plants were collected during the raining season between July – September 2016 at Ado-Ekiti in south-west Nigeria. A botanist from the forestry herbarium, (FRIN), Ibadan assisted with the identification and authentication of the plant sample (FHI NO: 110242) for future reference. Leaves from this plant were shade-dried and ground to a powder with mechanical mill (Mincer 9FQ-28, China). 100gm of powdered aqueous extract soaked in 1 L of distilled water and stirred, was left for 24h, at 24^{o}C - 25^{o}C. The resultant homogenate was filtered with Whatman no one filter paper. Its fraction was then evaporated to dryness at reduced pressure in a rotary evaporator (Buchi Rotavapor II, Buchi Germany) at 40^{o}C and lyophilized in a freeze-dryer (VirTis Genesis 25EL, SP Scientific, NY, USA) for sixteen hours; the dried extract was then preserved at 4^{o}C until ready for use [14].

5.2.3. Animal and Ethics Protocols

Animal ethics clearance was sought and obtained from the Research Ethics Committee, Health and Wellness Sciences Faculty, Cape Peninsula University of Technology (approval no: CPUT/HW-REC 2016/A1). Also, guidelines for the care and use of laboratory animals from the animal's ethics committee of the University of Ibadan were strictly followed in all the experimental protocols and the handling of the animals (approval No: UI-ACUREC/19/0017). Six (6) weeks old male Wistar rats, weighing 190±10gm were studied. They were procured and reared at the animal house, Department of Vet. Physiology and Biochemistry, UI., Nigeria where they were housed in individual cages under controlled, standard, laboratory conditions; humidity between 45% - 55%, and an ambient temperature between 22^{o}C - 26^{o}C and regular photoperiod (12-hours dark:12-hour light). They were fed two times in a day (09:00 am and 4:00 pm) with

rats' cubes of pelleted feed produced by Ladokun and Sons Livestock Feed, Nigeria Limited. The animals were afforded unrestricted access to clean water inside clean bowls.

5.2.3.1. Animal Grouping

Fifty (50) rats were arbitrarily distributed in five groups having ten rats each and marked: NC – Normal untreated Control; DC – Diabetic untreated Control; PA 200mg/kgBWT – Diabetic treated rats with *Phyllanthus amarus* (200mg per kg body weight); PA 400mg/kgBWT– Diabetic treated rats with *Phyllanthus amarus* (400mg per kg body weight); GLIBEN 0.2mg/kgBWT – Diabetic rats treated with glibenclamide, a conventional antidiabetic drug, (0.2mg per kg body weight).

5.2.3.2. Type 2 diabetes mellitus (T2D) in the animals

Animals were allowed one week after purchase to acclimatize to their new environment. After that, the animals had unrestricted access to 10% fructose water within another two weeks, and their weights were measured. After termination of the fructose water, a once-off, medium-dose streptozotocin injection (55mg/kgBWT) into cold citrate buffer pH 4.5 was administered intraperitoneally to induce diabetes. The non-diabetic control group received the same cold citrate buffer, intraperitoneally. Diabetes mellitus was established after 96h (4 days) of streptozotocin administration. We fasted the rats for 4h after which blood glucose was determined. Animals having glucose levels ≥ 11.1mmol/L were regarded as diabetic to be used in the experiment.

5.2.3.3. Treatment

Treatment to respective experimental groups with the extract and standard antidiabetic drug, glibenclamide (0.2mg per kg body weight per day) was commenced after 96h of diabetes confirmation. Again, body weights and blood glucose measurements were taken once weekly. Oral administration of the extract/glibenclamide then continued daily for four calendar weeks.

5.2.4. Oral Glucose Tolerance Test (OGTT) and Samples for Biochemical assays

A day before sacrifice, rats were fasted for eight hours, after which an OGTT was done on all the animals. Blood sample (0 min) was taken; then the rats received daily treatments. A dose of D-

glucose (50%) solution (2 g/kgBWT) was orally administered to each rat, and the successive glucose levels were determined at 30, 60, 90 and 180-minutes post-glucose ingestion. Blood (10µL) was withdrawn from the rats via tail prick method, then glucose level evaluated using Accu-chek$^{(R)}$ glucometer (Roche, Germany) based on the glucose oxidase method [15].

5.2.4.1. Liver, Kidney, Heart Tissues, and blood Samples Collection

After the four weeks' study period, the experiment was terminated, and the animals were sacrificed the following day. On the day of sacrifice, five rats (n=5) in each group were anaesthetised and euthanized using sodium pentobarbital, 60mg/kgBWT followed by cervical dislocation. 6mL of blood specimen was drawn and dispensed into respective blood tubes (plain, EDTA and lithium heparinized) for biochemical evaluations. The clotted, heparinized and EDTA blood samples were centrifuged at 3,500 revolutions per minute (rpm) for 20 minutes. The supernatant was separated labelled appropriately and stored in a -80^{o}C freezer until the time of analysis. Liver, kidney, and heart tissues were removed, washed with PBS then fixed by 10% formol-saline fixative, for histopathological assessment.

5.2.4.2. Homogenization

Heart, kidneys, and liver were immediately cut out, trimmed of fats, and washed in ice-cold PBS solution and weighed. The tissue samples were sliced into smaller pieces and homogenized in the buffer (1gm/2mL) using a homogenizer (Yellow^{line}D1 25 basic, IKA® -WERKE GMBH & CO, Germany); then briefly centrifuged at -4^{o}C for 20mins. The supernatant was separated and dispensed into 2mL cryovials and frozen at -80^{o}c until ready for endogenous antioxidant and biochemical evaluations. (Yellow^{line}D1 25 basic, IKA® -WERKE GMBH & CO, Germany).

5.2.4.3. Bodyweight change, organ weights and relative organ weights

Throughout the experimental procedures, body weights were monitored weekly. Changes in body weight of the animals were calculated as previously described [16], using the formula below:

$$\boldsymbol{Body\ weight\ change}\ (\boldsymbol{g}) = \boldsymbol{Final\ body\ weight}\ (\boldsymbol{g}) - \boldsymbol{Initial\ body\ weight}\ (\boldsymbol{g})$$

Where, the initial body weight = mean weight at day 4 (96h) post-streptozotocin administration and the final body weight = the mean weights at termination.

The cut and trimmed liver, kidneys and heart tissues of the rats were weighed on a tabletop weighing balance (Precisa 2200C SCS, Precisa Instruments AG, Switzerland) to get their absolute weights, while the organs' relative weights were calculated as follows:

$$\textbf{\textit{Relative organ weights}}\ (\%) = \frac{\textbf{\textit{Absolute weight}}\ (\boldsymbol{g})}{\textbf{\textit{Final body weight}}\ (\boldsymbol{g})}\ \boldsymbol{x}\ \mathbf{100}$$

$$\textbf{\textit{Combined relative kidney weight}}\ (\%) = \frac{\textbf{\textit{Left + right absolute kidney weights}}\ (\boldsymbol{g})}{\textbf{\textit{Final body weight}}\ (\boldsymbol{g})}\ \boldsymbol{x}\ \mathbf{100}$$

5.2.4.4. Blood Glucose and Serum Insulin

5.2.4.4.1. Blood Glucose

Fasting blood glucose (FBG) was measured based on the glucose dehydrogenase method [15], using a portable glucometer (Accu-Chek® Active, Roche, Germany). Glucose dehydrogenase in the strip converts the glucose in the blood sample to gluconolactone. This reaction liberates two electrons that react with a coenzyme (PQQ) electron acceptor. The complete reaction creates a harmless electrical current that the meter interprets as blood glucose. On a test strip that was slid in the measurement window, one drop (10µL) from the rat's tail prick was applied on the test field of the test strip, the meter then displays the concentration of glucose in mmol/L.

5.2.4.4.2. Serum Insulin

The level of insulin in the rat's serum was determined using Thermoscientific™ Pierce™ Rat insulin, ELISA Kit by carefully following its manufacturer's instruction as contained in the products insert. A calibration curve was plotted having the average absorbance for each standard concentration on the Y-axis against its corresponding insulin concentration on the X-axis. The level of insulin (µIU/mL) in each sample was determined by tracing the insulin concentration (X-axis) to the absorbance value on the Y-axis. Expressing the results in SI unit, 1µIU/mL = 6.945ρmol/L.

5.2.5. Assessment of serum markers of hepatic, renal and cardiac functions

5.2.5.1. Assessment of Hepatic Function

To assess the hepatic function in the rats' sera, the activities of the liver enzymes and the protein concentrations, as shown in figure 5.6 were determined colourimetrically on Roche/Hitachi Cobas c 311 analyser.

5.2.5.2. Assessment of Renal Function

5.2.5.2.1. Electrolytes

The rats' plasma sodium, potassium and chloride ions were determined using ion-selective electrode (ISE) on the Cobas c 311 analyser for clinical chemistry. At the same time, serum HCO_3^- was measured by titrimetric analysis.

5.2.5.2.2. Serum Urea

Urea in the rat's serum was determined kinetically using urease with glutamate dehydrogenase on Roche/Hitachi Cobas c 311 analysers.

5.2.5.2.3. Serum Creatinine

Creatinine in the rat's serum was determined using the Creatinine Jaffe Gen.2 (CREJ2) in-vitro test on Roche/Hitachi Cobas c systems.

5.2.5.2.4. Serum Uric acid

Uric acid (UA) level in the rat's serum was determined using a modified indirect equilibrium enzymatic (uricase) colourimetric test on Roche/Hitachi Cobas c 311 analysers.

5.2.5.3. Assessment of Cardiac Functions

5.2.5.3.1. Serum Lipid parameters

The rat's serum total cholesterol (TC), triglycerides (TG), high-density lipoprotein (HDL) and low-density lipoprotein cholesterol (LDL) were estimated using colourimetric assay procedures on Roche/Hitachi Cobas c 311 analysers.

5.2.5.3.2. Serum Troponin

Cardiac troponin I (cTnI) was determined in the rat's serum using the electrochemiluminescence immunoassay on Elecsys immunoassay analyser.

5.2.6. Endogenous Antioxidant Estimation

The activities of antioxidant enzymes in the liver, kidney and heart tissue homogenates were measured spectrophotometrically in a transparent 96-well plate with microplate ELISA reader (Multiskan Spectrum by Thermo Electron Corporation, MA, USA). The activity SOD in liver, kidney and heart tissues homogenate was determined by a modified method of Mistral and Fridovich [17]. Also, their glutathione-S-transferase activity was determined following a modified method (Prohaska ad Ganther [18]. Whereas, glutathione peroxidase (GPx) activity together with the level of reduced glutathione (GSH were measured according to a previously described method, Beutler et al. [19].

5.2.6.1. Tissue Lipid Peroxidation

Malondialdehyde (MDA) content was measured using the thiobarbiturate (TBA) test in the hepatic, renal and cardiac tissues according to a method described by Varshney and Kale [20]. MDA (nMol) as a function of lipid peroxidation was calculated by multiplying the absorbances by a molar extinction coefficient of 1.56×10^5/M/cm.

5.2.7. Anti-inflammatory Biomarkers

5.2.7.1. Myeloperoxidase Activity

Serum myeloperoxidase (MPO) activity was determined according to the method of Xia and Zweier [21]. In a cuvette was pipetted 200µL of O-dianisidine and H_2O_2 mixture followed by addition of 10µL of the serum sample. The mixture was measured at 0 seconds, 30 seconds, and 60 seconds respectively at 460nm wavelengths.

Calculation:

$$\boldsymbol{MPO\ generated} = \frac{\boldsymbol{change\ in\ activity}}{\boldsymbol{mg\ protein}}\ \boldsymbol{x\ 10}$$

T_1=30-0 and T_2 = 60 seconds.

5.2.8. Estimation of Serum Nitric Oxide

A modified method, as described by Maciel et al. [22], was followed to measure serum nitric oxide (NO) levels. In this method, serum NO levels were determined by the measurement of NO_X (nitrite and nitrate) after enzymatic reduction of nitrate to nitrite[23] by nitrate reductase, as previously described by Green et al., [24]. Briefly, 50µL of undiluted serum was incubated with the same volume of reductase buffer 0.1M potassium phosphate, p^H 7.5, containing 1mM nicotinamide adenine dinucleotide phosphate, 10mM flavin adenine dinucleotide and four units of nitrate reductase/mL for 20h at 37^{o}C [22]. The Nitrite concentration was then determined using the Griess reaction as described by Maciel et al. [22].

5.2.9. Histopathological Assessment of liver, kidney and heart using Haematoxylin and Eosin (H&E) stain

Liver, kidney, as well as heart tissues, were collected into 10% formol-saline; then, processed and embedded in paraffin wax. Afterwards, sections of 5µm in thickness were prepared and stained using haematoxylin and eosin (H&E) for histopathological assessment [25]. Histopathology evaluation was carried out by a qualified veterinary pathologist (Department of

Vet. Pathology, University of Ibadan, Nigeria) with no prior knowledge of the animal groupings and their treatments.

5.2.10. Statistical analysis

All graphical evaluations and representations were done with GraphPad Prism software, version 5.0 (GraphPad Software, San Diego, California, USA). Data were displayed as mean values (± SEM), n=5 [26]. A one-way ANOVA together with Bonferroni's multiple comparison evaluation (if $p < 0.05$) was used. Differences were significant if p is less than 0.05 [27].

5.3. Results

5.3.1. Body and organ weights of the animals

The results as depicted in figure 5.1, show a persistent body weight loss of the animals, except the healthy controls up until day 21 of the experiment, after which there was a visible sign of weight gain. Figure 5.2 revealed no significant difference in the absolute and relative weights of experimental animals' kidney and heart weights compared with the healthy untreated and diabetic controls. However, absolute, and relative liver weights' differences of the untreated diabetic rats versus the healthy controls are significant ($p < 0.0001$).

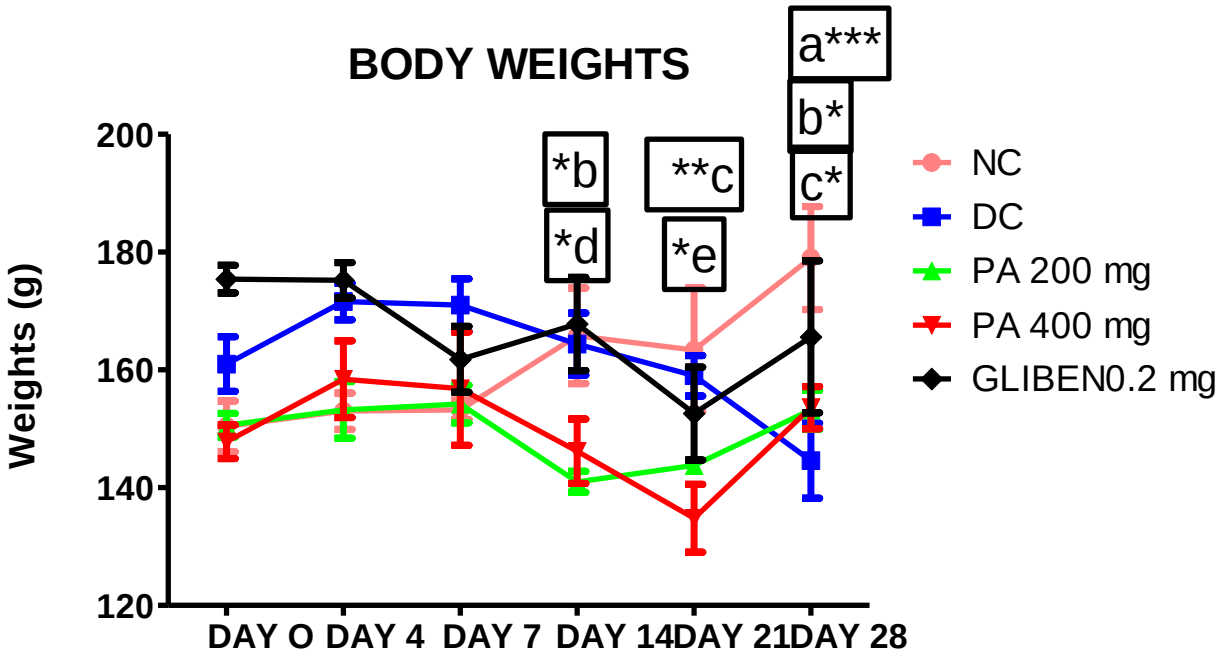


Figure 5.1: Graphical representation showing persistent body weight loss of the animals

[a***] $p < 0.001$ (NC vs DC at day 28), [b*] $p < 0.05$ (NC vs PA 200mg/kgBWT. at days 14 and 28)

[c**] $p < 0.01$ (NC vs PA 400mg/kgBWT. at 21), [c*] $p < 0.05$ (NC vs PA 400mg/kgBWT at 28)

[d*] $p < 0.05$ (DC vs PA 200mg/kgBWT at day 14); [e*] $p < 0.05$ (DC vs PA 400mg/kgBWT at day 21)

[f**] $p < 0.01$ (PA 200mg/kgBWT vs GLIBEN 0.2mg/kgBWT at day 14). Values are mean±SD (Two ways ANOVA followed by Bonferroni multiple comparison tests).

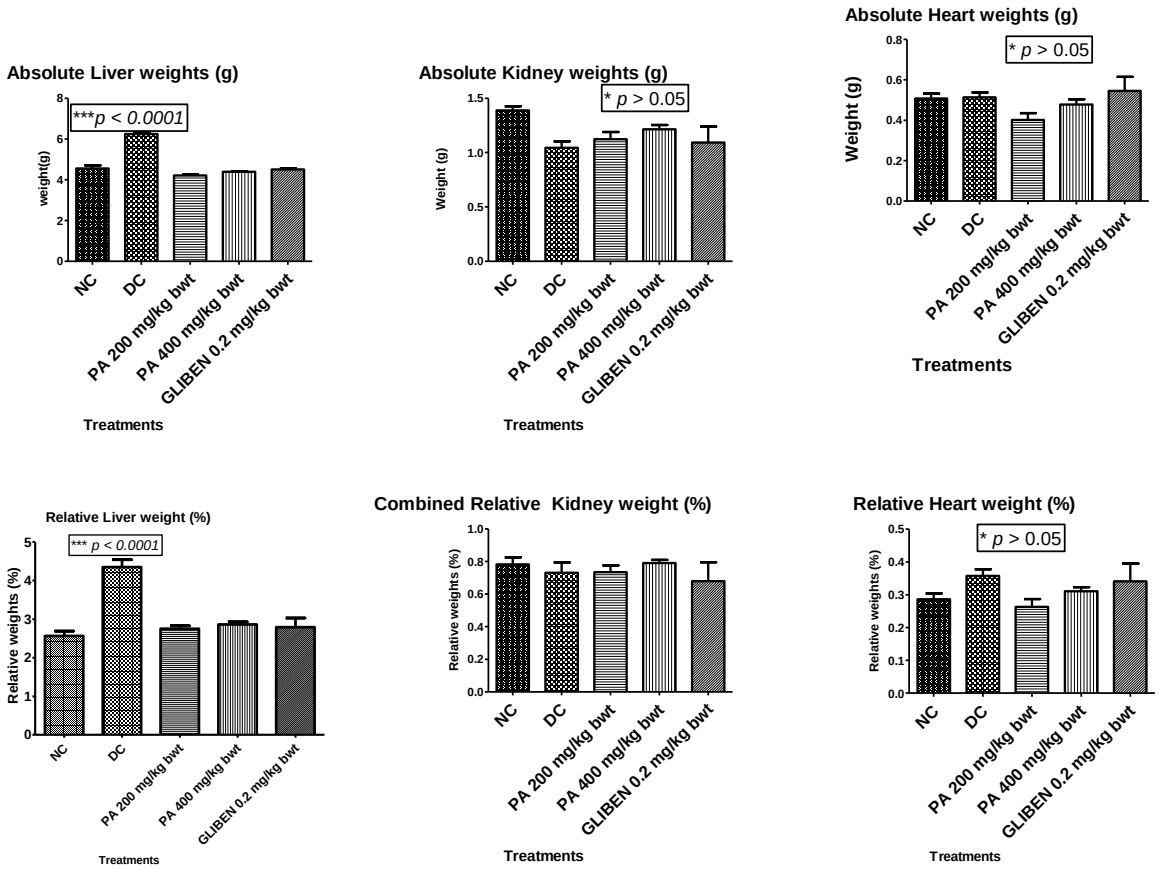


Figure 5.2: Absolute and Relative Liver, Kidney and Heart weights of the animals

For the absolute and Relative Liver weights, there was a significant weight enlargement in the diabetic liver compared to the normal controls, NC versus DC $^{***}p<0.001$, DC versus PA 200mg/kgBWT. $^{***}p<0.001$, DC versus PA 400mg/kgBWT, $^{***}p<0.001$, DC versus GLIBEN 0.2mg/kgBWT $^{***}p<0.001$ (Values are mean±SD, n=5 (Significant values were evaluated with one-way ANOVA afterwards, Bonferroni multiple comparisons were made)

Table 5.1: Mean± (SD) Body weight, % weight changes, absolute and relative Liver, Kidney and Heart weights in all the experimental groups

Groups	Bodyweights		Bodyweight change (%)	Liver wt		Combined Kidney wt		Heart wt	
	Initial	Final		ALW (g)	RLW (%)	AKW(g)	RKW (%)	AHW (g)	RHW (%)
NC	153±6.2	179 ±17.5	17.0	4.6±0.3	2.6±0.3	1.4 ±0.1	0.78±0.10	0.5±0.05	0.29±0.04
DC	171.6±10.	144.6 ± 14.3	-15.8	5.3±0.2	4.3±0.4	1.04±0.1	0.73±0.10	0.51±0.05	0.36±0.05
PA 200 mg/kg	153.3±9.6	153.2 ±6.6	0	4.2±0.1	2.8±0.2	1.12±0.2	0.73±0.10	0.40±0.08	0.26±0.05
PA 400 mg/kg	158.4±14.6	153.6 ±8.0	-2.5	4.4±0.1	2.9±0.2	1.21±0.1	0.79±0.04	0.48±0.06	0.31±0.03
GLIBEN 0.2mg/kg	175.2±6.0	165.6 ±25.8	-5.5	4.5±0.1	2.8±0.5	1.09±0.33	0.68±0.26	0.55±0.16	0.34±0.12

The result shows a significant loss in absolute and relative liver weights of the diabetic control rats compared to the normal controls (p<0.0001). ALW: Absolute liver wt., RLW: Relative liver wt., AKW: Absolute kidney wt., Combined kidney wt: Absolute Left kidney wt + Absolute Right kidney wt., RKW: Relative kidney wt. NC: normal control, DC: Diabetic control, PA 200mg/kgBWT: Diabetes + 200mg Phyllanthus amarus/kg body weight, PA 400mg/kgBWT: Diabetes + Phyllanthus amarus/kg body weight, GLIBEN 0.2mg/kgBWT: Diabetes+glibenclamide 0.2mg/kg body weight. Values are mean±SD, n=5 (Significant values were evaluated with one-way ANOVA afterwards, Bonferroni multiple comparisons were made)

Table 5.2: Mean ± (SEM) Fasting Blood Glucose, Oral Glucose Tolerance Test (OGTT), HB A1c and Serum Insulin

Parameters	NC	DC	PA 200 mg	PA 400 mg	GLIBEN. 0.2 mg
Initial FBG (mmo/L)	3.8 ± 0.5	10.3 ± 4.4	15.1 ± 1.1	11.1 ± 1.3	14.1 ± 4.6
Final FBG (mmo/L)	4.7 ± 0.9	13.3 ± 7.0	20.9 ± 2.2	21.2 ± 0.7	19.2 ± 1.8
Change	+0.9	+3.0	+5.8	+10.1	+5.1

In FBG (mmol/L)					
0'(mmo/L)	4.8 ± 1.5	14.9 ± 4.5	17.1 ± 5.4	15.5 ± 3.6	16.3 ± 2.2
30'(mmo/L)	8.5 ± 0.8	26.5 ± 1.8	30.4 ± 3.2	30.0 ± 3.4	25.3 ± 4.3
60'(mmo/L)	7.5 ± 1.2	28.8 ± 2.4	30.4 ± 2.8	25.8 ± 4.5	28.3 ± 3.6
90'(mmo/L)	5.8 ± 1.1	27.0 ± 4.0	31.8 ± 1.2	25.2 ± 5.1	25.8 ± 2.1
180'(mmo/L	4.5 ± 0.7	19.9 ± 3.9	17.2 ± 3.3	14.4 ± 5.2	20.8 ± 0.9
HBA1c (mmol/mol)	14.1±1.8	32.5 ± 3.1	29.4 ± 5.4	31.6 ± 3.3	31.3 ± 2.7
Serum Insulin (µU/mL)	8.6±0.9	9.0 ± 0.6	8.0 ± 1.3	12.8 ± 6.6	31.0 ± 7.5

This table indicates a. There is a varying degree of significant changes in the fasting blood glucose concentration of the normal/healthy control, diabetic controls, and the diabetic-treated rats with *Phyllanthus amarus*/glibenclamide four days after streptozotocin administration and at termination (day 28) of the experiment. b. The antihyperglycemic effect of PA extracts, demonstrated by the OGTT results. The blood glucose levels in the PA 200mg and PA 400mg rats tend to return to normal after 180 mins of oral glucose load when compared with the diabetic controls. c. The serum insulin levels of the PA 400mg and GLIBEN 0.2mg rats were raised compared to the normal control animals. NC=normal/healthy control rats; DC = Diabetic control rats without treatments; PA 200mg = Diabetic treated rats with *Phyllanthus amarus* 200mg extract per kg body weight/day; PA 400mg = Diabetic treated rats with *Phyllanthus amarus* 400mg/kg extract per kg body weight/day; GLIBEN. 0.2mg = Diabetic treated rats with glibenclamide 0.2mg drug per kg body weight/day.

5.3.2. Hepatic tissue oxidative stress biomarkers

Figure 5.4 shows that the PA 200mg/kgBWT and PA 400mg/kgBWT caused an elevation in the activity of GPx in the liver and reduced the level of lipid peroxidation compared to the rats treated with glibenclamide.

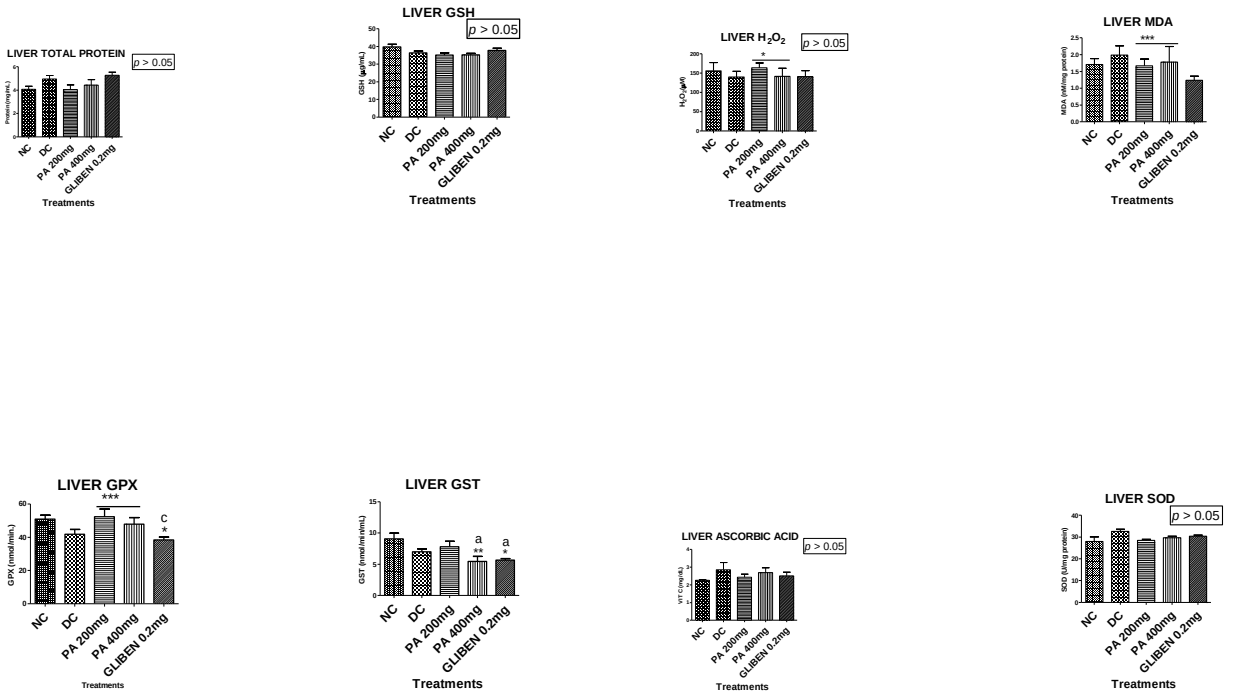


Figure 5.3: Hepatic oxidative stress biomarkers of the healthy untreated, diabetic controls and the diabetic treated animals.

Both 200mg/kgBWT and 400mg/kgBWT reduced liver MDA (p<0.0001) and H_2O_2 (p>0.05) but increased liver GPx (p<0.0001). Values are mean±SD, n=5 (analysed by on-way ANOVA afterwards, Bonferroni multiple comparisons were made); a = compared to NC, b = compared to DC, c = compared to PA 200mg. *p<0.05, ***p<0.0001. NC = normal/healthy control rats; DC = Diabetic control rats without treatments; PA 200mg = Diabetic treated rats with *Phyllanthus amarus* 200mg extract per kg body weight./day; PA 400 mg = Diabetic treated rats with *Phyllanthus amarus* 400 mg extract per kg body weight/day; GLIBEN. 0.2mg = Diabetic treated rats with glibenclamide 0.2mg drug per kg body weight/day.

5.3.3. Renal tissue oxidative stress biomarkers

Figure 5.5 below shows that PA 200mg/kgBWT and PA 400mg/kgBWT significantly reduced lipid peroxidation in the rats' kidney in a similar way to glibenclamide compared to the diabetic untreated animals (p<0.0001).

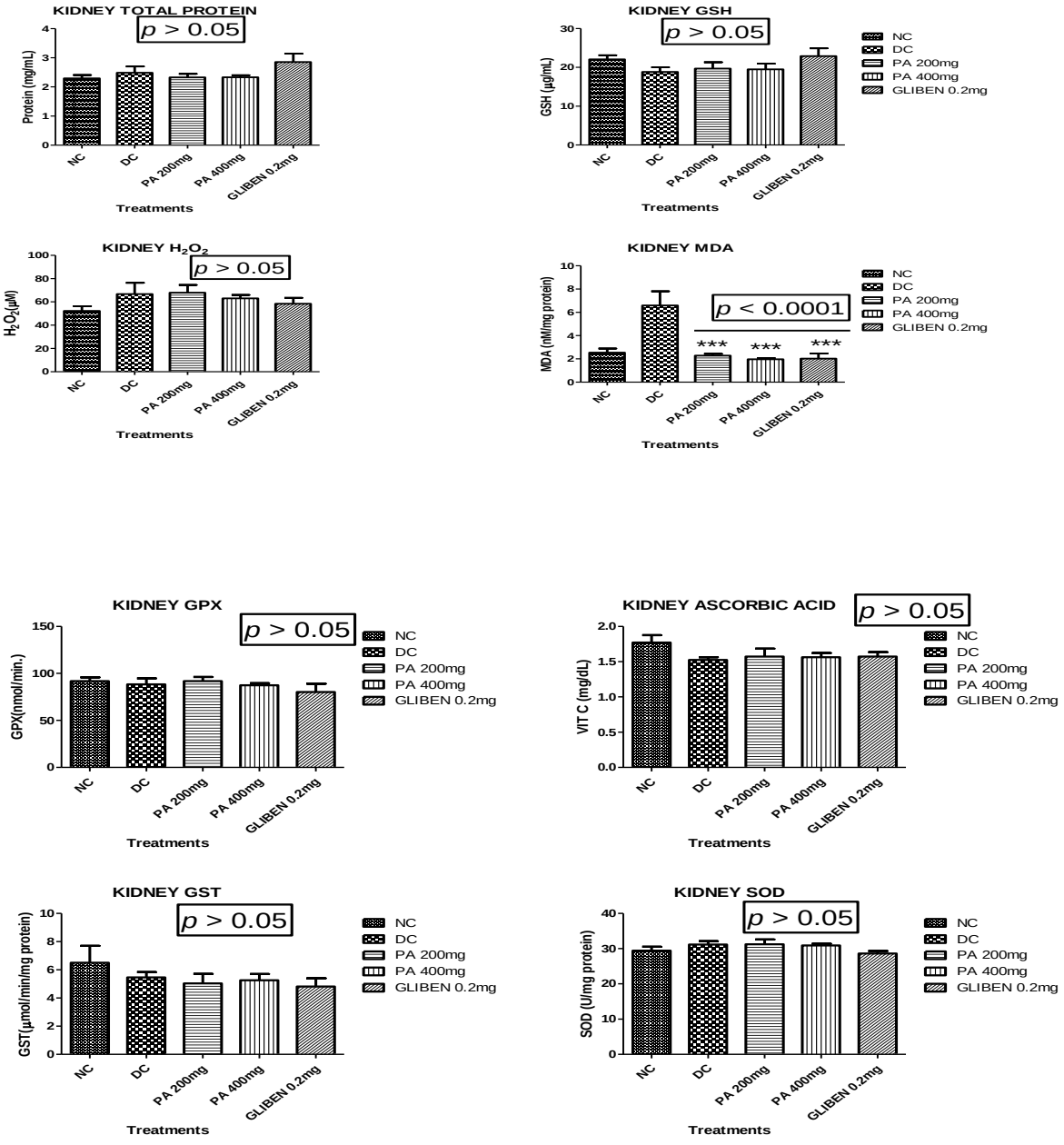


Figure 5.4: Renal oxidative stress biomarkers of the healthy untreated, diabetic controls and the diabetic treated animals

There is a significantly reduced lipid peroxidation in the diabetic-treated rats' kidney, but higher lipid peroxidation in diabetic-untreated animals, as demonstrated by the kidney malondialdehyde (MDA) levels. Values are mean±SD, n=5 (Data were evaluated by one-way ANOVA afterwards, Bonferroni multiple comparisons were made); a = compared to NC, b = compared to DC, c = compared to PA 200mg. **p less than* 0.05, ***p less than* 0.01; ***p less than* 0.001.

5.3.4. Cardiac tissue oxidative stress biomarkers

Figure 5.6 below shows that PA 200mg/kgBWT and PA 400mg/kgBWT reduced lipid peroxidation

in the rats' heart in a similar way to glibenclamide compared with the diabetic untreated rats.

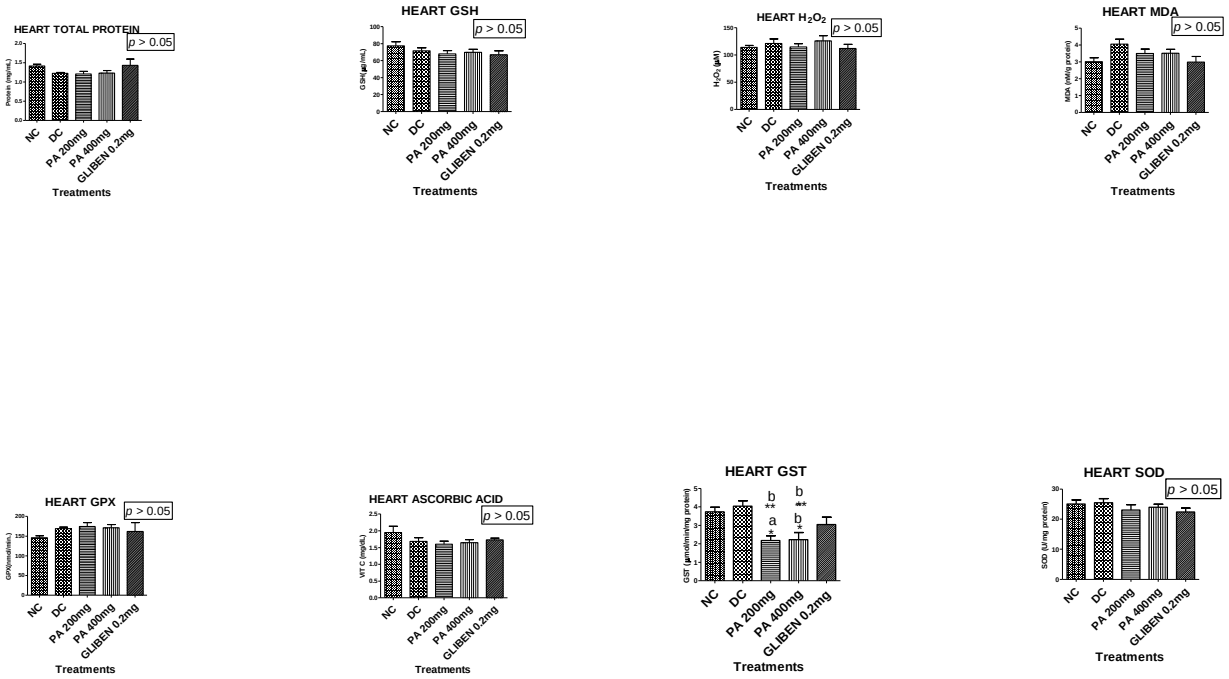


Figure 5.5: Cardiac oxidative stress biomarkers of healthy untreated, diabetic controls and the diabetic treated animals.

Significant reduction glutathione transferase (GST) in the rats' heart of the diabetic-treated rats with PA 200mg/kgBWT and PA 400mg/kgBWT. Values are mean±SD, n=5 (evaluated by one-way ANOVA afterwards, Bonferroni multiple comparisons were made); a = compared to NC, b = compared to DC, c = compared to PA 200mg. * if $p<0.05$, ** if $p<0.01$; ** if $p<0.001$.

5.3.5. Hepatic (Liver) Function tests

Figure 5.7: showing PA 200 mg/kgBWT and PA 400mg/kgBWT normalised the activities of ALP and AST, respectively compared to the diabetic untreated rats. PA 200mg/kgBWT normalised the activity of GGT compared to the diabetic untreated rats. Significant hypoproteinemia was observed in all the diabetic groups (p<0.0001).

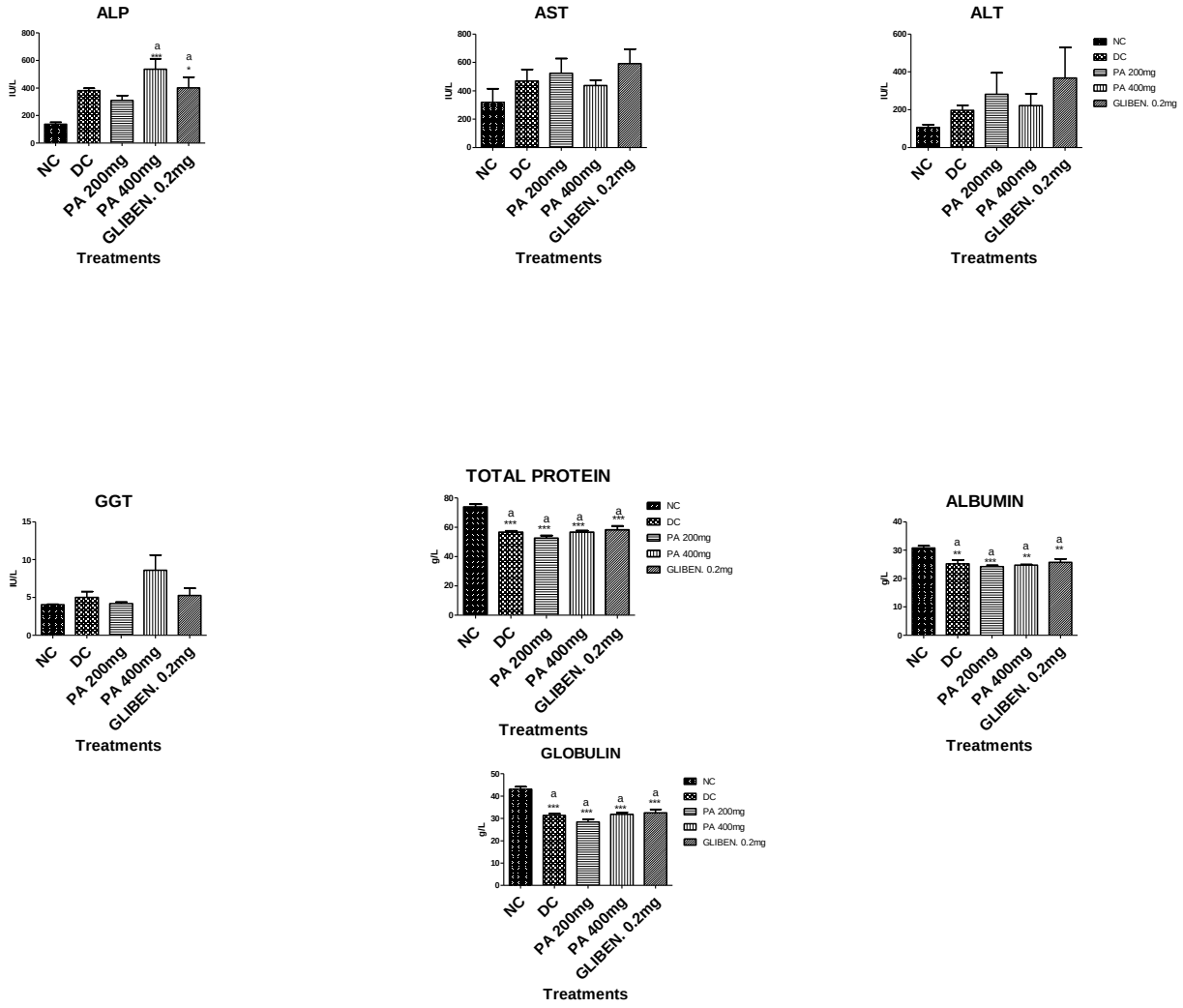


Figure 5.6: Hepatic (Liver) Function tests of the healthy untreated, diabetic controls and the diabetic treated animals.

PA 200mg/kgBWT. and PA 400mg/kgBWT normalised the activities of ALP and AST, respectively compared to the diabetic untreated rats. PA 200mg/kgBWT normalised the activity of GGT compared to the diabetic untreated rats. There was significant hypoproteinemia in all the diabetic groups (p<0.0001). PA 400mg = Diabetic treated rats with *Phyllanthus amarus* 400mg extract per kg body weight/day; GLIBEN. 0.2mg = Diabetic treated rats with glibenclamide 0.2mg drug per kg body weight/day. Values are mean±SD, n=5 (evaluated by one-way ANOVA afterwards, Bonferroni multiple comparisons were

made); a = compared to NC, b = compared to DC, c = compared to PA 200 mg. * *if* p<0.05, ** if p<0.01; ** if p<0.001.

5.3.6. Renal function tests

Figure 5.8 shows that there was no abnormality in the renal functions of the healthy untreated and diabetic control rats as well as the diabetic treated rats.

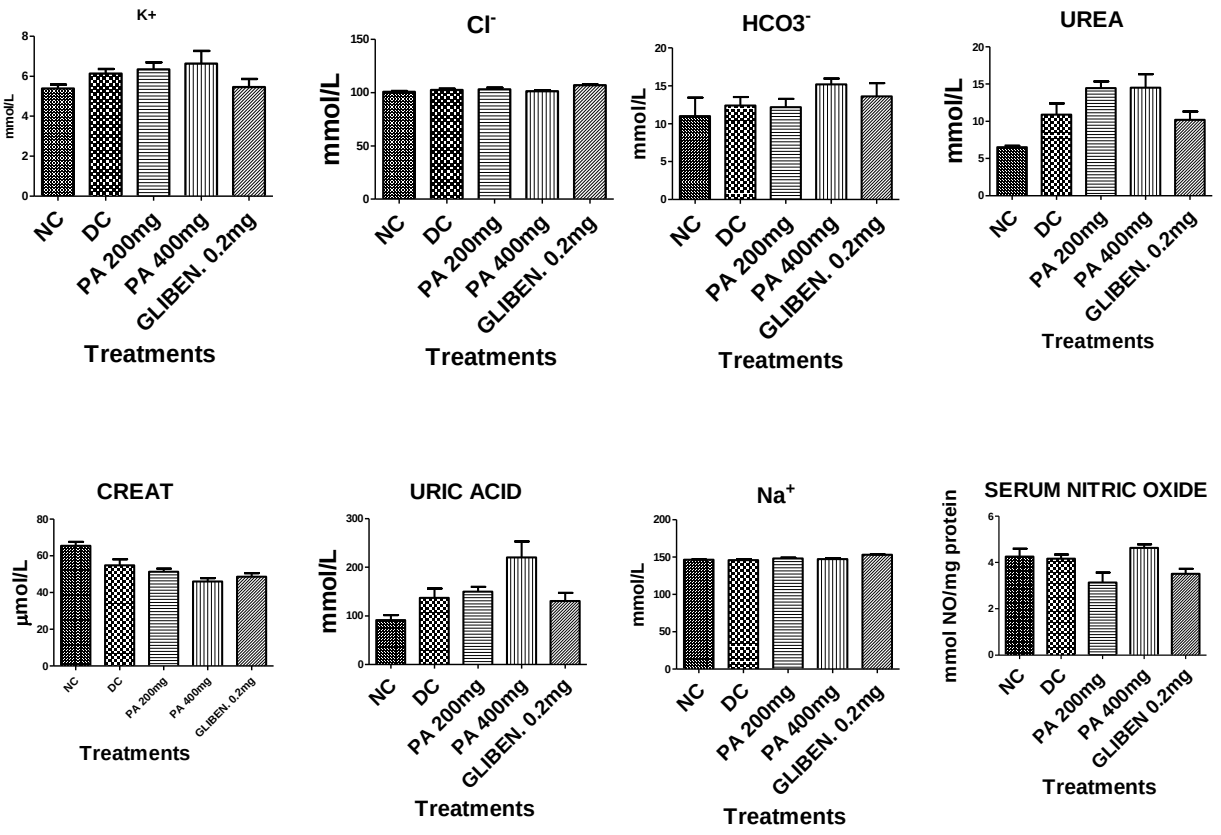


Figure 5.7: Renal function tests of the healthy untreated, diabetic controls and the diabetic treated animals.

NC = normal control rats; DC = Diabetic control rats without treatments; PA 200mg = Diabetic treated rats with *Phyllanthus amarus* 200mg extract per kg body weight/day; PA 400mg = Diabetic treated rats with *Phyllanthus amarus* 400mg extract per kg body weight/day; GLIBEN. 0.2mg = Diabetic treated rats with glibenclamide 0.2mg drug per kg body weight/day. Values are mean±SD, n=5 (evaluated by one-way ANOVA afterwards, Bonferroni multiple comparisons were made); a = compared to NC, b = compared to DC, c = compared to PA 200 mg. * *if* p<0.05, ** if p<0.01; ** if p<0.001.

5.3.7. Cardiac functions

Figure 5.9 shows that PA 200 mg/kgBWT significantly reduced serum triglyceride and cholesterol levels (p<0.0001) compared to the normal controls. PA 200mg/kgBWT, PA 400mg/kgBWT and GLIBEN 0.2mg/kgBWT significantly reduced serum MPO level when compared with the diabetic controls.

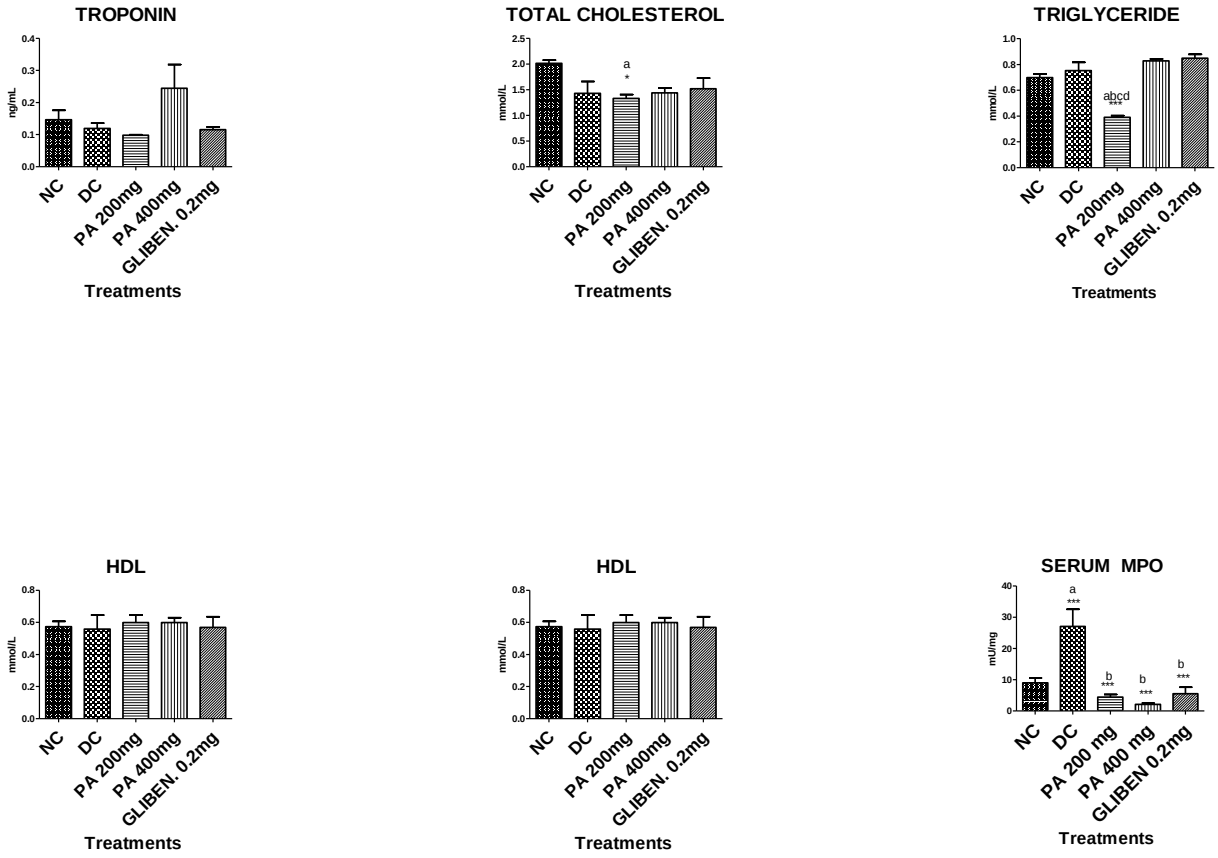
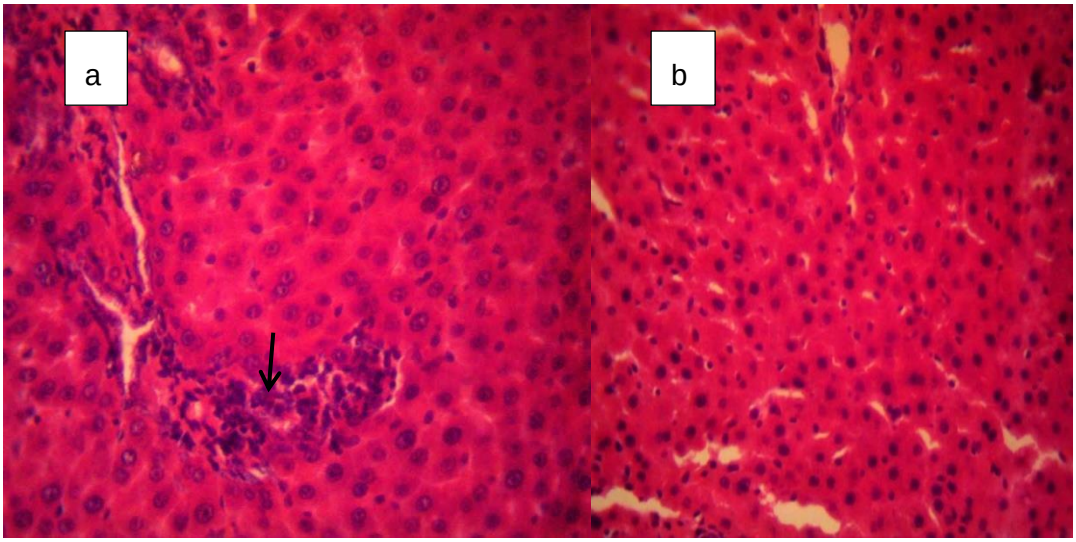
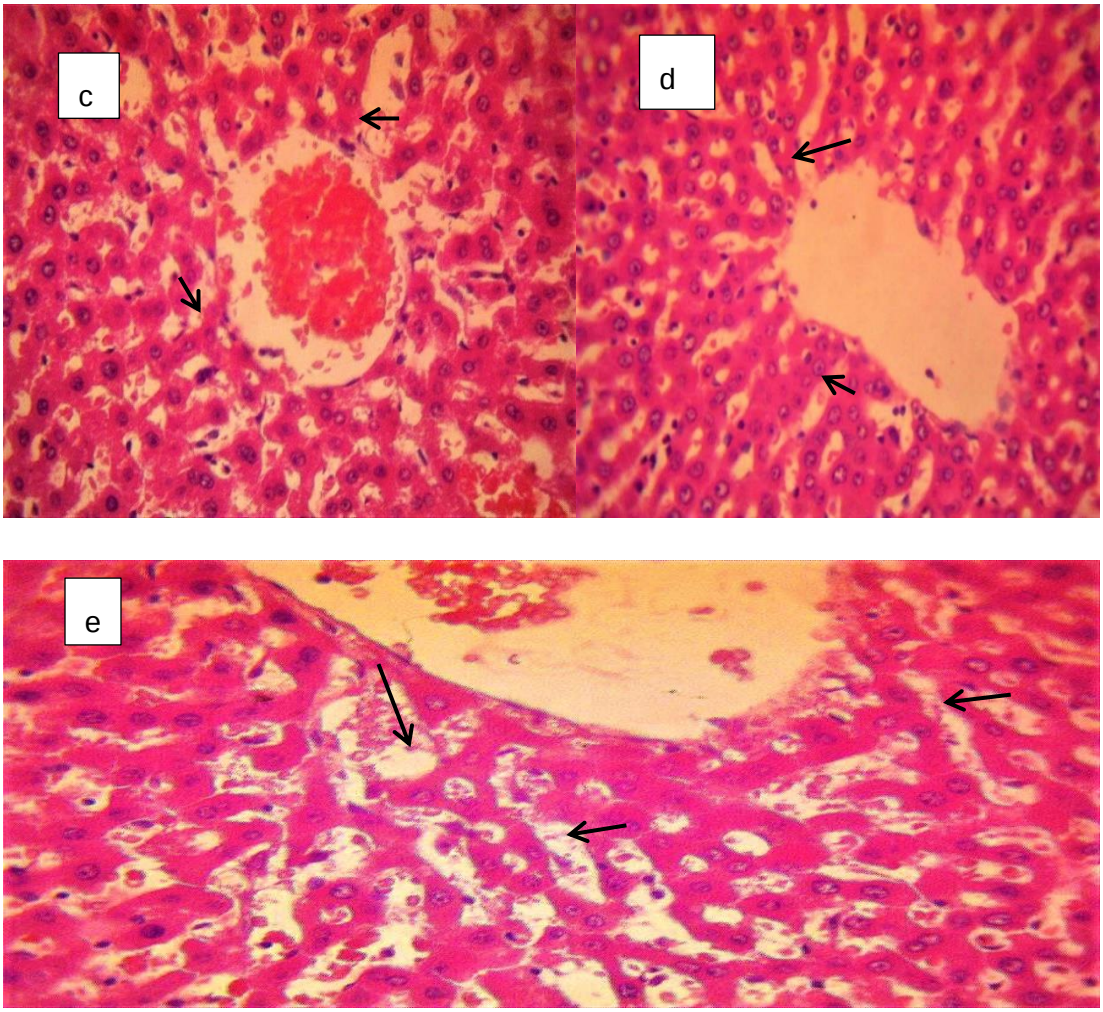


Figure 5.8: Cardiac function tests of the healthy untreated, diabetic controls and the diabetic treated animals.

PA 200mg/kgBWT significantly brought down the serum levels of triglyceride, while the PA 200mg/kgBWT, PA 400mg/kgBWT and GLIBEN 0.2mg/kgBWT significantly reduced serum MPO level. Values are mean±SD, n=5 (evaluated by one-way ANOVA afterwards, Bonferroni multiple comparisons were made); a = compared to NC, b = compared to DC, c = compared to PA 200 mg. * *if* $p<0.05$, ** if $p<0.01$; ** if $p<0.001$.

5.3.8. Histopathology Assessment

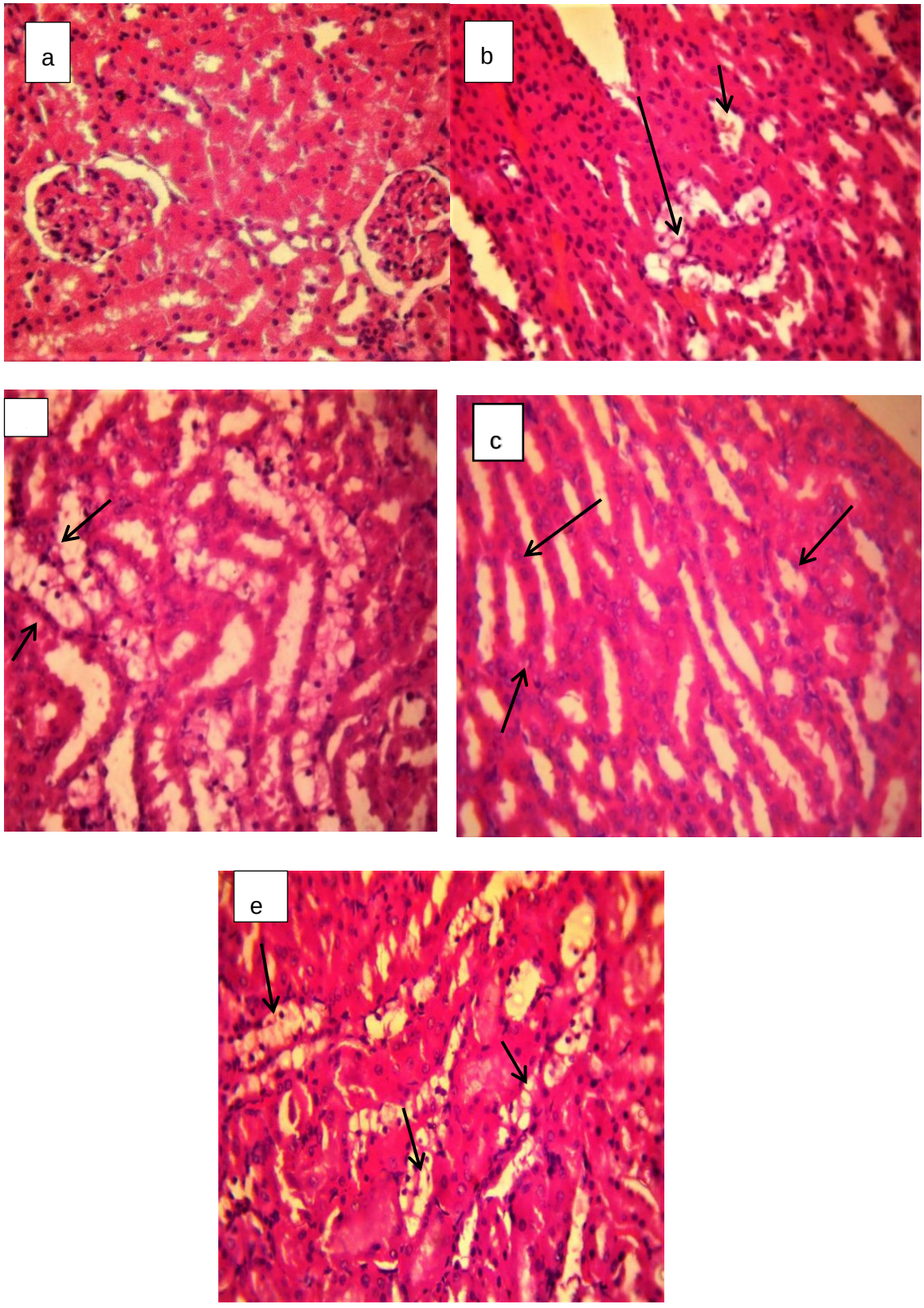




a. Healthy Liver, H&E, x600. Aggregates of lymphocytes in the portal area (arrow).

b. Diabetic Liver, H&E, x600. No visible lesions c. PA 200mg/kgBWT Liver. H&E, x600. Moderate atrophy of centrilobular hepatic cords (arrows) with prominent sinusoids. d. PA 400mg/kgBWT Liver. H&E, x600. Moderate atrophy of centrilobular hepatic cords(arrows) with prominent sinusoids. e. Glibenclamide 0.2mg/kgBWT Liver. H&E, x600. Moderate atrophy of centrilobular hepatic cords(arrows) with prominent sinusoids.

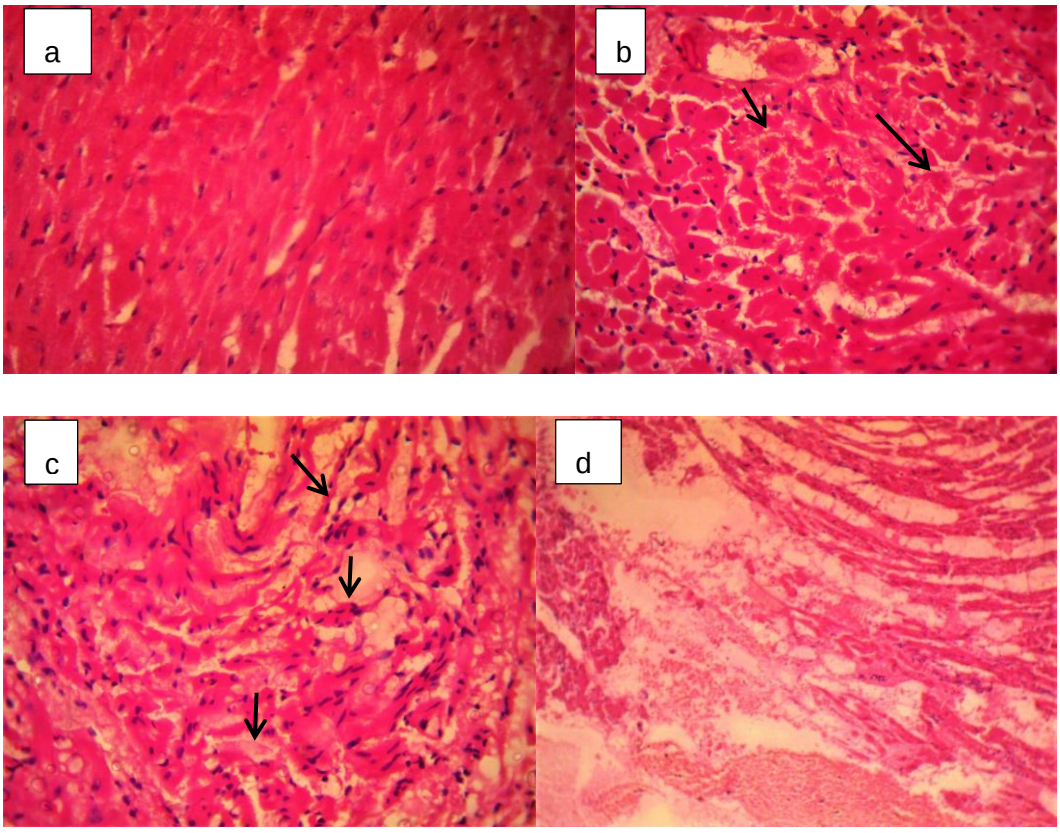
Figure 5.9: Liver Histopathology of the healthy untreated, diabetic controls and the diabetic treated animals.

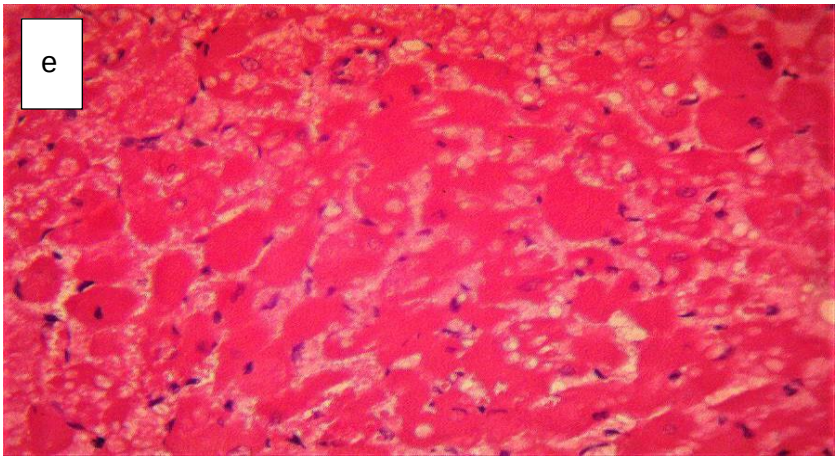

a
b
c
d
e

a. Normal Kidney. H&E, x600. No visible lesion b. Diabetic Kidney. X600, H&E.

Vacuoles degenerated in the renal tubular epithelium (arrows). c. PA 200mg/kgBWT Kidney. X600, H&E. Vacuoles degenerated in the renal tubular epithelium (arrows d. PA 400mg/kgBWT Kidney. X600, H&E. Flattening (attenuation) of the tubular epithelium (arrows). e. Glibenclamide 0.2mg/kgBWT Kidney. X600, H&E. Vacuoles degenerated in the renal tubular epithelium (arrows).

Figure 5.10: Kidney Histopathology of the healthy untreated, diabetic controls and the diabetic treated animals.





a. Normal Heart. H&E. x600. No visible lesion b. Diabetic Heart. H&E. x600. Focal coagulative necrosis and fragmentation of cardiomyocytes (arrows) c. PA 200mg/kgBWT Heart. H&E. x600. Thinning of the muscle fibres (arrows) d.PA 400mg/kgBWT Heart. H&E. x600. Extensive necrosis and fragmentation of cardiomyofibres (arrows) with haemorrhage and thinning of the muscle fibres. e. Glibenclamide 0.2mg/kgBWT Heart. H&E. x600. Vacuolation of the cardiomyocytes

Figure 5.11: Heart Histopathology of the normal, diabetic controls and the diabetic treated animals.

5.4. Discussion

Diabetes mellitus is a complex metabolic abnormality often seen with hyperglycaemia, excessive urination, thirst and hunger [28]. Another cardinal symptom of diabetes is unexplained weight loss [29]. In people with diabetes, lack of or no insulin secretion prevents glucose influx into the body cells from the blood to be used as energy [30]. This condition is termed insulin resistance. Insulin resistance causes the body to burn fat and muscle for energy, leading to reduced overall body weight [31]. In the present study, there was a persistent body weight loss of the animals, except the healthy controls up until day 21 of the experiment, after which there was a visible sign of weight gain (figure 5.1). As depicted in figure 5.2, no significant difference was observed in the experimental animals' liver, kidney and heart weights compared with the healthy untreated and diabetic controls.

In the present study, we observed that the final fasting blood glucose (FBG) values of the experimental animals that received treatments remained persistently higher compared to the normal/healthy and diabetic controls at the termination of the experiment (Table 5.2). It was this discovery that prompted us to escalate further with a more precise technique, oral glucose tolerance test (OGTT), thereby, evaluating insulin release and sensitivity [32]. OGTT is a parameter recommended by the World Health Organisation to assess the body's ability to

handle glucose after a period of oral glucose load [33;34]. Karuna and colleagues [35] examined the antihyperglycemic, hypolipidemic and antioxidant activities of PA aqueous extract in renal oxidative stress-induced diabetic rats. The study demonstrated how *Phyllanthus amarus* treatment rectified the significant decrease in body weight, hyperglycaemia and hyperlipidaemia seen in diabetic rats. There was also a significantly reduced renal lipid peroxidation, protein oxidation and a considerably increased glutathione content and glutathione reductase, glutathione peroxidase and glutathione-S-transferase activities when compared to the diabetic group. In another study by Putakala et al. [36], the usefulness of PA aqueous extract on insulin resistance with oxidative stress using high fructose-fed male Wistar rats was investigated. In their study, ‘’66% fructose plus *Phyllanthus amarus* (200 mg/kg body weight/day) were administered simultaneously to the animals for 60 days. The fructose-fed rats demonstrated weight gain, hyperglycaemia, hyperinsulinemia, impaired glucose tolerance, impaired insulin sensitivity, dyslipidaemia, hyperleptinemia, and hypoadiponectinemia after 60 days. Co-administration *Phyllanthus amarus,* along with the high fructose diet, significantly improved all the alterations’’ [36].

In comparison, the present study confirmed the antihyperglycemic effect of PA extracts demonstrated by the OGTT results and, as reported by Ogar et al., [37]. In our study, the PA 400 mg/kgBWT tends to return blood glucose to normal level after 180 mins of oral glucose load when compared with the diabetic controls, which suggest improved glucose tolerance. However, both the glycosylated haemoglobin (HbA1c) and serum insulin remained persistently high. In our opinion, the failure of the present study to improve these alterations may be because of the short duration of this study (four weeks) compared to the works of Karuna and co-workers [35] and that of Putakala et al. [36]. Their studies were prolonged to approximately eight weeks each.

Moreover, research indicates that chronic hyperglycaemia seen in people with diabetes mellitus elevates the production of free radicals, resulting in oxidative stress and related dysfunctions like cardiovascular diseases, compromised renal and hepatic functions [38]. The present study revealed that PA 200mg/kgBWT and PA 400 mg/kgBWT caused an increase in the glutathione peroxidase (GPx) activities in the liver and reduced the tissue lipid peroxidation level compared with the animals administered with glibenclamide. This finding corroborates the beneficial use of *Phyllanthus amarus* extract to attenuate metabolic syndrome and hepatic oxidative stress in fructose-fed rats [36].

A complication of T2D is diabetic nephropathy (DN) [39]. It is a complication that arises from uncontrolled diabetes. Besides, it is suggested that DN is a leading cause of illness and untimely

death in T2D patients [36]. In the present study, PA 200mg/kgBWT and PA 400mg/kgBWT significantly reduced lipid peroxidation in rats' kidney similarly to glibenclamide when compared with the diabetic untreated rats *(p*<0.0001). The results agree with that of Karuna and co-workers [35] who investigated the antihyperglycaemic, lipid-lowering together with antioxidant activities of *Phyllanthus amarus* aqueous extract in streptozotocin-induced diabetic rats for eight weeks.

The present study shows that PA 200mg/kgBWT and PA 400mg/kgBWT reduced lipid peroxidation in the rats' heart in a similar way to glibenclamide compared to the diabetic untreated rats. The finding corroborates the study that investigated the cardioprotective effect of *Phyllanthus amarus* extract in a myocardial dysfunction-induced animal model [40]. In that study, the plant extracts together with *Momordica charantia* (bitter gourd) was reported to possess a modulatory effect on enzymes linked with cardiac function in heart tissue of doxorubicin-stressed rats, thus, making the plant a potential therapeutic agent for the management of cardiovascular complications arisen from drugs like doxorubicin [41].

Besides, PA 200mg/kgBWT as well as PA 400mg/kgBWT used in the present study tend to normalise the activities of alkaline phosphatase (ALP) and aspartate aminotransferase (AST), respectively compared with the diabetic untreated rats. The PA 200mg/kgBWT normalised the activity of gamma-glutamyl transferase (GGT compared to the diabetic untreated rats. Findings emanating from this study corroborate the useful hepatoprotective ability of *Phyllanthus amarus* plant extract [42;43].

Hyperuricemia, high serum urea and hypoproteinaemia are observed risk factors in diabetes [44]. In the present study, there is uraemia and hyperuricemia in all the diabetic experimental rats compared with the healthy control animals. Also, there is significant hypoproteinaemia in all the diabetic groups (*p*<0.0001) compared to normal/healthy rats. From this study, the hepatoprotective role of *Phyllanthus amarus* against oxidative stress in experimental animals was established. However, its ability to recover renal damage due to diabetes could not be ascertained.

Research suggests that patients with T2D and animal models of T2D may display a deranged lipid profile [45]. Additionally, diabetes presents the most remarkable risk for atherosclerosis which arises from injury to the endothelial lining of the artery; this allows infiltration of cholesterol particles into the lining of the artery wall, causing inflammation. In the present study, total cholesterol, high-density lipoproteins (HDL), and triglyceride levels were measured in experimental diabetic and control rats. PA 200mg/kgBWT significantly brought down the levels

of total cholesterol and triglyceride (p<0.0001). This finding correlates with the work of Ogar and colleagues [37].

From the current study, rats' liver from the PA 200mg/kgBWT, the 400 mg/kgBWT, as well as the GLIBEN 0.2mg/kgBWT groups, showed moderate atrophy of the centrilobular hepatic cords and widening of the sinusoids. The kidneys of rats in the PA 200mg/kgBWT, GLIBEN 0.2mg/kgBWT as well as the diabetic group showed vacuolar degeneration of renal tubular epithelium. However, the kidneys of rats in the PA 400mg/kgBWT group only showed mild flattening of the renal tubular epithelium. The heart of rats in the diabetic group, as well as the PA 200mg/kgBWT and 400mg/kgBWT, showed necrosis of cardiomyocytes. There was thinning of the muscle fibres in the PA 200mg/kgBWT as well as in the 400mg/kgBWT groups. The GLIBEN 0.2mg/kgBWT group showed vacuolation of the cardiomyocytes. Besides, PA 200mg/kgBWT, the 400mg/kgBWT, as well as GLIBEN 0.2mg/kgBWT, did not have any outstanding protection on the hepatocytes. In the same vein, PA did not improve the lesions observed in the heart of the diabetic group. However, in the GLIBEN 0.2mg/kgBWT group, only mild lesions (vacuolation of cardiomyocytes) were observed. However, PA at 400mg/kgBWT ameliorated the renal lesions that were seen in the diabetic group.

5.5. Conclusions

Results from this study demonstrated the antihyperglycemic, antioxidant and hypolipidaemic activities of Phyllanthus amarus extract. These properties of the extract corroborate its ethnobotanical use, thus making it a potential natural product for the development of antidiabetic drugs. However, its ability to recover renal, hepatic, and cardiac lesions under diabetic conditions may need further investigation with more sensitive renal, hepatic, and cardiac biomarkers.

5.6. Author Contributions:

Conceptualization O.O.O.; methodology F.A.O., A.A.O; software, F.A.O.; validation, O.O.O. & F.A.O.; formal analysis F.A.O & AAO., M.T.; investigation, F.A.O.; resources O.O.O.; data curation, F.A.O.; writing—original draft preparation, F.A.O.; review and editing, O.O.O. A.A.A., A.A.O., M.T; visualization, O.O.O. F.A.O. & Y.G.; supervision, O.O.O. A.A.A & Y.G.; project administration, O.O.O.; funding acquisition, O.O.O.

5.7. Funding:

The Cape Peninsula University of Technology's Research Fund (RJ-24) and the National Research Foundation Grant (NRF-RO22) awarded to O.O.O., funded this work, for which the authors are very grateful. The funders were not involved in the experimental formulation, analysis, manuscript writing and the journal to which it must be submitted.

5.8. Acknowledgements:

The authors acknowledge and appreciate Mr Fanie Rautenbach for his technical assistance.

5.9. Conflicts of Interest:

None to declare.

References

[1]. Schoenfelder, T.; Cirimbelli, T.M. and Citadini-Zanette, V. Acute effect of Trema micrantha (Ulmaceae) on serum glucose levels in normal and diabetic rats. *J. Ethnopharmacol.* 2006; *107*(3), pp.456-459.

[2]. American Diabetes Association. Diagnosis and classification of diabetes mellitus. Diab. Care. 2013;36(1):67–74.

[3]. Matough, F.A.; Budin, S.B.; Hamid, Z.A.; Alwahaibi, N. & Mohamed, J. The role of oxidative stress and antioxidants in diabetic complications. Sultan Qaboos Univ. Med J. 2012;12(1):5–18.

[4]. Ayeleso, A.O.; Matumba, M.G.; Ntambi, J.M. & Mukwevho, E. Insights into the metabolism of lipids in obesity and diabetes. In *Lipid Signaling & Metab.* 2020; (pp. 345-

357). Academic Press

[5]. Tesfaye, S. & Gill, G. Chronic diabetic complications in Africa. African J Diabetes Med. 2011;19(1):4–8.

[6]. Patel, C.A.; Pathak, N.L.; Bhatt, N.; Marya, B.; Gavania, M. & Trivedi, H. Cardiovascular complications of diabetes mellitus. J Appl Pharm Sci. 2011;1(4):1–6.

[7]. Kiran, D.; Rohilla, A.; Rohilla, S. & Khan, M.U. Pleiotropic multifaceted therapeutic potential of Phyllanthus amarus. Int J Pharm Biol Arch. 2011; 2(2):610–4.

[8]. Verma, S.; Sharma, H. & Garg, M. Phyllanthus amarus: A review. J Pharmacogn Phytochem. 2014;3(2):18–22.

[9]. Handa, S. & Kapoor, V. Textbook of pharmacognosy. 2003. Vallabh Prakashan. ISBN: 139788185731261.

[10]. Tripathi, A.K.; Verma, R.K.; Gupta, A.K.; Gupta, M.M. & Khanuja, S.P.S. Quantitative determination of phyllanthin and hypophyllanthin in Phyllanthus species by high-performance thin-layer chromatography. Phytochem Anal. 2006;17(6):394–7.

[11]. Khabiya, R.; Upadhyay, D.; Srivastava, A. & Anandjiwala, S. Simultaneous quantification of three bioactive lignans, viz., phyllanthin, hypophyllanthin, and niranthin from Phyllanthus amarus using high-performance thin-layer. J Planar Chromatogr. 2014;27(4):281–6.

[12]. Rohilla, A.; Rohilla, Kiran, D.; S. & Khan, M.U. Pleiotropic multifaceted therapeutic potential of Phyllanthus amarus. Int J Pharm Biol Arch. 2011;2(2):610–4.

[13]. Dhongade, H. & Chandewar, A.V. A review on pharmacognostical, phytochemical, pharmacological properties of Phyllanthus amarus. Int J Biomed Adv Res. 2013;4(5):280–8.

[14]. Olabiyi, F.A.; Aboua, Y.G.; Popoola, O.K; Monsees, T.K & Oguntibeju, O.O. Evaluation of antioxidant, antityrosinase activities and cytotoxic effects of Phyllanthus amarus extracts. Nat Prod J. 2020;10(2):130–8.

[15]. Owiredu, W.K.B.; Amegatcher, G. & Amidu, N. Precision and accuracy of three blood glucose meters: Accu-chek advantage, one-touch horizon and sensocard. J Med Sci. 2009;9(4):185–93.

[16]. Belviranlı, M.; Gökbel, H.; Okudan, N. & Başaralı, K. Effects of grape seed extract

supplementation on exercise-induced oxidative stress in rats. *Brit. J. Nutr.* 2012; *108*(2), pp.249-256

[17]. Misra, H. & Fridovich, I. The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. J Biol Chem. 1972;217:3170–5.

[18]. Prohaska, J.R. & Ganther, HE. Glutathione peroxidase activity of glutathione-S-transferases purified from rat liver. Biochem Biophys Res Commun. 1977;76(2):437–45.

[19]. Beutler, E.; Duron, O. & Kelly, B. Improved method for the determination of blood glutathione. J Lab Clin Med. 1963;6:882–8.

[20]. Varshney, R. & Kale, RK. Effects of calmodulin antagonists on radiation-induced lipid peroxidation in microsomes. Int J Radiat Biol. 1990;58(5):733–43.

[21]. Xia, Y. & Zweier, J.L. Measurement of myeloperoxidase in leukocyte-containing tissues. Anal Biochem. 1997;245(1):93–6.

[22]. Maciel, E.A.P.; Athanazio, D.A.; Reis, E.A.G.; Cunha, F.Q.; Queiroz, A.; Almeida, D.; McBride, A.J.; Ko, A.I. & Reis. High serum nitric oxide levels in patients with severe leptospirosis. Acta Trop. 2006;100(3):256–60.

[23]. Sarchielli, P.; Pini, L.A.; Coppola, F.; Rossi, C.; Baldi, A.; Mancini, M.L. and Calabresi, P. Endocannabinoids in chronic migraine: CSF findings suggest a system failure. Neuropsychopharmacol, 2007; 32(6), pp.1384-1390)

[24]. Green, L.C.; Ruiz de Luzuriaga, K.; Wagner, D.A.; Rand, W.; Istfan, N.; Young, V.R. and Tannenbaum, S.R. Nitrate biosynthesis in man. Proc Natl Acad Sci U S A. 1981;78(12):7764–8.

[25]. Drury, R. & Wellington, E. Carlton's histopathological techniques: Oxford University Press. London, New York. 1976.

[26]. Park, H.; Oh, J.; Shim, G.; Cho, B.; Chang, Y.; Kim, S.; Baek, S.; Kim, H.; Shin, J.; Choi, H. & Yoo, J. In vivo neuronal gene editing via CRISPR–Cas9 amphiphilic nano complexes alleviates deficits in mouse models of Alzheimer's disease. *Nat Neurosci. 2019,* **22,** 524–528. https://doi.org/10.1038/s41593-019-0352-0

[27]. Ekwueme, D.U.; Zhao, J.; Rim, S.H.; de Moor, J.S.; Zheng, Z.; Khushalani, J.S.; Han, X.; Kent, E.E. & Yabroff, K.R. Annual out-of-pocket expenditures and financial hardship among cancer survivors aged 18–64 years—the United States, 2011–2016. *Morbidity and Mortality Weekly Report*, 2019; *68*(22), p.494).

[28]. Jerra Vidya Sagar. “Antidiabetic activity of Ethanolic extract of Portulaca qudrifda leaves", International J. Curr. Res. 2016; 8, (04), 29777-29779.

[29]. Okon, U.; Owo, D.U.; Udokang, N.E.; Udobang, J.A. & Ekpenyong, C.E. Oral administration of aqueous leaf extract of Ocimum gratissimum ameliorates polyphagia, polydipsia and weight loss in streptozotocin-induced diabetic rats. Am J Med Med Sci. 2012;2(3):45–9.

[30]. Fu, Z. R.; Gilbert, E. & Liu, D. Regulation of insulin synthesis and secretion and pancreatic Beta-cell dysfunction in diabetes. Curr Diabetes Rev. 2013;9(1):25–53.

[31]. Oike, Y.; Akao, M.; Yasunaga, K.; Yamauchi, T.; Morisada, T.; Ito, Y.; Urano, T.; Kimura, Y.; Kubota, Y.; Maekawa, H. & Miyamoto, T. Angiopoietin-related growth factor antagonizes obesity and insulin resistance. Nat Med. 2005;11(4):400–8.

[32]. Stumvoll, M.; Mitrakou, A.; Pimenta, W.; Jenssen, T.; Yki-Järvinen, H.A.N.N.E.L.E.; Van Haeften, T.; Renn, W.P.H.D. & Gerich, J. Use of the oral glucose tolerance test to assess insulin release and insulin sensitivity. *Diabetes care*, 2000, *23*(3), 295-301.

[33]. Morris, C.; O’Grada, C.; Ryan, M.; Roche, H.M.; Gibney, M.J.; Gibney, E.R. & Brennan, L. Identification of differential responses to an oral glucose tolerance test in healthy adults. PLoS One. 2013;8(8):1–9.

[34]. Islam, M.A.; Akhtar, M.A.; Khan, M.R.I.; Hossain, M.S.; Alam, A.H.M.K.; Wahed, M.I.I.; Amran, M.S.; Rahman, B.M. & Ahmed, M. Oral glucose tolerance test (OGTT) in normal control and glucose-induced hyperglycemic rats with Coccinia cordifolia L. and Catharanthus roseus L. Pak J Pharm Sci. 2009;22(4):402–4.

[35]. Karuna, R.; Bharathi, V.G.; Reddy, S.S.; Ramesh, B. & Saralakumari, D. Protective effects of Phyllanthus amarus aqueous extract against renal oxidative stress in Streptozotocin-induced diabetic rats. Indian J Pharmacol. 2011;43(4):414–8.

[36]. Putakala, M.; Gujjala, S.; Nukala, S. & Desireddy, S. Beneficial effects of Phyllanthus amarus against high fructose diet-induced insulin resistance and hepatic oxidative stress in male Wistar rats. Appl Biochem Biotechnol. 2017;183(3):744–64.

[37]. Ogar, I.; Egbung, G.E.; Nna, V.U.; Atangwho, I.J. & Itam, E.H. Hyptis verticillata attenuates dyslipidaemia, oxidative stress and hepato-renal damage in streptozotocin-induced diabetic rats. Life Sci. 2019;219:283–93. Available from: https://doi.org/10.1016/j.lfs.2019.01.027

[38]. Pan, H.Z.; Zhang, L.; Guo, M.Y.; Sui, H.; Li, H.; Wu, W., et al. The oxidative stress status in diabetes mellitus and diabetic nephropathy. Acta Diabetol. 2010 Dec 28;47(1):71–76.

[39]. Baynes, J. Role of oxidative stress in the development of complications in diabetes. Diabetes. 1991;40(4):405–12.

[40]. Putakala, M.; Sudhakara, G.; Nukala, S.; Bongu, S.B.R.; Chintakunta, N. & Desireddy, S. Cardioprotective effect of Phyllanthus amarus against high fructose diet-induced myocardial and aortic stress in a rat model. Biomed Pharmacother. 2017;95:1359–68.

[41]. Saliu, J.A.; Oyeleye, S.I.; Olasehinde, T.A. & Oboh, G. Modulatory effects of stonebreaker (Phyllanthus amarus) and bitter gourd (Momordica charantia) on enzymes linked with cardiac function in heart tissue of doxorubicin-stressed rats. Drug Chem Toxicol. 2019;1–9.

[42]. Yadav, N.P.; Pal, A.; Shanker, K.; Bawankule, D.U.; Gupta, A.K.; Darokar, M.P. & Khanuja, S.P. Synergistic effect of silymarin and standardized extract of Phyllanthus amarus against CCl4-induced hepatotoxicity in Rattus norvegicus. Phytomedicine. 2008;15(12):1053–61

[43]. Londhe, J.S.; Devasagayam, T.P.A.; Foo, L.Y.; Shastry, P. & Ghaskadbi, S.S. Geraniin and amariin, ellagitannins from Phyllanthus amarus, protect liver cells against ethanol-induced cytotoxicity. Fitoterapia. 2012;83(8):1562–8.

[44]. Lal, S.S.; Suk, Y.; Andriyas, E.A. & Lall, A.M. Hyperuricemia, high serum urea and hypoproteinemia are the risk factor for diabetes. Asian J Med Sci. 2009;1(2):33–4.

45]. Bardini, G.; Rotella, C.M. & Giannini, S. Dyslipidemia and diabetes: reciprocal impact of impaired lipid metabolism and Beta-cell dysfunction on micro- and macrovascular complications. Rev Diabet Stud. 2012;9(2–3):82–93.

CHAPTER SIX: *Phyllanthus amarus* ameliorates sperm abnormality and testicular oxidative stressed diabetic rats *via* phytoestrogenic as well as radical-scavenging actions

FA Olabiyi[1,7], AA Oyagbemi[2], MO Tijani[3], YG Aboua[4], TK Monsees[5], AA Adedapo[6], OO Oguntibeju[1*]

[1]Phytomedicine and Phytochemistry Group, Oxidative Stress Research Centre, Department of Biomedical Sciences, Cape Peninsula University of Technology, Bellville 7535, South Africa, [2]Department of Veterinary Physiology and Biochemistry, University of Ibadan, Nigeria, [3]Department of Veterinary Pathology, University of Ibadan, Nigeria, [4]Department of Health Sciences, Namibia University of Science and Technology, Windhoek, Namibia, [5]Department of Medical Biosciences, University of the Western Cape, Bellville, 7535, South Africa, [6]Department of Veterinary Toxicology and Pharmacology, University of Ibadan, Nigeria. [7]Department of Medical Laboratory Science, Afe Babalola University, P.M.B 5454 Ado-Ekiti, Nigeria.

*Corresponding author: Oluwafemi Omoniyi Oguntibeju

E-mail address: oguntibejuo@cput.ac.za Fax: +27(0)219538490

NB: Preparation of the manuscript for submission from this chapter is according to the ANDROLOGIA journal author's guidelines.

ABSTRACT

This study evaluated the potential estrogenic and antiandrogenic actions of *Phyllanthus amarus* (PA) in a model of type 2 diabetes-mediated male reproductive dysfunction. Rats were orally administered daily with PA (200mg and 400mg/kg BWT) or glibenclamide (GLIBEN 0.2mg/kg BWT) for four weeks. Diabetes confirmation was achieved with fasting blood glucose estimation; besides, oral glucose tolerance test was done. Androgenicity was evaluated by estimating serum testosterone and sperm parameters. Besides, serum oestradiol and insulin levels were

measured. Oxidative stress was assessed by measuring serum nitric oxide (NO), myeloperoxidase (MPO), testicular hydrogen peroxide (H_2O_2) generation, malondialdehyde (MDA) contents and antioxidant enzymes. *In-vitro* phytoestrogenic property was assessed by MTT, E-Screen assays and cell counts in breast cancer (MCF-7) cell lines. PA at 200mg/kg BWT together with 400mg/kg BWT improved serum testosterone, insulin, and oestradiol correspondingly to glibenclamide compared to the diabetic controls. PA 200mg/kg BWT significantly improved sperm count compared to the diabetic control and PA 400mg/kg BWT groups. PA 200mg/kg BWT reduced serum NO levels compared to the diabetic controls while GLIBEN 0.2mg/kg BWT significantly reduced serum NO levels compared to the normal and diabetic controls. PA 200mg/kg BWT, PA 400mg/kg BWT and GLIBEN 0.2mg/kg BWT significantly reduced serum MPO. PA 200mg/kg BWT and PA 400mg/kg BWT reduced MDA as well as H_2O_2 compared to diabetic control. PA 400mg/kg BWT significantly increased glutathione peroxidase (GPx) activity when compared with the diabetic controls. PA 200mg/kg BWT group showed a reduction in seminiferous tubular diameter and an expanded interstitium. The PA 400mg/kg BWT group showed similar changes, but the PA 400mg/kg showed spermatogenic arrest, depletion and vacuolation of the seminiferous tubular epithelium. The extract produced significant proliferative effects on MCF-7 cells, thus, confirming its estrogenic activity. Thus, PA at 200 mg/kg BWT extract may improve reproductive functions through increasing spermatogenesis and decreasing testicular free radicals in diabetic rats.

Keywords: *Phyllanthus amarus*, phytoestrogens, male reproductive function, diabetes mellitus, oxidative stress

6.1. Introduction

Globally, diabetes mellitus (DM) (Almalki, Alghamdi & Al-Attar, 2019) is a significant threat to human wellness in recent time. The prevalence of DM is vastly increasing among children as well as youngsters. This dysfunction is linked to a reproductive abnormality in both men as well as women (Niwas & Chand, 2014). It may affect male reproductive functions due to its impacts on the endocrine regulation of spermatogenesis, steroidogenesis, sperm maturation, impairment

of penile erection with ejaculation (Shi et al., 2017). The abnormality emanates when the pancreas fails to secrete adequate insulin or due to the body's ineffective utilisation of the insulin being secreted. The anomaly results in insulin resistance and dysfunction in glucose uptake by liver cells, muscles as well as adipose tissues leading to a disruption in blood glucose homeostasis, thereby leaving excess glucose in the bloodstream, a condition called hyperglycaemia (Pugliese & Miceli, 2002; Rafacho, Ortsäter, Nadal, & Quesada, 2014; Rains & Jain, 2011). Hyperglycaemia or raised blood glucose in uncontrolled diabetes often leads to astringent impairment to the body, particularly on the nerves and blood vessels (WHO, 2016). Besides, hyperglycaemia has been reported to induce oxidative stress in people with diabetes (Niedowicz & Daleke, 2005). Among the different types of diabetes, over 90% of the diabetic cases are type 2 diabetes (T2D) which becomes a primary public health concern globally. Of all these cases, research showed that testosterone levels are lower in an adult male with metabolic syndrome; a dysfunction of biochemical and physiological processes that is evident with the occurrence of cardiovascular disease and type 2 diabetes. The testosterone level is also reduced in T2D patients due to impaired Leydig cell function (Zuo et al., 2014). More so, insulin resistance (IR), a characteristic of T2D, is a primary pathogenic feature of this disorder which serves as an autonomic causative for cardiovascular disease (CVD) (Laakso, 2015; Patel et al., 2016). It has also been suggested that 17β-oestradiol (E2) levels above or below the physiological range may promote IR and T2D. Diabetes can damage body nerves when blood glucose and blood pressure are elevated; this leads to digestive and erectile dysfunction (International Diabetes Federation, 2019) and loss of libido which could result in infertility in some males (Lotti & Maggi, 2018)

Hyperglycaemia elicits oxidative stress which essentially may be responsible in the pathophysiology of diabetes-related male reproductive dysfunction (Amaral, Oliveira, & Ramalho-Santos, 2008; Karimi, Goodarzi, Tavilani, Khodadadi, & Amiri, 2011; Mallidis et al., 2009). Oxidative stress comes in when reactive oxygen species (ROS) counterbalances antioxidant defence mechanisms; leading to cellular damage (Tremellen, 2008). Research suggests that hyperglycaemia induces oxidative stress in people with diabetes, during which ROS are produced by oxidative phosphorylation (Ansley & Wang, 2013). Several endogenous and exogenous antioxidants lessen the damaging effects of free radicals due to glucose auto-oxidation in hyperglycaemia.

At the endogenous level, antioxidant properties of steroid hormones have been demonstrated in different cells and tissues (Ahmed & Hassanein, 2012). Besides, researchers suggested that

oestrogens, a female sex hormone, possesses a remarkable modulating impact on systemic glucose homeostasis (Barros, Gabbi, Morani, Warner, & Gustafsson, 2009; Foryst-Ludwig & Kintscher, 2010). Research also shows that antioxidant supplementation reduces glycaemic index and diabetic complications, hence protection from cellular damage due to free radical-induced oxidative stress (Mohasseb, Ebied, Yehia, & Hussein, 2011; Rahimi, Nikfar, Larijani, & Abdollahi, 2005). Moreover, some of these antioxidants are nowadays available as supplements or can be obtained naturally from dietary sources.

Phyllanthus amarus (PA) is a herb used by traditional Hindu people in southern India; it is also readily available in the Philippines, Cuba (Verma, Sharma, & Garg, 2014) and some places in West African countries like Nigeria (Adeneye, 2012) and Togo (Lawson-Evi et al., 2011). In Nigeria, *Phyllanthus amarus* is referred to as ''ehin olobe'' or ''ehin olubisowo'' among the Yoruba tribe (Samson, 2016). The herb is reputably useful in folk medicine for several diseases. The plant is otherwise called stone breaker or windbreaker.

Phytochemical studies revealed that the herb has many constituents like polyphenols and tetracyclic triterpenoids (Kassuya et al., 2006), flavonoids (Leite et al., 2006) tannins (Houghton, Woldemariam, O'Shea, & Thyagarajan, 1996; Kiemer, Hartung, Huber, & Vollmar, 2003), lignans (Adedapo, Adegbayibi, & Emikpe, 2005; Kassuya et al., 2006; J. R. Patel, Tripathi, Sharma, Chauhan, & Dixit, 2011) and alkaloids (Houghton et al., 1996).

Phyllanthus amarus is composed of phyllanthin and hypophyllanthin, which are its two significant phytoestrogens (Islam, Naskar, Mazumder, Gupta, & Ghosal, 2008). Phytoestrogens are a naturally occurring group of compounds present in various plant foods. They are polyphenols having structural similarity with the endogenous female sex hormone oestrogens, hence, their estrogenic/anti-estrogenic property.

Both animal and pre-clinical models have provided strong evidence that phytoestrogens may have antidiabetic function via both oestrogen-dependent and oestrogen-independent pathways. Besides, scientists have linked the major phytoestrogens, isoflavones and lignans to T2D from epidemiological and clinical trials (Talaei & An, 2015). However, lignans, widely distributed in fruits as well as vegetables were less investigated.

The benefits of phytoestrogens cannot be overemphasized, as several studies have shown that phytoestrogen supplements may provide health benefits. For instance, resveratrol and quercetin supplementations may reduce blood pressure (Y. Liu, Ma, Zhang, He, & Huang, 2015; Serban et al., 2016), resveratrol, flaxseed lignans, and soy isoflavones may improve glycaemic control (K. Liu, Zhou, Wang, & Mi, 2014; Pan et al., 2007; Ricci, Cipriani, Chiaffarino, Malvezzi, & Parazzini,

2010), isoflavone herbal extract may lower the chances of prostate cancer(Van Die, Bone, Williams, & Pirotta, 2014), soy isoflavone supplementation reduces serum total and low-density lipoprotein cholesterol levels (Taku et al., 2007), and soy isoflavones and lignans may reduce the levels of C-reactive protein, an inflammatory biomarker, in postmenopausal women having high levels of this protein (Dong, Wang, He, & Qin, 2011; Hallund, Tetens, Bügel, Tholstrup, & Bruun, 2008). Despite the beneficial health-promoting potentials of phytoestrogens, their (anti) oestrogenic properties have raised questions. They are seen as endocrine disruptors or chemicals interfering with sex hormone action, suggesting that they might produce adverse effects (Rietjens, Louisse, & Beekmann, 2017).

Over a period of a few decades now, there are reports of rising male reproductive disorders in several countries. It was these reports that led to the hypothesis that estrogens during fetal life at the time of sex differentiation may cause reduced sperm counts, cryptorchidism, hypospadias, and testicular cancer (Monsees, Franz, Gebhardt et al., 2000; Storgaard, Bonde & Olsen, 2005). However, Storgaard and colleagues believed the hypothesis was from animal experiments and are reported from the wildlife. Hence, the need for epidemiological literature search for evidence in male humans. They said that possibly apart from testicular cancer, there was no strong epidemiological evidence to show that prenatal exposure to estrogen is related to disruptive development of the male reproductive organs (Storgaard, Bonde & Olsen, 2005). Reports have also indicated an adverse correlation between exposure to certain chemicals, environmental toxicants, and male fertility. These so-called disruptors are environmental toxins that may mimic the effect of estrogens (Giwercman, 2011; Mima, Greenwald, & Ohlander, 2018). Endocrine disruptive chemicals or EDCs, Polychlorinated biphenyl or PCBs, phthalate, pesticides and herbicides, Organophosphate, Dichlorodiphenyl-Dichloroethylene or DDT, Heavy metals such as lead, cadmium & mercury, as well as Bisphenol A are common culprits (Mima, Greenwald, & Ohlander, 2018; Cariati, F., D'Uonno, N., Borrillo, F. *et al.* 2019). Other forms of environmental exposures that have a potential negative impact on male infertility are air pollution, hyperthermia, the heat generated from cellphones and portable laptop computers when these devices are placed close to the scrotal area could create testicular stress, thus, disrupt spermatogenesis (Mima, Greenwald, & Ohlander, 2018). Some of these toxicants were reported mainly targeting Sertoli cells that play a crucial role in spermatogenesis (Monsees, Franz, Gebhardt et al., 2000). Also, testicular connexin 43 has been reported as a precocious molecular target from environmental toxicants that adversely impact male fertility (Pointis, Gilleron, Carette, & Segretain, 2011). Besides, it has been shown that lifestyle-related and ecological factors that act

on fetal life cause dysfunction of Leydig cells and Sertoli cells thereby lead to testicular dysgenesis, including poor semen quality, testicular germ cell cancer (TGCC), hypospadias and or cryptorchidism (Giwercman, 2011).

Moreover, our previous report revealed that the *Phyllanthus amarus* aqueous extract has the highest antioxidant capacity, and a lower extract concentration (<10 µg/mL) stimulated Sertoli cell proliferation, which might be due to the phytoestrogenic activities of *Phyllanthus amarus,* contributed by its active compositions such as phyllanthin and hypophyllanthin (Olabiyi, Aboua, Popoola, Monsees, & Oguntibeju, 2020). Also, in a previous study, PA extract exhibited anti-diabetic activity in graded doses of 200 and 400mg/kg body weight/day in alloxan-induced diabetic rats, with the 400mg/kg dosage being the effective concentration (Adedapo, Ofuegbe, & Adeyemi, 2013) but its potential to modulate the complex responses associated with oxidative stress and male reproduction is not fully elucidated.

Therefore, this study investigated the effects of *Phyllanthus amarus* aqueous extracts on reproductive functions in diabetic male Wistar rats and its potential in-vitro phytoestrogenic activity.

6.2. Materials and Methods

6.2.1. Chemicals

Streptozotocin (18883-66-4), 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), Oestradiol (E2758), Dextran and Activated charcoal were purchased from Merck Group/Sigma-Aldrich Chemical Company, South Africa. Foetal bovine serum was purchased from Celtic Molecular Diagnostics (South Africa), D-Fructose was purchased from Loba Chemie PVT. LTD, Mumbai, India. Human breast cancer cell line (MCF-7) was purchased from ATCC® (USA, no HTB-22). Rat Insulin ELISA kits were purchased from Thermo Scientific (South Africa). Dulbecco's Modified Eagles Medium/F – 12 with phenol red (GIBCO, Cat No 2053123), Dulbecco's Modified Eagles Medium/F – 12 without phenol red (HYCLONE, Cat No SH30272.02), 10% Foetal Bovine Serum, 1% Penicillin (1000 U/mL) and 1% Streptomycin (1000 U/mL) (LONZA, Germany) and Tris-HCL (AppliChem, Germany) were used.

6.2.2. Biological Compound

Freshly *Phyllanthus amarus* (PA) whole plants were gotten from Afe Babalola University (ABUAD) campus, Ado-Ekiti, Nigeria. PA was identified and authenticated at the taxonomy section of the forestry herbarium, FRIN, Ibadan in Nigeria where a voucher specimen was kept (FHI NO: 110242). Shade-dried leaves of PA were blended to powder with a blender (Mincer 9FQ-28, China). The PA aqueous extract was prepared by soaking 100 gm powder in 1 Litre distilled water, stirred, and left at room temperature for 24 hrs. The homogenate was filtered using Whatman no-1 filter paper. The fraction was evaporated to dryness (Ivanova, Kolarova, Aleksieva, et al., 2013) under reduced pressure in a rotary evaporator (Buchi Rotavapor II, Buchi Germany) at 40^{o}C and lyophilized in a freeze-dryer (VirTis Genesis 25EL, SP Scientific, NY, USA) for 16 hours; the product was stored at 4^{o}C for further use.

6.2.2.1. Animals

Six weeks old male Wistar rats, weighing between 180-200 gm were investigated. They were bought and kept at the experimental animal house, Department of Veterinary Physiology and Biochemistry, University of Ibadan, Nigeria where they were kept in specially designed animal cages under controlled, standard, laboratory conditions; humidity between 45% to 55%, and room temperature and 12-hrs dark: 12-hrs light cycle. They were fed twice daily (mornings and evenings) for four weeks, with rats' cubes, a pelleted feed purchased from Ladokun and Sons Livestock Feed, Nigeria Limited. They were always allowed access to clean water in clean bowls.

6.2.2.2. Ethical Considerations

Ethical clearance was obtained from the Health and Wellness Sciences Faculty's Research Ethics Committee, Cape Peninsula University of Technology (CPUT/HW-REC 2016/A1). The University of Ibadan animal use and care research ethics committee approved the animal handling protocol (UI-ACUREC/19/0017).

6.2.2.3. Type 2 Diabetes Mellitus Induction in the Animals

Animals were allowed one week after purchase to acclimatize to their environment. After that, the animals had free access to 10% fructose water for another two weeks, and their weights were measured. A single dose of streptozotocin injection (55mg/kg body weight) in cold citrate buffer pH 4.5 was administered intraperitoneally (i.p) to induce diabetes. The non-diabetic control group received cold citrate buffer pH 4.5, also via i.p route. Treatment to respective experimental groups with the extract and standard antidiabetic drug, glibenclamide (0.2 mg/kg

BWT commenced after 96 hrs (4 days) of DM confirmation with hyperglycaemia (blood glucose ≥ 11.11 mmol/L). Body weights and blood glucose measurements were again taken once weekly. Treatments were continued for 4wks.

6.2.2.4. Animal grouping and Experimental Design

Rats were randomly grouped into five as follows; NC: Normal control; DC: Diabetic control; PA 200 mg: diabetic rats + *Phyllanthus amarus* 200mg/kg BWT; PA 400mg: diabetic rats + PA 400mg/kg BWT; and GLIBEN 0.2mg: diabetic rats + standard antidiabetic drug, glibenclamide 0.2mg/kg BWT, where each group was made up of 10 rats (n = 10). All animals were maintained under standard laboratory conditions and fed ad libitum with standard rat chow (SRC) and water. Group NC rats received SRC and vehicle only (distilled water), and on the day of DM induction, they received a single intraperitoneal injection of cold citrate buffer pH 4.5; The remaining experimental animals were allowed free access to 10% fructose for two weeks, followed by intraperitoneal injection with a single dose of streptozotocin (STZ) 55mg/kg BWT dissolved in 0.1M cold citrate buffer pH 4.5. Group DC served as diabetic control rats. The PA 200mg group comprised of rats that were fed with 200mg/kg BWT day of *Phyllanthus amarus* extract for 4 wks (28 days), selected according to a previous study (Ogar, Egbung, Nna, Atangwho, & Itam, 2019). The PA 400mg group comprised of rats that were also fed with PA 400 mg/kg BWT/day for 4wks. GLIBEN 0.2mg group was the diabetic rats treated with 0.2mg/kg BWT/day of standard drug, glibenclamide.

6.2.2.4.1. Sample Collection, Preparation and Homogenization

On the day of sacrifice to terminate the experiment, five rats (n=5) in each group were anaesthetised and euthanized using i.p injection of sodium pentobarbital, 60 mg/kg BWT. They were then sacrificed by cervical dislocation. 6 mL blood samples were drawn and dispensed into

respective blood tubes (plain, EDTA and lithium heparin) for biochemical evaluations. The heparinized and EDTA blood samples were centrifuged at 2000 x *g* for 10 minutes. Supernatant plasma/serum was separated and stored in a -80^{o}C freezer until the time of analysis. Right testicular tissues were collected and fixed by 10% formol-saline fixative, for histopathological assessment, while the left testes were quickly excised and washed in ice-cold phosphate-buffered saline solution after which they were blotted with filter paper and weighed. The tissue samples were homogenized in the buffer (1gm/2mL) in a homogenizer (Yellow^{line}D1 25 basic, IKA® -WERKE GMBH & CO, Germany); then briefly centrifuged for 20mins. The resultant supernatant was collected and used for further biochemical evaluations.

6.2.2.5. Bodyweight change, Right Epididymal and Testicular Relative organ weights

Body weight was monitored weekly with the aid of a portable, battery-operated weighing scale. The body weight change during the study period was calculated using the formula:

$$\boldsymbol{Bodyweight\ change}\ (\boldsymbol{g}) = \boldsymbol{Final\ bodyweight}\ (\boldsymbol{g}) - \boldsymbol{Initial\ bodyweight}\ (\boldsymbol{g})$$

The epididymis and right testes of the experimental animals were excised and weighed using a precision weighing balance to obtain their absolute weights, while the relative weights of the right epididymis and testes were calculated using the formula:

$$\boldsymbol{Relative\ organ\ weights}\ (\%) = \frac{\boldsymbol{Absolute\ organ\ weight}\ (\boldsymbol{g})}{\boldsymbol{Final\ body\ weight}\ (\boldsymbol{g})}\ \boldsymbol{x\ 100}$$

6.2.3. Measurement of Sex Hormones, Serum Insulin and Oxidative Stress Biomarkers in Testes

6.2.3.1. Serum Testosterone

Serum testosterone was determined by electrochemiluminescence immunoassay technique using Elecsys Testosterone II assay for Cobas e 411 analysers. The test uses a competitive method using a high-affinity monoclonal antibody (sheep) directed explicitly to target testosterone. The testosterone released from the sample by 2-bromoestradiol competes with the added testosterone derivative labelled with a ruthenium complex for the binding sites on the biotinylated antibody.

6.2.3.2. Serum Oestradiol

Serum Oestradiol was determined by electrochemiluminescence immunoassay technique using Elecsys Oestradiol II assay for Cobas e 411 analysers. The test uses a competitive method on a polyclonal antibody directed explicitly against 17β-oestradiol. Endogenous oestradiol from the sample then competes with the added oestradiol derivative labelled with a ruthenium complex for the binding sites on the biotinylated antibody.

6.2.3.3. Serum Insulin

Serum insulin was determined using Thermoscientific™ Pierce™ Rat insulin, ELISA Kit (Al-Hindi, Yusoff, Atangwho, et al., 2016). All reagents and samples were allowed to attain room temperature (18 – 25ºC) before use., following the manufacturer's instructions. The concentration of insulin (µU/mL) in each sample was determined from a prepared standard curve by interpolating the Insulin concentration to the absorbance value. Expressing the results in SI unit, one µU/mL = 6.945ρmol/L.

6.2.3.4. Oxidative Stress Biomarkers in the Testes

The activity of superoxide dismutase (SOD) in testicular tissue homogenate was determined by a modified method of Misra & Fridovich (1972). In contrast, the glutathione-S-transferase activity was determined according to a modified method of Prohaska & Ganther (1977). Glutathione peroxidase activity and Reduced Glutathione levels were measured according to the method of Beutler et al. (1963). Malondialdehyde (MDA) content as an indicator of lipid peroxidation was measured in the testicular tissues following a method described by Varshney & Kale (1990). A modified procedure, as defined by Maciel et al. (2007), was observed to measure serum nitric oxide (NO) levels. Whereas, serum myeloperoxidase (MPO) activity was determined according to the method of Xia & Zweier (1997).

6.2.4. Sperm Parameters Evaluations

6.2.4.1. Collection of Semen Sample

The caudal epididymal semen sample collection was performed according to the method reported by Younglai et al. (2001). The seminal fluid was collected from the supernatant and used for further analysis.

6.2.4.2. Sperm Count, Motility, and Abnormal Sperm

The seminal fluid evaluation was done using a haemocytometer and trypan blue solution. Briefly, 20µL of semen was added to 20µL of trypan blue solution, and the number of spermatozoa, motility and abnormal sperm were enumerated using the haemocytometer and light microscope (Yokoi, Uthus, & Nielsen, 2003).

6.2.5. Cell Culture Studies

6.2.5.1. MCF-7 Breast Cancer Cell Culture

According to a method, as described with slight modification from a previous study (Olabiyi, Aboua, Popoola, Monsees & Oguntibeju, 2020), MCF-7 breast cancer cells were bought from ATCC® (USA, control no HTB-22). They were seeded for 24 hours and 72h at 1,500 cells/ 200µL/well and 800 cells/ 200µL/well, respectively in a 96 well plate. The cells were first allowed to attach and grow for 24h in a complete culture medium comprising of Dulbecco's Modified Eagle medium (DMEM) without phenol red, supplemented with 10% charcoal-stripped foetal bovine serum and 1% Penicillin (1000U/mL) and 1% Streptomycin (1000U/mL combination antibiotics, for treatment the following day. *Phyllanthus amarus* extracts were dissolved in phenol red-free DMEM to a final concentration of 1mg/mL. Serial dilutions from 1000µg/mL to 0.1µg/mL of the extracts were prepared using the complete growth medium. Four wells were used for each concentration (Chen, Chang, Kuo, Huang, Hu, et al., 2016) of the extracts. Another four wells were used for untreated negative control (supplemented growth medium only) and four wells for 1 nM 17β-oestradiol in the growth medium as a positive control. They were incubated for 24h, after which the media were discarded from the plates and cells were washed with phosphate buffer (PBS) (100µL / well).

6.2.5.2. MTT Assay to Determine Cytotoxicity

MTT will measure the activity of mitochondrial dehydrogenases in vital cells via spectroscopy. The amount of MTT conversion into blue formazan is dependent on (a) the number of living cells (no toxic effect present, enzyme active unchanged), and (b) the enzyme activity (which can be activated if the cell is stressed and the mitochondria have to work more to produce energy; when the toxic effect of a substance get stronger, the cells starts dying and the enzyme activity goes down). Thus, the MTT assay can be used to reflect the number of living cells (as in E-Screen) or to measure cytotoxicity following the modified MTT method (Lappalainen, Jääskeläinen, Syrjänen, Urtti, & Syrjänen, 1994; Mosmann, 1983).

On order to determine cytotoxicity using colourimetric MTT assay, 200µL of fresh complete culture media was dispensed to each well, followed by 20µL of MTT (1mg/mL) which was dissolved in PBS solution and incubate at 37^{o}C, 5% CO_2 and 95% humidified air for 5h. Afterwards, the supernatant was discarded, and the plates were drained of residual fluids by briefly placing them upside down on paper towels. 100µL of non-sterile, undiluted DMSO was dispensed to each well to dissolve the blue formazan, and the absorbance was measured with microplate (MTP) reader (POLAR STAR OMEGA, BMG LABTECH) at a wavelength of 570nm with a reference wavelength of 690nm to correct the effect of cell debris and precipitated proteins which may be produced during the dissolving process. The optical density was calculated by subtracting the absorbance of the reference wavelength from the absorbance of the test wavelength. The percentage mitochondrial dehydrogenase activity of MCF-7cells, a measure of the proliferation of the cells (Monsees et al., 2000, Olabiyi, Aboua, Popoola, Monsees & Oguntibeju, 2020) was calculated following the equation below :

$$\%\, mDehydrogenase\ activity = \left\{\frac{Absorbance\ of\ treated\ cells}{Absorbance\ of\ untreated\ cells}\right\} x\ 100$$

6.2.5.3. Morphology Evaluation

Cells that were seeded at a density of 1500 cells/200µL/well in a 96 well plate were used to analyse morphological changes produced by the plant extracts. We allowed the seeded cells to attach and grow for 24hrs in complete culture medium to be treated with varying concentrations of the extracts for another 24hrs. The cells were observed under an inverted microscope (200x and 400x magnifications), and microphotographs were taken. Minimum of five random

microscopic visible fields for each negative control, positive control and the concentrations of the extract were analysed for cell counts as described previously (Olabiyi, Aboua, Popoola, Monsees & Oguntibeju, 2020).

6.2.5.4. E-Screen Assay to Determine the Phytoestrogens activity in *Phyllanthus* amarus

To determine the phytoestrogenic activities of the *Phyllanthus amarus* extracts by E-Screen assay, the protocol of Dang & Lowik (2005) to remove the steroid hormones from the foetal bovine serum (FBS) was followed. Breast cancer cells (MCF - 7 cells) that were seeded with DMEM (phenol red-free) growth medium supplemented with 10% FBS and 1% penicillin-streptomycin in 96 well plates at a density of 1500 cells/ 200µL/well and 800 cells/200µL/well for 24hrs and 72hrs experiments respectively. The plates were incubated in 5% CO_2, 95% air (Lin & Costa, 1994) and 37°C incubator for 24hrs and 72hrs, respectively. After that, the media was discarded, and the cells were washed with PBS. Different concentrations (0.1µg/mL, 1µg/mL, 10µg/mL and 100µg/mL) of the *Phyllanthus amarus* extract were prepared using phenol red-free DMEM, 10% Charcoal-Dextran-FBS (charcoal-stripped FBS) and 1% Penicillin-Streptomycin. Furthermore, 1nM oestradiol was used as a positive control and phenol red-free DMEM with charcoal-stripped FBS as a negative control. After 24 and 72hrs, the MCF-7 cells were assessed using MTT assay as previously described.

6.2.6. Histopathology using Haematoxylin and Eosin Staining

Harvested testes from the rats were preserved in 10% formalin saline buffer fixative. The tissues were processed and embedded in paraffin wax. Tissue sections of 5µm in thickness were made and stained with haematoxylin and eosin (H&E) for histopathological assessment (Drury & Wellington, 1976). A qualified veterinary pathologist with no prior knowledge of the animal groupings and their treatments carried out the histopathology evaluation.

6.2.6.1. The Diameter of Seminiferous Tubules and Height of Seminiferous Tubular Epithelium

The diameter of seminiferous tubules and height of seminiferous tubular epithelium of 20 randomly selected seminiferous tubules in each of the five groups (N=100) were measured using the AmScope Toupview software (United Scope LLC, Irvine, California, USA). The measurements were tabulated and subjected to descriptive statistical analysis using IBM SPSS Statistics for Windows, version 23 software (IBM Corp., Armonk, N.Y., USA).

6.2.7. Statistical Analysis

GraphPad Prism software, version 5.0 (GraphPad Software, San Diego, California, USA), was used for all graphical evaluations and representations. Data were expressed as mean ± SEM. A one-way ANOVA test with Bonferroni's multiple comparison test if $p<0.05$ was used. Differences were considered statistically significant if $p<0.05$ (Ekwueme, Zhao, Rim, et al., 2019)

6.3. Results

Table 6.1: Mean ± (SEM) Fasting Blood Glucose (FBG) in diabetic and non-diabetic animals

Test	NC	DC	PA 200mg	PA 400mg	GLIBEN. 0.2mg
Initial FBG (mmo/L)	3.8 ± 0.5	10.3 ± 4.4	15.1 ± 1.1	11.1 ± 1.3	14.1 ± 4.6
Final FBG (mmo/L)	4.7 ± 0.9	13.3 ± 7.0	20.9 ± 2.2	21.2 ± 0.7	19.2 ± 1.8
Change In FBG (mmol/L)	+0.9	+3.0	+5.8	+10.1	+5.1

The table reveals the mean fasting blood glucose concentrations of the experimental animals and normal controls at day 4 (initial FBG) and at day 28 (final FBG) after streptozotocin

administration. As shown, there was persistent hyperglycaemia at the termination of the experiments. NC = normal control rats; DC = Diabetic control rats without treatments; PA 200mg = Diabetic treated rats with *Phyllanthus amarus* 200mg extract per kg BWT./day; PA 400mg = Diabetic treated rats with *Phyllanthus amarus* 400mg extract per kg BWT/day; GLIBEN. 0.2mg = Diabetic treated rats with glibenclamide 0.2mg drug per kg BWT/day.

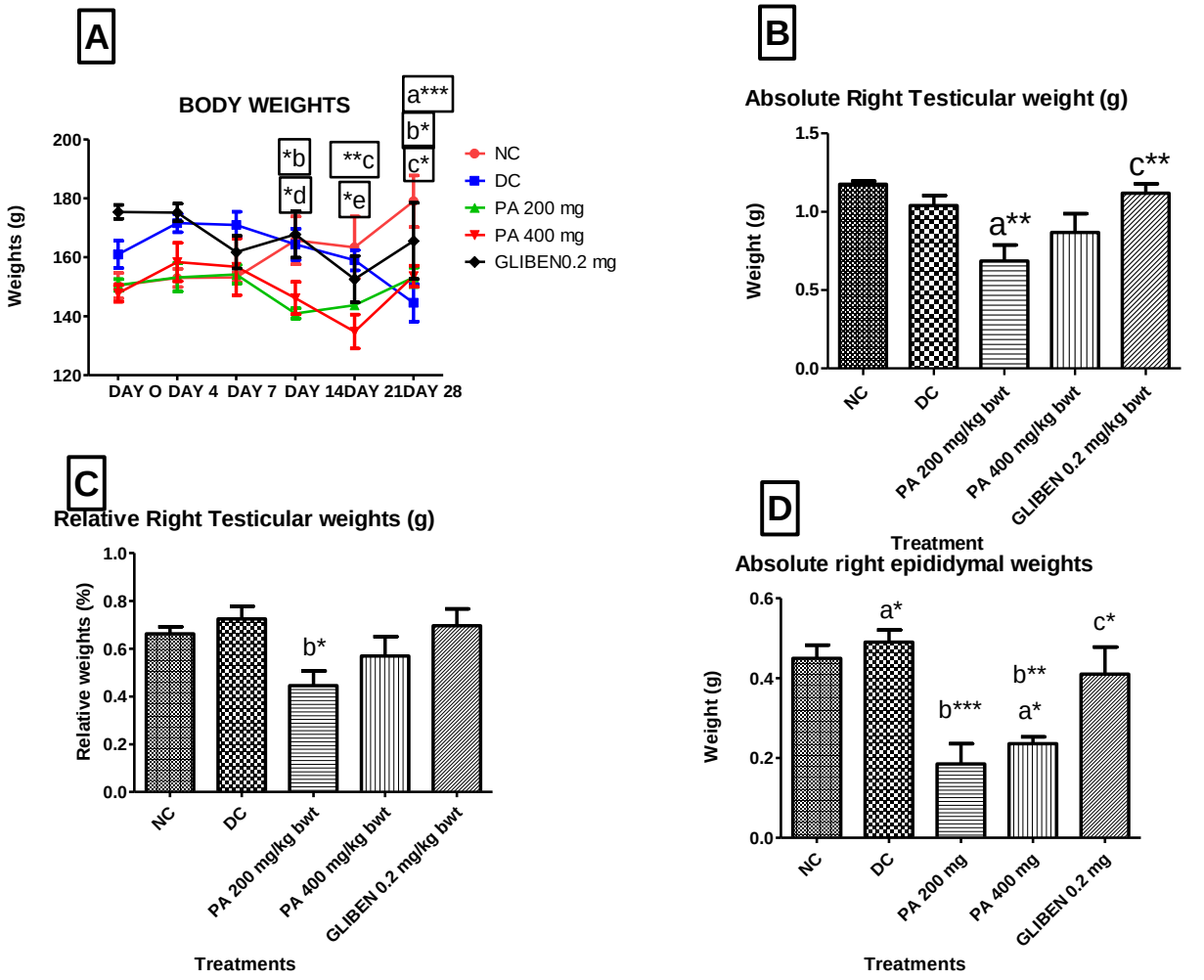


Figure 6.1: Effect of Phyllanthus amarus extracts on the body, testicular and epididymal weights

A. There is a significant difference in the bodyweights of the DC vs NC at day 28 ($^{a***}p<0.001$); (NC vs PA 200mg at days 14 and 28 ($^{b*}p<0.05$); NC vs PA 400mg at 21 ($^{c**}p<0.01$); NC vs PA 400mg at 28 ($^{c*}p<0.05$); DC vs PA 200mg at day 14 ($^{d*}p<0.05$); DC vs PA 400mg/kg at day 21 ($^{e*}p<0.05$) and PA 200mg vs GLIBEN 0.2mg/kg at day 14 ($^{f**}p<0.01$). B. There is a significant difference in the absolute right testicular weights, NC vs PA 200mg ($^{a**}p<0.01$); PA 200mg vs

GLIBEN 0.2mg ($^{c**}p<0.01$). C. There is a significant difference in the right relative testicular weights DC vs 200mg ($^{b*}p<0.05$). and D. Showing a significant difference in the right epididymal weight of NC vs PA 200mg ($^{a*}p<0.01$); NC vs PA 400mg ($^{a*}p<0.05$); DC vs PA 200mg ($^{b***}p<0.0001$); DC vs PA 400mg ($^{b**}p<0.01$); PA 200mg vs GLIBEN 0.2mg ($^{c*}p<0.05$). Data are presented as mean ± SEM, n = 5 (analysed by on-way ANOVA followed by Bonferroni multiple comparison test); a = compared to NC, b = compared to DC, c = compared to PA 200mg. *$p<0.05$, $^{**}p<0.01$; **$p<0.001$. NC = normal control rats; DC = Diabetic control rats without treatments; PA 200mg = Diabetic treated rats with *Phyllanthus amarus* 200mg extract per kg BWT/day; PA 400mg = Diabetic treated rats with *Phyllanthus amarus* 400mg extract per kg BWT/day; GLIBEN. 0.2mg = Diabetic treated rats with glibenclamide 0.2mg drug per kg BWT/day.

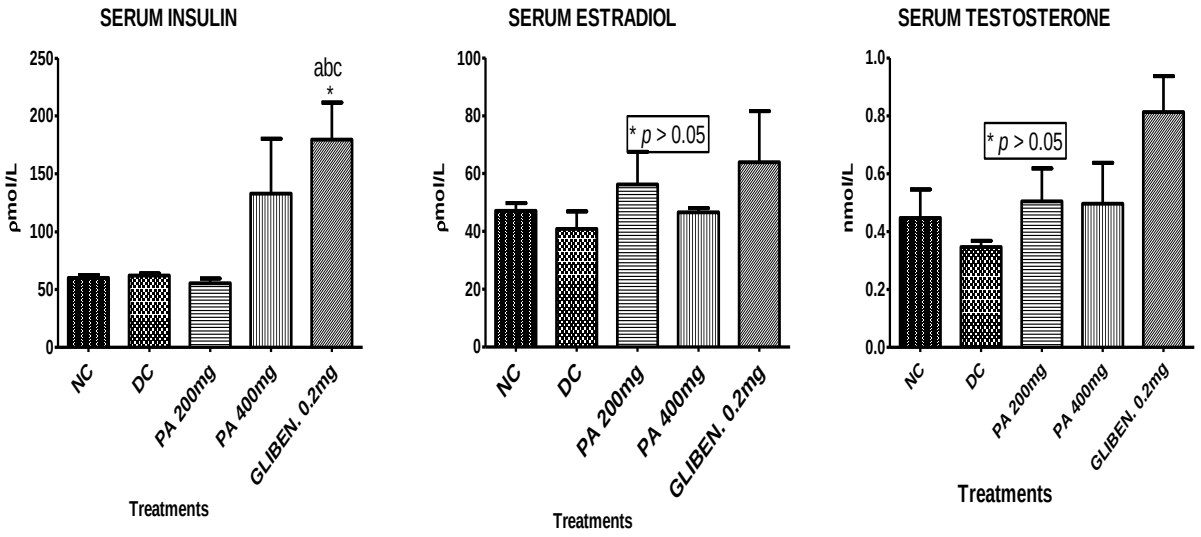


Figure 6.2: Effect of Phyllanthus amarus extracts on Serum insulin, oestradiol and testosterone levels

As depicted in the figure above, PA 200mg/kg BWT improved serum testosterone by 45.66% in a similar manner to glibenclamide (134.83%) compared to the diabetic controls. It also raised serum oestradiol by 37.67% in a similar manner to glibenclamide (56.46%) compared to the diabetic control. PA 400mg/kg BWT increased serum insulin by 113% similarly to glibenclamide (188.6%) compared to the non-treated diabetic controls, and by 122.7% when compared to the normal controls ($p>0.05$). $^{a*}p<0.05$ (NC vs GLIBEN 0.2mg); $^{b*}p<0.05$ (NC vs GLIBEN 0.2 mg); $^{c*}p<0.05$ (NC vs GLIBEN 0.2 mg). Data are presented as mean ± SEM, n = 5, a = compared to NC, b = compared to DC; c = compared to PA 200mg *$p<0.05$, **$p<0.001$. NC=normal control rats; DC = Diabetic control rats without treatments; PA 200 mg = Diabetic treated rats with

Phyllanthus amarus 200 mg extract per kg BWT./day; PA 400mg = Diabetic treated rats with *Phyllanthus amarus* 400 mg extract per kg BWT/day; GLIBEN. 0.2mg = Diabetic treated rats with glibenclamide 0.2mg drug per kg BWT/day.

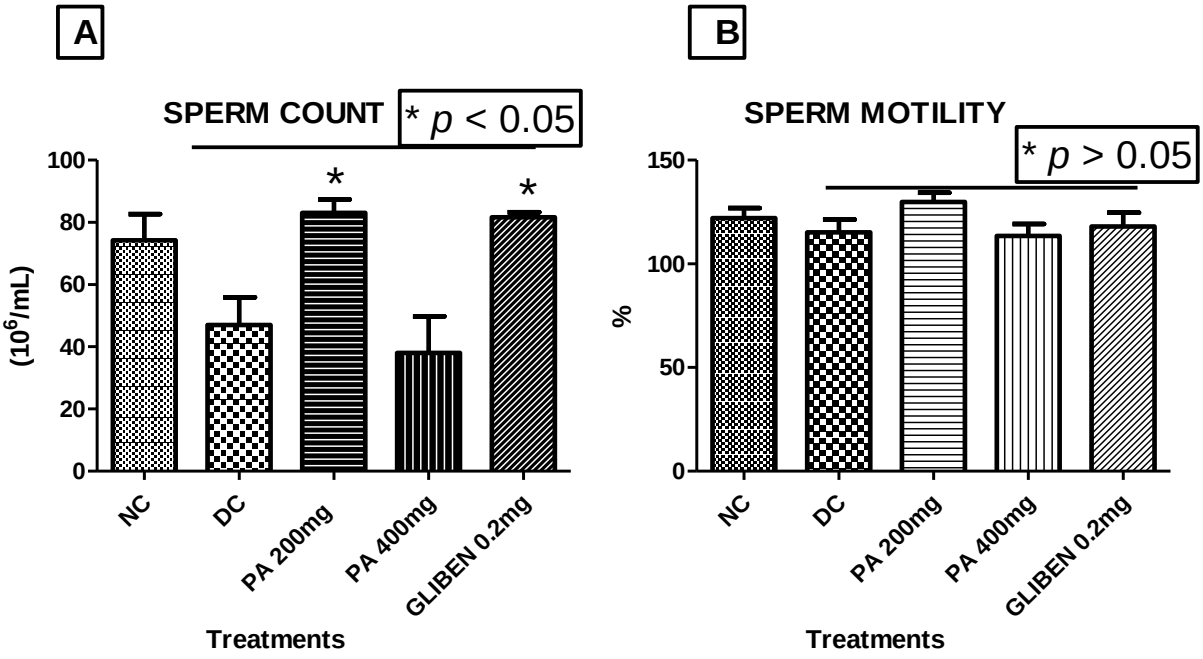


Figure 6.3: Effect of Phyllanthus amarus extracts on Sperm counts and motility

PA 200mg/kg BWT significantly normalised sperm count by 11.86% in a similar manner to glibenclamide (10.07%) (p = 0.0033) when compared to the diabetic untreated control rats and PA 400mg/kg BWT. Data are presented as mean ± SEM, n = 5, a = p > 0.05 compared to NC, b = p > 0.05 compared to DC, c = p < 0.05 compared to PA 400mg. *p < 0.05, NC = normal control rats; DC = Diabetic control rats without treatments; PA 200mg = Diabetic treated rats with *Phyllanthus amarus* 200mg extract per kg BWT/day; PA 400mg = Diabetic treated rats with *Phyllanthus amarus* 400mg extract per kg BWT /day; GLIBEN. 0.2mg = Diabetic treated rats with glibenclamide 0.2mg drug per kg BWT/day.

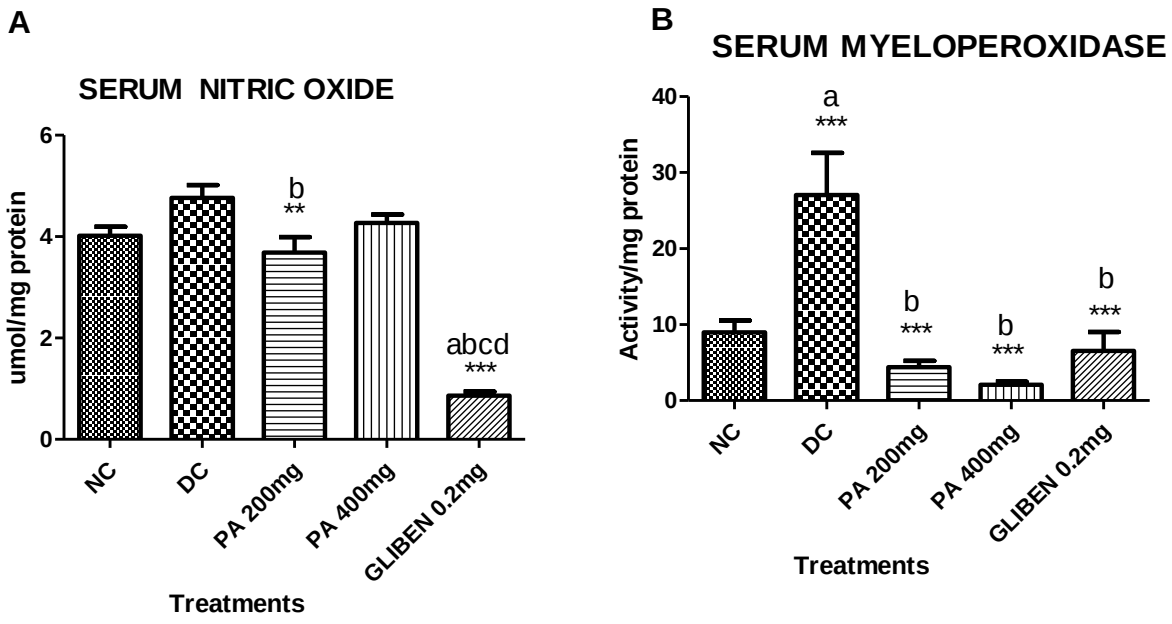


Figure 6.4: Effect of Phyllanthus amarus extracts on Serum Nitric oxide and myeloperoxidase

PA 200mg/kg BWT reduced serum nitric oxide by -22.61% ($p < 0.0001$) compared to the diabetic control while the GLIBEN 0.2mg/kg BWT significantly reduced serum nitric oxide compared to the normal and diabetic controls, PA 200mg/kg BWT and PA 400mg/kg BWT ($p < 0.0001$). PA 200mg/kg BWT, PA 400 mg/kg BWT and GLIBEN 0.2mg/kg BWT significantly reduced serum MPO by 83.63%, 92.34% and 75.82% respectively ($p < 0.0001$). Data are presented as mean ± SEM, n = 5, a = compared to NC, b = compared to DC, c = compared to PA 200 mg/kg BWT, d = compared to 400 mg/kg BWT *$p < 0.05$, **$p < 0.001$, ***$p < 0.0001$. NC = normal control rats; DC = Diabetic control rats without treatments; PA 200 mg = Diabetic treated rats with *Phyllanthus amarus* 200 mg extract per kg body weight/day; PA 400mg = Diabetic treated rats with *Phyllanthus amarus* 400mg extract per kg body weight/day; GLIBEN. 0.2mg = Diabetic treated rats with glibenclamide 0.2 mg drug per kg body weight/day.

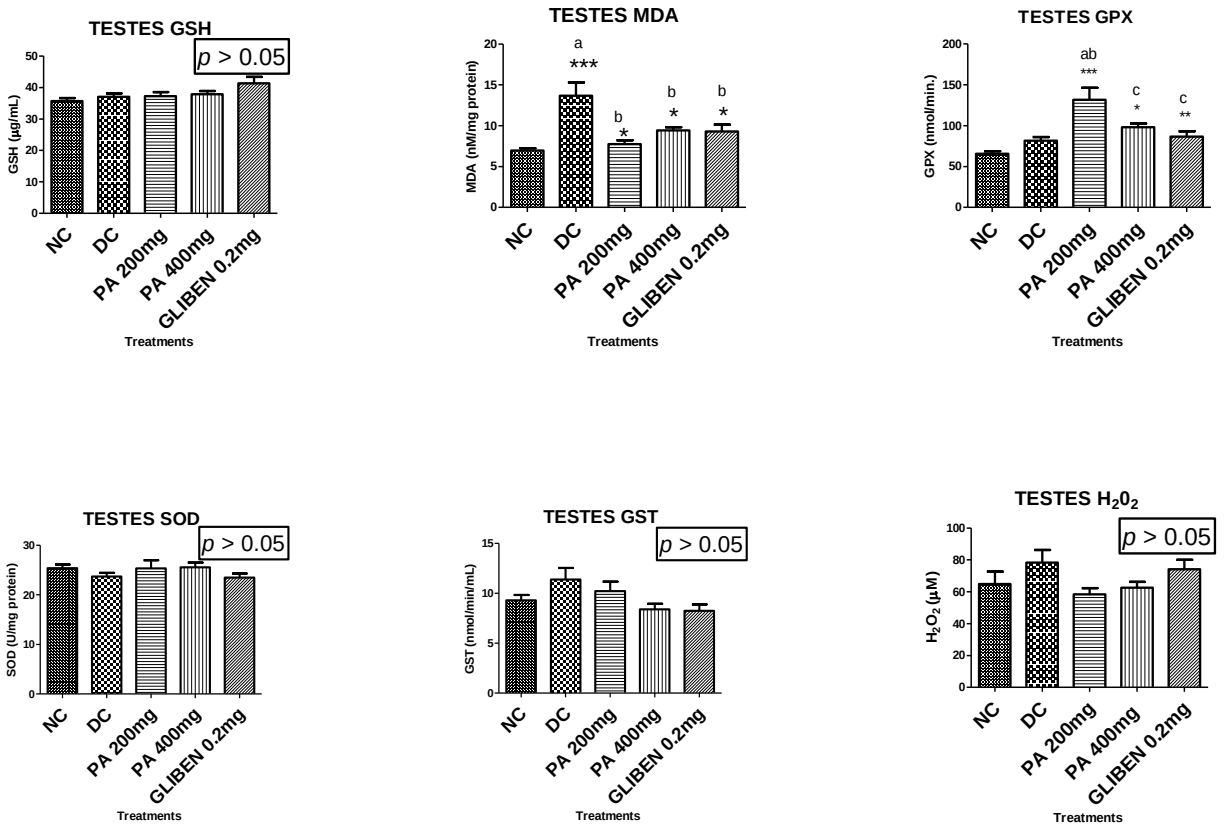


Figure 6.5: Effect of Phyllanthus amarus extracts on Testicular biomarkers of oxidative stress

Here, both PA 200mg/kg BWT together with PA 400mg/kg BWT reduced H_2O_2 by -25.38% and -20.15% respectively compared to the diabetic control but not significant ($p > 0.05$). But they significantly reduced MDA levels when compared with the diabetic controls ($p < 0.0001$, $p < 0.05$). PA 400mg/kg BWT. significantly increased glutathione peroxidase activity by 61.42% ($p < 0.0001$) compared to the diabetic controls. Data are presented as mean ± SEM, n = 5, a = compared to NC, b = compared to DC, c = compared to PA 200mg/kg BWT, d = compared to 400mg/kg BWT. *$p < 0.05$, **$p < 0.001$, ***$p < 0.0001$. NC = normal control rats; DC = Diabetic control rats without treatments; PA 200mg = Diabetic treated rats with *Phyllanthus amarus* 200mg extract per kg body weight/day; PA 400mg = Diabetic treated rats with *Phyllanthus amarus* 400mg extract per kg body weight/day; GLIBEN. 0.2mg = Diabetic treated rats with glibenclamide 0.2mg drug per kg body weight/day.

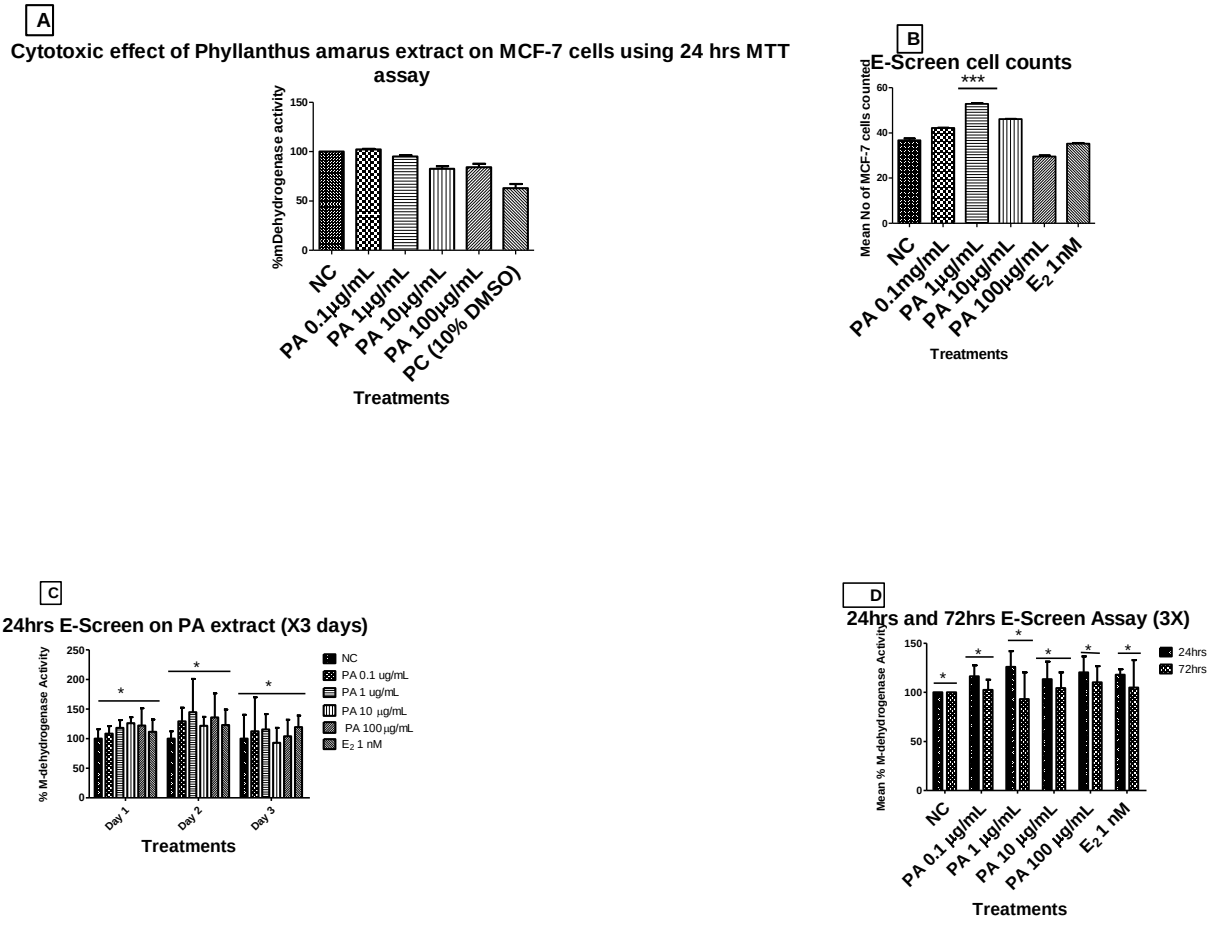


Figure 6.6: Effect of Phyllanthus amarus extracts on 24hrs MTT, cell counts and E-Screen assay

The figure reveals the phytoestrogenic activity of *Phyllanthus amarus* plant extract on MCF-7 breast cancer cell line (a). 24 hrs MTT on MCF-7 cells: This indicates that PA extracts not cytotoxic at the doses tested; NC – Negative control; PC – Positive control (10% DMSO) (b). Significant proliferation of MCF-7 cells depicted by E-Screen cell counts at 1µg/mL ($p < 0.0001$), (c). 24 hrs E-Screen assay on PA extract repeated for three different days(X3) showing no significant difference ($p > 0.05$). (d). 24 hrs and 72 hrs E-Screen on PA extract at varying concentrations vs 17β Oestradiol (E_2) as the positive control (no significant difference for the 24 hrs vs 72hrs, $p > 0.05$).

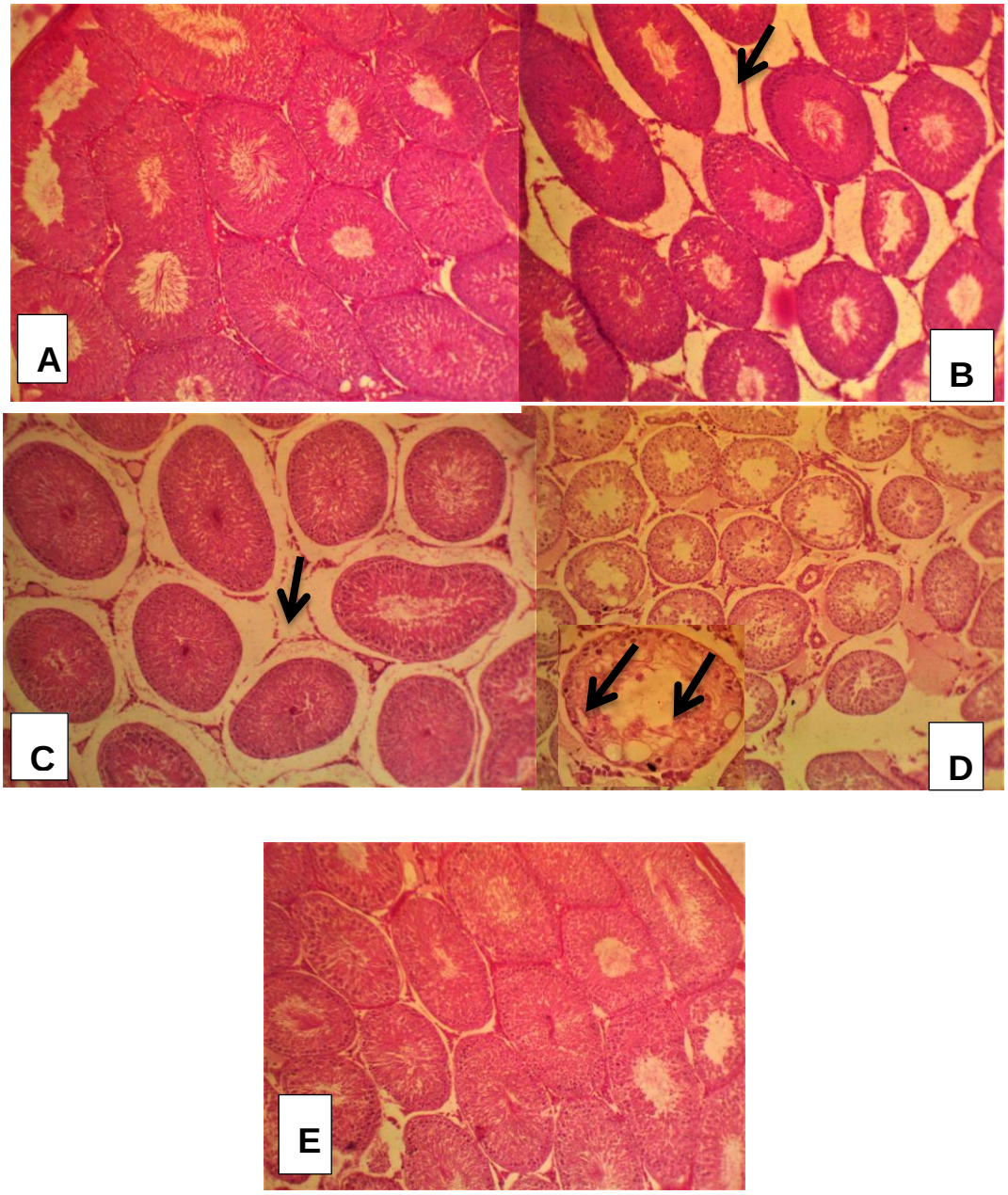


Figure 6.7: Effect of Phyllanthus amarus extracts on Testicular histology

The microphotographs show that there was: A. No visible lesion in Normal Control: H&E, x150. B. Reduction in the diameter of seminiferous tubules with an expansion of the interstitial space (arrow) in the Diabetic Control: H&E, x150. C. Observed reduction in the diameter of seminiferous tubules (Saxena, Dhungel, Bhattacharya, Jha, & Srivastava, 2004) with an expansion of the interstitial space (arrow) in the PA 200mg: H&E, x150. D. Reduction in seminiferous tubular diameter, depletion of germinal epithelium and spermatogenic arrest. Inset shows seminiferous tubule with spermatogenic arrest, depleted germinal epithelium, disruption

and vacuolation of the epithelium (arrows) in the PA 400mg: H&E, x150, and E. There was No visible lesion in the GLIBEN 0.2mg: H&E, x150.

Table 6.2: Mean (± SD) Diameters of the seminiferous tubules (mm) and height of the seminiferous tubular epithelium (mm)

GROUPS	Diameters (mm)	P value	Height (mm)	P value
NC	0.30 ± 0.05	0.000	0.10 ± 0.03	0.000
DC	0.25 ± 0.03		0.07 ± 0.01	
PA 200mg/kg BWT	0.25 ± 0.02		0.08 ± 0.01	
PA 400mg/kg BWT	0.18 ± 0.03		0.05 ± 0.01	
GLIBEN 0.2mg/kg BWT	0.33 ± 0.04		0.12 ± 0.02	

From the results, there was a reduction in the mean diameters of the seminiferous tubules and height of the seminiferous tubular epithelium in groups B (Diabetic Control), C (PA 200mg/kg BWT) and D (PA 400mg/kg BWT) when compared with the mean observed in the normal/healthy control group (Group A). A slight increase in the mean diameters of the seminiferous tubules and height of the seminiferous tubular epithelium in group E (GLIBEN 0.2mg/kg BWT) was observed. Moreover, one-way ANOVA to compare the means between the different groups at 95% confidence interval and significance level of $p < 0.05$ was done. From the result, the changes observed in the means of the diameters of seminiferous tubules and height of seminiferous tubular epithelium between the groups was significant as P value in both cases was 0.000.

6.4. Discussion

A report by the international diabetes federation shows that hyperglycaemia and hypertension can cause damage to the nerves throughout the body, thus, leading to digestive problems, the supply of blood and nutrients to several parts of the body thereby, leading to erectile dysfunction (ED) via the nitric oxide pathway (International Diabetes Federation, 2019). Besides, ED and loss of libido could be a cause of male infertility (Lotti & Maggi, 2018). In the recent past, emerging evidence has shown that diabetes can cause male reproductive dysfunctions (Sisman et al., 2014). In this study, T2D was induced in male Wistar rats by giving 10% fructose water to rats to drink for two weeks, followed by intraperitoneal injection of streptozotocin 55mg/kg BWT and diabetes was confirmed after 96 hrs. Findings from this study show persistent hyperglycaemia up to day 28 (end of experiments) (Table 6.1). In the present study, we observed there was a loss in the body, epididymal and testicular weight in the experimental groups due to diabetes (figure 6.1). We also noted that this loss could not be restored by treatment with *Phyllanthus amarus* as reported by previous investigators (Lawson-Evi et al., 2011). In our opinion, the strain of the animals, abundant tannins in the extract and the short period of 28 days of this study could be responsible for the failure of the extract to restore the body and organ weight loss in the groups.

In contrast, the PA 400mg/kg BWT increased serum insulin by 113% in a similar manner to glibenclamide (188.6%) compared to the non-treated diabetic controls, and by 122.7% compared to the healthy controls. This finding supports the antihyperglycaemic activity of the plant extract (Karuna, Bharathi, Reddy, Ramesh, & Saralakumari, 2011). Both PA 200mg/kg BWT and 400 mg/kg BWT tend to normalise serum testosterone when compared with diabetic controls (figure 6.2). As depicted in figure 6.3A, the PA 200mg/kg BWT dose extract significantly normalised sperm count similarly to glibenclamide ($p = 0.0033$) compared with the diabetic untreated control animals, thus, supporting its beneficial androgenic role.

In our previous report, it was indicated that antioxidant substances in *Phyllanthus amarus* extracts showed biphasic characteristics, such that at low concentrations, it is an antioxidant and at high levels, it is a pro-oxidant (Olabiyi, Aboua, Popoola, Monsees & Oguntibeju, 2020). The characteristic feature observed with this extract perhaps serves to protect against oxidative stress and lipid peroxidation such that it could promote Sertoli cell proliferation, particularly at lower concentrations, as earlier reported (Catala, 2007; Young et al., 2008). As shown in figure 6.4, the PA 200mg/kg BWT significantly ($p < 0.0001$) reduced serum nitric oxide compared with diabetic control while GLIBEN 0.2mg/kg BWT lowered serum nitric oxide ($p < 0.0001$) when compared with the untreated healthy and diabetic controls, PA 200 mg and the PA 400mg groups. Besides, both PA 200mg/kg BWT and PA 400 mg/kg BWT

extract doses brought down the elevated serum myeloperoxidase (MPO) levels in a similar manner to glibenclamide compared to the diabetic untreated rats. As shown in figure 6.5, PA 200 mg/kg BWT significantly increased glutathione peroxidase activity ($p < 0.0001$) and significantly reduced malondialdehyde ($p < 0.0001$) compared with the diabetic controls. These findings suggest its beneficial defence against free radical cellular damage by mitigating the inflammatory process and oxidative stress.

We have previously reported that *Phyllanthus amarus* stimulated significant proliferation of Sertoli (TM4) cells, hence, its phytoestrogenic ability (Olabiyi, Aboua, Popoola, Monsees & Oguntibeju, 2020). In the current study, we found that the aqueous extract of *Phyllanthus amarus* produced proliferative effects on breast cancer, MCF - 7 cells in a similar manner to the 17β-oestradiol control ($p > 0.05$), suggesting its estrogenic activity (figure 6.6). Furthermore, a cytotoxicity assay using 24 hrs MTT revealed that the extract proliferates MCF - 7 cells at lower concentrations (0.1-10µg/mL) but reduced the cell numbers at concentrations above 10µg/mL as depicted by the E- Screen cell counts. The E-screen result signifies its antitumour potential against breast cancer due to its component hypophyllanthin and phyllanthin (Mohamed, Jantan, Nafiah, & Seyed, 2018; Parvathaneni, Battu, Gray, & Gummalla, 2014).

Histologically, the most significant changes seen in the current study are in the PA 400mg/kg BWT group, while the PA 200mg/kg BWT group showed mild changes. There was a total arrest of spermatogenesis in the PA 400 mg/kg BWT group (figure 6.7D). This dosage could produce a spermatogenic failure during spermatogenesis leading to disruptions, azoospermia, severe oligozoospermia, asthenozoospermia or teratozoospermia (Jan et al., 2012). Additionally, the germinal epithelium was depleted and vacuolated in this group. The changes observed in the PA 200 mg group were milder, suggesting less disruption to spermatogenesis. Here, there was a reduction in the diameter of seminiferous tubule and expansion of the interstitial space. The two groups (PA 200 mg/kg BWT and 400 mg/kg BWT) showed a reduction in the mean diameters of the seminiferous tubules as well as the mean heights of the seminiferous tubular epithelium (Table 6.2). From the result, the changes observed in the means of the diameters of seminiferous tubules and height of seminiferous tubular epithelium between the groups was significant ($p < 0.05$). These findings suggest a dose-dependent induction of testicular degeneration. Besides, these findings mimic those of Adedapo et al. (2003) who reported a reduction in the mean seminiferous tubular diameter as well as varying degrees of testicular degeneration in eight-weeks-old sexually matured albino rats administered with aqueous crude extract of *Phyllanthus amarus.* However, in the present study, glibenclamide did not have any significant effect on testicular histology.

Our findings corroborate the beneficial role of this potential Phyto estrogenic herb. However, several reports linking phytoestrogens and infertility are controversial; thus, debates are still on-going. Therefore, because of a lack of consensus, more studies are yet required to substantiate for their negative impact on male reproductive health. Besides, at what level of exposure is considered hazardous or harmful needs to be addressed, and a dose-response established.

6.5. Conclusion

Phyllanthus amarus extract could modulate diabetes and exert its antidiabetic effect via oestrogen-dependent pathways and oxidative stress.

6.6. Acknowledgements

The financial support from the NRF South Africa through the grant (R022) awarded to Professor OO Oguntibeju and the University Research Fund of CPUT are duly appreciated. Our thanks also to the management of Afe Babalola University, Ado-Ekiti, Nigeria for the financial support in the form of the Higher Degree Fellowship awarded to Mr FA Olabiyi

6.7. ORCID

Folorunso Olabiyi http://orcid.org/0000-0003-0715-1357

Ademola Oyagbemi http://orcid.org/0000-0002-8996-8610

Monsuru Tijani http://orcid.org/0000-0001-9521-675X

Yapo Aboua http://orcid.org/0000-0001-7725-5536

Thomas Monsees http://orcid.org/0000-0003-3004-826X

Oluwafemi Oguntibeju http://orcid.org/0000-0002-7299-1128

References

Adedapo, A. A., Abatan, M. O., Akinloye, A. K., Idowu, S. O., & Olorunsogo, O. O. (2003). Morphometric and histopathological studies on the effects of some chromatographic fractions of Phyllanthus amarus and Euphorbia hirta on the male reproductive organs of rats. *Journal of Veterinary Science*, *4*(2), 181–185.

Adedapo, Adeolu A., Adegbayibi, A. Y., & Emikpe, B. O. (2005). Some clinicopathological changes associated with the aqueous extract of the leaves of Phyllanthus amarus in rats. *Phytotherapy Research*, *19*(11), 971–976. https://doi.org/10.1002/ptr.1768

Adedapo, Adeolu A., Ofuegbe, S. O., & Adeyemi, A. A. (2013). The anti-diabetic activities of the methanol leaf extract of Phyllanthus amarus in some laboratory animals. *Asian Journal of Medical Sciences*, *4*(3), 23–34. https://doi.org/10.1515/jbcpp-2013-0084

Adeneye, A. A. (2012). The leaf and seed aqueous extract of Phyllanthus amarus improves insulin resistance diabetes in experimental animal studies. *Journal of Ethnopharmacology*, *144*(3), 705–711. https://doi.org/10.1016/j.jep.2012.10.017

Ahmed, M. A., & Hassanein, K. M. A. (2012). Effects of estrogen on hyperglycemia and liver dysfunction in diabetic male rats. *International Journal of Physiology, Pathophysiology and Pharmacology*, *4*(3), 156–166.

Al-Hindi, B., Yusoff, N.A., Atangwho, I.J., Ahmad, M., Asmawi, M.Z., & Yam, M.F. (2016). A soxhlet extract of Gongronema latifolium retains moderate blood glucose-lowering effect and produces structural recovery in the pancreas of STZ-induced diabetic rats. *Medical Sciences*, *4*(2), 9

Almalki, D.A., Alghamdi, S.A., & Al-Attar, A.M. (2019). Comparative study on the influence of some medicinal plants on diabetes induced by streptozotocin in male rats. *BioMed Research International*, *2019*

Amaral, S., Oliveira, P., & Ramalho-Santos, J. (2008). Diabetes and the impairment of reproductive function: possible role of mitochondria and reactive oxygen species. *Current Diabetes Reviews*, *4*, 46–54.

Ansley, D. M., & Wang, B. (2013). Oxidative stress and myocardial injury in the diabetic heart. *Journal of Pathology*, *229*(2), 232–241. https://doi.org/10.1002/path.4113

Barros, R. P. A., Gabbi, C., Morani, A., Warner, M., & Gustafsson, J. Å. (2009). Participation of ERα and ERβ in glucose homeostasis in skeletal muscle and white adipose tissue.

American Journal of Physiology-Endocrinology and Metabolism, *297*(1), 124–133. https://doi.org/10.1152/ajpendo.00189.2009

Beutler, E., Duron, O., & Kelly, B. (1963). An improved method for the determination of blood glutathione. *Journal of Laboratory Clinical Medicine*, *6*, 882–888.

Catala, A. (2007). The ability of melatonin to counteract lipid peroxidation in biological membranes. *Current Molecular Medicine*, *7*(7), 638–649.

Cariati, F., D'Uonno, N., Borrillo, Iervolino, S., Galdiero, G., & Tomaiuolo R. (2019) "Bisphenol A: an emerging threat to male fertility". *Reproductive Biology and Endocrinology* **17, (1),** 6. https://doi.org/10.1186/s12958-018-0447-6

Chen, Y.H., Chang, G.K., Kuo, S.M., Huang, S.Y., Hu, I.C., Lo, Y.L., & Shih, S.R. (2016). Well-tolerated Spirulina extract inhibits influenza virus replication and reduces virus-induced mortality. *Scientific Reports*, *6*(1), 1-11.

Dang, Z. C., & Lowik, C. W. G. M. (2005). Removal of serum factors by charcoal treatment promotes adipogenesis via a MAPK-dependent pathway. *Molecular and Cellular Biochemistry*, *268*(1–2), 159–167. https://doi.org/10.1007/s11010-005-3857-7

Dong, J., Wang, P., He, K., & Qin, L. (2011). Effect of soy isoflavones on circulating C-reactive protein in postmenopausal women: a meta-analysis of randomized controlled trials. *Menopause*, *18*(11), 1256–1262.

Drury, R., & Wellington, E. (1976). Carlton's histopathological techniques: Oxford University Press.

Ekwueme, D.U., Zhao, J., Rim, S.H., de Moor, J.S., Zheng, Z., Khushalani, J.S., Han, X., Kent, E.E., & Yabroff, K.R. (2019). Annual out-of-pocket expenditures and financial hardship among cancer survivors aged 18–64 years—the United States, 2011–2016. *Morbidity and Mortality Weekly Report*, *68*(22), 494

Figueiredo, M.M., Amorim, I.F., Pinto, A.J., Barbosa, V.S., de Jesus Pinheiro, L., Deoti, B., Faria, A.M., & Tafuri, W.L. (2013). Expression of Toll-like receptors 2 and 9 in cells of dog jejunum and colon naturally infected with Leishmania infantum. *BMC Immunology*, *14*(1), 1-12.

Foryst-Ludwig, A., & Kintscher, U. (2010). Metabolic impact of estrogen signalling through ERalpha and ERbeta. *Journal of Steroid Biochemistry and Molecular Biology*, *122*(1–3), 74–81. https://doi.org/10.1016/j.jsbmb.2010.06.012

Giwercman, A. (2011). Estrogens and phytoestrogens in male infertility. *Current Opinion in Urology*, *21*(6), 519–526. https://doi.org/10.1097/MOU.0b013e32834b7e7c

Hallund, J., Tetens, I., Bügel, S., Tholstrup, T., & Bruun, J. M. (2008). The effect of a lignan

complex isolated from flaxseed on inflammation markers in healthy postmenopausal women. *Nutrition, Metabolism and Cardiovascular Diseases*, *18*(7), 497–502. https://doi.org/10.1016/j.numecd.2007.05.007

Houghton, P. J., Woldemariam, T. Z., O'Shea, S., & Thyagarajan, S. P. (1996). Two securinega-type alkaloids from Phyllanthus amarus. *Phytochemistry*, *43*(3), 715–717. https://doi.org/10.1016/0031-9422(96)00345-7

International Diabetes Federation. (2019). *Complications in diabetes*. *About diabetes*. Retrieved from https://www.idf.org/aboutdiabetes/complications.html

Islam, A., Naskar, S., Mazumder, U. K., Gupta, M., & Ghosal, S. (2008). Estrogenic properties of Phyllanthin and hypophyllanthin from Phyllanthus amarus against carbofuran induced toxicity in female rats. *Pharmacologyonline*, *3*, 1006–1016.

Ivanova, V., Kolarova, M., Aleksieva, K., Schlegel, R., Schumann, P., & Graefe, U. (2013). Octadeca-8, 11-dienoic acid methyl ester, a new fatty acid metabolite from Fistulina hepatica. *Journal of Modern Medicinal Chemistry*, *1*(1), 43-48.

Jan, S. Z., Hamer, G., Repping, S., de Rooij, D. G., Van Pelt, A. M. M., & Vormer, T. L. (2012). Molecular control of rodent spermatogenesis. *Biochimica et Biophysica Acta*, *1822*(12), 1838–1850. https://doi.org/10.1016/j.bbadis.2012.02.008

Karimi, J., Goodarzi, M. T., Tavilani, H., Khodadadi, I., & Amiri, I. (2011). Relationship between advanced glycation end products and increased lipid peroxidation in the semen of diabetic men. *Diabetes Research and Clinical Practice*, *91*(1), 61–66. https://doi.org/10.1016/j.diabres.2010.09.024

Karuna, R., Bharathi, V. G., Reddy, S. S., Ramesh, B., & Saralakumari, D. (2011). Protective effects of Phyllanthus amarus aqueous extract against renal oxidative stress in Streptozotocin-induced diabetic rats. *Indian Journal of Pharmacology*, *43*(4), 414–418.

Kassuya, C. A. L., Silvestre, A., Menezes-de-Lima, O., Marotta, D. M., Rehder, V. L. G., & Calixto, J. B. (2006). Antiinflammatory and antiallodynic actions of the lignan niranthin isolated from Phyllanthus amarus. Evidence for interaction with platelet-activating factor receptor. *European Journal of Pharmacology*, *546*(1–3), 182–188. https://doi.org/10.1016/j.ejphar.2006.07.025

Kiemer, A. K., Hartung, T., Huber, C., & Vollmar, A. M. (2003). Phyllanthus amarus has anti-inflammatory potential by inhibition of iNOS, COX-2, and cytokines via the NF-kB pathway. *Journal of Hepatology*, *38*, 289–297. https://doi.org/10.1016/S0

Laakso, M. (2015). Is insulin resistance a feature of or a primary risk factor for cardiovascular disease? *Current Diabetes Reports*, *15*(105), 1–9. https://doi.org/10.1007/s11892-015-

0684-4

Lappalainen, K., Jääskeläinen, I., Syrjänen, K., Urtti, A., & Syrjänen, S. (1994). Comparison of cell proliferation and toxicity assays using two cationic liposomes. *Pharmaceutical Research: An Official Journal of the American Association of Pharmaceutical Scientists*. https://doi.org/10.1023/A:1018932714745

Lawson-Evi, P., Eklu-Gadeg, K., Agbonon, A., Aklikokou, K., Creppy, E., & Gbeassor, M. (2011). Antidiabetic activity of Phyllanthus amarus schum and Thonn (Euphorbiaceae) on alloxan-induced diabetes in male Wistar rats. *Journal of Applied Sciences*, *11*(16), 2968–2973. https://doi.org/10.3923/jas.2011.2968.2973

Lee, Y. A., You, J. K., Eun, J. C., & Yokozawa, T. (2007). Ameliorative effects of proanthocyanidin on oxidative stress and inflammation in streptozotocin-induced diabetic rats. *Journal of Agricultural and Food Chemistry*, *55*(23), 9395–9400. https://doi.org/10.1021/jf071523u

Leite, D. F. P., Kassuya, C. A. L., Mazzuco, T. L., Silvestre, A., De Melo, L. V., Rehder, V. L. G., & Calixto, J. B. (2006). The cytotoxic effect and the multidrug resistance reversing action of lignans from Phyllanthus amarus. *Planta Medica*, *72*(15), 1353–1358. https://doi.org/10.1055/s-2006-951708

Lin, X., & Costa, M. (1994). Transformation of human osteoblasts to anchorage-independent growth by insoluble nickel particles. *Environmental Health Perspectives*, *102*(suppl 3), 289-292.

Liu, K., Zhou, R., Wang, B., & Mi, M. T. (2014). Effect of resveratrol on glucose control and insulin sensitivity: A meta-analysis of 11 randomized controlled trials. *American Journal of Clinical Nutrition*, *99*(6), 1510–1519. https://doi.org/10.3945/ajcn.113.082024

Liu, Y., Ma, W., Zhang, P., He, S., & Huang, D. (2015). Effect of resveratrol on blood pressure: A meta-analysis of randomized controlled trials. *Clinical Nutrition*, *34*(1), 27–34. https://doi.org/10.1016/j.clnu.2014.03.009

Lotti, F., & Maggi, M. (2018). Sexual dysfunction and male infertility. *Nature Reviews Urology*, *15*(5), 287–307. https://doi.org/10.1038/nrurol.2018.20

Maciel, M. A. M., Cunha, A. F., & Dantas, T. N. C. (2007). NMR Characterization of Bioactive Lignans from Phyllanthus amarus Schum &. *Annals of Magnetic Resonance*, *6*(3), 76–82.

Mallidis, C., Agbaje, I. M., Rogers, D. A., Glenn, J. V., Pringle, R., Atkinson, A. B., & McClure, N. (2009). Advanced glycation end-products accumulate in the reproductive tract of men with diabetes. *International Journal of Andrology*, *32*(4), 295–305.

https://doi.org/10.1111/j.1365-2605.2007.00849.x

Mima, M., Greenwald, D., & Ohlander, S. (2018). Environmental Toxins and Male Fertility. *Current Urology Report,* **19,** 50. https://doi.org/10.1007/s11934-018-0804-1

Misra, H., & Fridovich, I. (1972). The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. *Journal of Biological Chemistry*, *217*, 3170–3175.

Mohamed, S. I. A., Jantan, I., Nafiah, M. A., & Seyed, M. A. (2018). Dendritic cells pulsed with generated tumour cell lysate from Phyllanthus amarus immune response. *BMC Complementary and Alternative Medicine*, *18*(232), 1–14.

Mohasseb, M., Ebied, S., Yehia, M. A. H., & Hussein, N. (2011). Testicular oxidative damage and the role of combined antioxidant supplementation in experimental diabetic rats. *Journal of Physiology and Biochemistry*, *67*(2), 185–194. https://doi.org/10.1007/s13105-010-0062-2

Monsees, T. K., Franz, M., Gebhardt, S., Winterstein, U., Schill, W. B., & Hayatpour, J. (2000). Sertoli cells as a target for reproductive hazards. *Andrologia*, *32*(4–5), 239–246. https://doi.org/10.1046/j.1439-0272.2000.00391.x

Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *Journal of Immunological Methods*, *65*(1–2), 55–63. https://doi.org/10.1016/0022-1759(83)90303-4

Niedowicz, D. M., & Daleke, D. L. (2005). The role of oxidative stress in diabetic complications. *Cell Biochemistry and Biophysics*, *43*(2), 289–330. https://doi.org/10.1385/CBB:43:2:289

Niwas, J., & Chand, J. (2014). Diabetes mellitus induced impairment of male reproductive functions: a review. *Current Diabetes Reviews*, *10*(3), 147–157.

Ogar, I., Egbung, G. E., Nna, V. U., Atangwho, I. J., & Itam, E. H. (2019). Hyptis verticillata attenuates dyslipidaemia, oxidative stress and hepato-renal damage in streptozotocin-induced diabetic rats. *Life Sciences*, *219*, 283–293. https://doi.org/10.1016/j.lfs.2019.01.027

Olabiyi, F. A., Aboua, Y. G., Popoola, O. K., Monsees, T. K., & Oguntibeju, O. O. (2020). Evaluation of antioxidant, antityrosinase activities and cytotoxic effects of Phyllanthus amarus extracts. *The Natural Products Journal*, *10*(2), 130–138. https://doi.org/10.2174/2210315509666190405100745

Pan, A., Sun, J., Chen, Y., Ye, X., Li, H., Yu, Z., & Lin, X. (2007). Effects of a flaxseed-derived lignan supplement in type 2 diabetic patients: A randomized, double-blind,

cross-over trial. *PLoS ONE*, *2*(11), 1–7. https://doi.org/10.1371/journal.pone.0001148

Parvathaneni, M., Battu, G. R., Gray, A., & Gummalla, P. (2014). Investigation of the anticancer potential of hypophyllanthin and phyllanthin against breast cancer by in vitro and in vivo methods. *Asian Pacific Journal of Tropical Disease*, *4*, 71–76.

Patel, J. R., Tripathi, P., Sharma, V., Chauhan, N. S., & Dixit, V. K. (2011). Phyllanthus amarus: Ethnomedicinal uses, phytochemistry and pharmacology: A review. *Journal of Ethnopharmacology*, *138*(2), 286–313. https://doi.org/10.1016/j.jep.2011.09.040

Patel, T. P., Rawal, K., Bagchi, A. K., Akolkar, G., Bernardes, N., Dias, D. da S., & Singal, P. K. (2016). Insulin resistance: an additional risk factor in the pathogenesis of cardiovascular disease in type 2 diabetes. *Heart Failure Reviews*, *21*(1), 11–23. https://doi.org/10.1007/s10741-015-9515-6

Pointis, G., Gilleron, J., Carette, D., & Segretain, D. (2011). Testicular connexin 43, a precocious molecular target for the effect of environmental toxicants on male fertility. *Spermatogenesis*, *1*(4), 303-317. https://doi.org/10.4161/spmg.1.4.18392

Prohaska, J. R., & Ganther, H. E. (1977). Glutathione peroxidase activity of glutathione-S-transferases purified from rat liver. *Biochemical and Biophysical Research Communications*, *76*(2), 437–445.

Pugliese, A., & Miceli, D. (2002). The insulin gene in type 1 diabetes. *Diabetes/Metabolism Research and Reviews*, *18*(7), 13–25. https://doi.org/10.1080/15216540500163301

Rafacho, A., Ortsäter, H., Nadal, A., & Quesada, I. (2014). Glucocorticoid treatment and endocrine pancreas function: Implications for glucose homeostasis, insulin resistance and diabetes. *Journal of Endocrinology*, *223*(3), R49–R62. https://doi.org/10.1530/JOE-14-0373

Rahimi, R., Nikfar, S., Larijani, B., & Abdollahi, M. (2005). A review of the role of antioxidants in the management of diabetes and its complications. *Biomedicine and Pharmacotherapy*, *59*(7), 365–373. https://doi.org/10.1016/j.biopha.2005.07.002

Rains, J. L., & Jain, S. K. (2011). Oxidative stress, insulin signalling, and diabetes. *Free Radical Biology and Medicine*, *50*(5), 567–575. https://doi.org/10.1016/j.freeradbiomed.2010.12.006

Ricci, E., Cipriani, S., Chiaffarino, F., Malvezzi, M., & Parazzini, F. (2010). Effects of soy isoflavones and genistein on glucose metabolism in perimenopausal and postmenopausal non-Asian women: a meta-analysis of randomized controlled. *Menopause*, *17*(5), 1080–1086.

Rietjens, I. M. C. M., Louisse, J., & Beekmann, K. (2017). The potential health effects of

dietary phytoestrogens. *British Journal of Pharmacology*, *174*(11), 1263–1280. https://doi.org/10.1111/bph.13622

Samson, A. M. (2016). *In vivo antiplasmodial potential of combinations of three Nigerian antimalarial plants*. Ladoke Akintola University of Technology, Ogbomoso. 1-67.

Saxena, A.K., Dhungel, S., Bhattacharya, S., Jha, C.B. & Srivastava, A.K. (2004). Effect of chronic low dose of methotrexate on cellular proliferation during spermatogenesis in rats. *Archives of Andrology*, *50*(1), 33-35.

Serban, M. C., Sahebkar, A., Zanchetti, A., Mikhailidis, D. P., Howard, G., Antal, D., & Banach, M. (2016). Effects of quercetin on blood pressure: A systematic review and meta-analysis of randomized controlled trials. *Journal of the American Heart Association*, *5*(7), 1–15. https://doi.org/10.1161/JAHA.115.002713

Shi, G. J., Li, Z. M., Zheng, J., Chen, J., Han, X. X., Wu, J., & Yu, J. Q. (2017). Diabetes associated with male reproductive system damages: Onset of presentation, pathophysiological mechanisms and drug intervention. *Biomedicine and Pharmacotherapy*, *90*, 562–574. https://doi.org/10.1016/j.biopha.2017.03.074

Sisman, A. R., Kiray, M., Camsari, U. M., Evren, M., Ates, M., Baykara, B., & Uysal, N. (2014). Potential novel biomarkers for diabetic testicular damage in streptozotocin-induced diabetic rats: Nerve growth factor-beta and vascular endothelial growth factor. *Disease Markers*, *2014*, 1–7. https://doi.org/10.1155/2014/108106

Storgaard, L., Bonde, J.P. & Olsen, J., (2006.) Male reproductive disorders in humans and prenatal indicators of estrogen exposure: a review of published epidemiological studies. *Reproductive Toxicology*, *21*(1), 4-15.

Taku, K., Umegaki, K., Sato, Y., Taki, Y., Endoh, K., & Watanabe, S. (2007). Soy isoflavones lower serum total and LDL cholesterol in humans: A meta-analysis of 11 randomized controlled trials. *American Journal of Clinical Nutrition*, *85*(3), 1148–1156.

Talaei, M., & An, P. (2015). Role of phytoestrogens in the prevention and management of type 2 diabetes. *World Journal of Diabetes*, *6*(2), 271–283. https://doi.org/10.4239/wjd.v6.i2.271

Tremellen, K. (2008). Oxidative stress and male infertility: A clinical perspective. In: *Studies on Men's Health and Fertility* (Vol. 14, pp. 243–258). Totowa, NJ: Humana Press. https://doi.org/10.1007/978-1-61779-776-7_16

Van Die, M. D., Bone, K. M., Williams, S. G., & Pirotta, M. V. (2014). Soy and soy isoflavones in prostate cancer: A systematic review and meta-analysis of randomized controlled trials. *BJU International*, *113*(5 B), 119–130. https://doi.org/10.1111/bju.12435

Varshney, R., & Kale, R. K. (1990). Effects of calmodulin antagonists on radiation-induced lipid peroxidation in microsomes. *International Journal of Radiation Biology*, *58*(5), 733–743. https://doi.org/10.1080/09553009014552121

Verma, S., Sharma, H., & Garg, M. (2014). Phyllanthus amarus: A review. *Journal of Pharmacognosy and Phytochemistry*, *3*(2), 18–22.

World Health Organization. (2016). Global Report on Diabetes.1-88. ISBN 978 92 4 156525 7

Xia, Y., & Zweier, J. L. (1997). Measurement of myeloperoxidase in leukocyte-containing tissues. *Analytical Biochemistry*, *245*(1), 93–96. https://doi.org/10.1006/abio.1996.9940

Yokoi, K., Uthus, E. O., & Nielsen, F. H. (2003). Nickel deficiency diminishes sperm quantity and movement in rats. *Biological Trace Element Research*, *93*(1–3), 141–153. https://doi.org/10.1385/BTER:93:1-3:141

Young, J., Wahle, K. W. J., & Boyle, S. P. (2008). Cytoprotective effects of phenolic antioxidants and essential fatty acids in human blood monocyte and neuroblastoma cell lines: Surrogates for neurological damage in vivo. *Prostaglandins, Leukotrienes and Essential Fatty Acids*, *78*(1), 45–59. https://doi.org/10.1016/j.plefa.2007.10.005

Younglai, E. V., Holt, D., Brown, P., Jurisicova, A., & Casper, R. F. (2001). Sperm swim-up techniques and DNA fragmentation. *Human Reproduction*, *16*(9), 1950–1953. https://doi.org/10.1093/humrep/16.9.1950

Zuo, Z., Wu, T., Lin, M., Zhang, S., Yan, F., Yang, Z., & Wang, C. (2014). Chronic exposure to tributyltin chloride induces pancreatic islet cell apoptosis and disrupts glucose homeostasis in male mice. *Environmental Science and Technology*, *48*(9), 5179–5186. https://doi.org/10.1021/es404729p.

CHAPTER SEVEN: General Discussion

7.1. General Discussion

Diabetes mellitus is a complex and heterogenous dysfunction of multiple aetiology affecting carbohydrate, protein and lipid metabolism due to impaired insulin secretion from pancreatic β-cells (Gouri & Dekaken, 2013) or its action on the peripheral tissues (Kahn, 1985; Scheen, 2003; Robertson, 2006; Gezginci-Oktayoglu et al., 2009; American Diabetes Association, 2013; DeFronzo et al., 2015; Bene et al., 2018). Generally, diabetes is a top ten cause of mortality, and the number of Type 2 diabetes mellitus (T2D) patients is increasing in both developed and developing countries; 80% contribution is from low- and middle-income countries, with an estimated global prevalence sitting at 9.3% (International Diabetes Federation, 2019). Although many synthetic drugs have been formulated to treat and manage diabetes mellitus, their high cost, some intolerable side effects and increasing failure rates make them undesirable; hence, the use of alternative or complementary therapy becomes a ready option. Also, the use of traditional medicinal plants to treat various ailments is widely known, but there is still very scanty empirical information about the health benefits of some of these plants. Besides, their mechanism of antidiabetic action, as well as their toxicity and interference with male reproduction needs further investigation.

In the present study, effects of *Phyllanthus amarus*, a herb of the Euphorbiaceous family was thoroughly investigated in both in vitro and in vivo (Ngo et al., 2017) studies. The study whose findings were reported in this thesis is divided into two parts; Part A is an *In-vitro* study that investigated the concentrations of the total polyphenol and individual phytochemicals present in the plant extract, thereby given an insight into its antioxidant capacity. The inhibitory activities of the extract on tyrosinase, α-amylase and α-glucosidase enzymes were examined to determine the mechanism of its depigmentation and antidiabetic actions, respectively. The effect of the extract on male reproductive functions using mouse Sertoli (TM4), as well as the phytoestrogenic activity of the extract on breast cancer (MCF-7) cell lines were investigated using MTT and the E-Screen assay, respectively. To the best of our knowledge, this is the first in vitro study that examined the effect of *Phyllanthus amarus* extracts on these two cell lines, and its inhibitory activities on tyrosinase and α-glucosidase enzymes. Part B, an *In-vivo* study that investigated the effects of a graded dose of 200mg

and 400mg/kgbwt/day of aqueous plant extract and glibenclamide 0.2mg/kgbwt on the kidney, liver, heart and testes of diabetes-induced male Wistar rats.

Type 2 diabetes (T2D) was induced in the rats by letting them have free access to 10% fructose water to consume for two weeks. A single medium dose of streptozotocin (55mg/kgbwt) was then injected intraperitoneally. Rats with fasting blood glucose level ≥11.1 mmol/L were considered diabetic and were continued with respective treatments for four weeks. At the end of the treatment or experimental period, the rats were anaesthetized using i.p sodium pentobarbital, 60mg/kgbwt They were euthanized by cervical dislocation, after which blood samples and organs were harvested, stored until they were used for analysis.

In the *in vitro* study, ELISA-based methods were used for the quantitative determination of polyphenols, flavanol, flavonol, flavonoids, oxygen reducing antioxidant capacity (ORAC), trolox equivalent antioxidant capacity (TEAC), α-amylase and α-glucosidase activities on the aqueous, methanol and hexane extracts of *Phyllanthus amarus*. Besides, tyrosinase inhibitory efficacy of the hexane, methanol, and aqueous extracts of *Phyllanthus amarus* was examined. Our findings revealed that *Phyllanthus amarus* has a higher concentration of polyphenolic acids; these are naturally occurring antioxidants with potentials to scavenge free radicals because of their hydroxyl group (Jalal-Hosseinimehr et al., 2007). Studies showed that they contributed to increased antioxidant activity as demonstrated in some extracts via several mechanisms which they exert by different phenolics or through synergistic effects with other non-phenolic compounds (Abbasi et al., 2010; Kumar et al., 2014; Putakala et al., 2017[a]). In the present study, the aqueous extract shows the highest antioxidant capacity, which is in agreement with the reports by Mazlan et al. (2013). Higher levels of ferric reducing antioxidant power and trolox equivalent antioxidant capacity were observed in the aqueous extract, as opposed to the methanol extract that shows higher oxygen reducing antioxidant capacity results. These results thus confirmed the high antioxidant capacity of the aqueous extract.

Furthermore, scientific evidence demonstrated that samples with high antioxidant activity are associated with significant antityrosinase activity, as they play essential roles in preventing free radical-related skin damage (Ko et al., 2008; Mazlan et al., 2013; Chang & Teo, 2016). In the present study, both aqueous and hexane extract fractions displayed significant tyrosinase inhibitory activity, with the aqueous extract being the most active having the least inhibitory concentration at 50%. This report correlates with the ethnobotanical use of the plant to treat various skin lesions. *In vitro* antidiabetic effect of the aqueous extract using α-glucosidase indicates that *Phyllanthus amarus* possesses a significant inhibitory effect on α-glucosidase, which could modulate oxidative stress caused by diabetes. The mechanism of action exhibited in this study, clearly shows that *Phyllanthus amarus* extract's activity by

inhibition of α-glucosidase could delay the absorption of glucose from the intestinal tract; hence, its antihyperglycemic effect. Besides, the present study corroborates the previous investigators who reported that *Phyllanthus amarus* extracts at various doses, significantly reduced blood glucose levels (Lawson-Evi et al., 2011; Adedapo et al., 2013).

Cytotoxicity studies were done on mouse Sertoli (TM4) and Breast cancer (MCF-7) cell lines with MTT, E-Screen assays and cell counts. Our findings revealed that the aqueous fraction of *Phyllanthus amarus* extracts demonstrated the highest mitochondrial dehydrogenase activity at lower concentrations. In this instance, too, TM4 cell numbers were significantly higher when compared with the untreated controls. Besides, Sertoli cell viability was impaired after exposure to higher extract concentrations which could lead to impaired spermatogenesis. The significant stimulation of Sertoli cell proliferation could be because of the estrogenic properties exhibited by the *Phyllanthus amarus* extracts. Earlier investigators previously pointed out that some of the constituents, such as Phyllanthin and hypophyllanthin, are active phytoestrogens (Islam et al., 2008; Iranloye et al., 2010). It is noteworthy that the TM4 (Sertoli) cells are isolated from immature rats; at this age, Sertoli cells express oestrogen receptors and cell proliferation can be activated by oestrogen exposure (Lucas et al., 2011). More so, a scientific report suggests bioflavonoids, which are natural antioxidants widely distributed in fruits and vegetables as having a protective effect on the deoxyribonucleic acid damage precipitated by the hydroxyl radicals (Russo et al., 2000; Nimse & Pal, 2015). In our opinion, the observations in the present study could be as a result of the antioxidant substances in the extracts that are known to display a dual-phasic property; they are antioxidants at low concentrations, and at high levels, they are pro-oxidants. This property may protect against oxidative stress and lipid peroxidation, such that it promotes cell proliferation, at the lower concentrations, as previous investigators have reported (Catala, 2007; Young et al., 2008). Besides, in the present study, the extract produced a significant proliferative effect on breast cancer (MCF-7) cells, thus, confirming its estrogenic activity.

A cardinal symptom of diabetes is unexplained weight loss (Okon et al., 2012). The in vivo part of this study revealed a persistent body weight loss in the animals, except in the healthy controls up to the day 21 of the experiment. There was a marked difference in the absolute and relative liver weights of diabetic untreated animals versus the healthy controls ($p<0.0001$). The absolute and relative testicular weight of the diabetic untreated rats and the person with diabetes + PA 200mg/kgbwt was markedly significant ($p<0.05$). Although it was earlier reported that there was weight gain in diabetic animals after treatment with *Phyllanthus amarus* extract (Adeneye, 2012), findings from the present study did not, however, indicate restoration in the weight loss by the animals when treated either with *Phyllanthus amarus* or glibenclamide. The reason for this difference could be due to the short

duration of the treatment or the strain of the animals or even the abundant tannin that is present in the extract. It has been shown that organ hypertrophy and hyperplasia occur in disease states to compensate for decreased functionality or due to inflammatory responses (Mestry et al., 2017).

Adeneye (2012) demonstrated that *Phyllanthus amarus* could effectively control glucose level in diabetes mellitus mediated via improvement in insulin resistance, thus supporting its ethnomedicinal use in the local management of diabetes mellitus. In the same vein, our study shows that PA at 400mg/kgbwt increased serum insulin similarly to the glibenclamide when compared to the non-treated diabetic and healthy controls. This finding also agrees with a previous report that *Phyllanthus amarus* at 400mg/kg body weight effectively controlled blood glucose in rats (Adedapo et al., 2014). Scientific research revealed that Inflammation is closely linked with insulin resistance and endothelial dysfunction, which are regarded as important factors in the pathogenesis of T2D (Cersosimo & DeFronzo, 2006; Van Den Oever et al., 2010; Janus et al., 2016; Chen et al., 2016). In the present study, *Phyllanthus amarus* 200mg/kgbwt significantly reduced serum nitric oxide compared to the diabetic control while GLIBEN 0.2mg/kgbwt significantly reduced serum nitric oxide versus the healthy and diabetic controls, PA 200 mg and PA 400mg. PA 200mg/kgbwt, PA 400 mg/kgbwt and GLIBEN 0.2mg/kgbwt significantly reduced serum myeloperoxidase (MPO) activity when versus the diabetic controls. These findings agree with Ren et al. (2016) which shows that both phytoestrogens, apigenin and naringenin can ameliorate glucose and lipid metabolism, as well as endothelial dysfunction in T2D rats at least in part by down-regulating oxidative stress and inflammation(Ren et al., 2016). PA 200mg/kgbwt, as well as PA 400mg/kgbwt, reduced H_2O_2 contents and malondialdehyde (MDA) levels compared to the diabetic controls. This finding also corroborates the antioxidant property of the extract as previously reported (Giribabu et al., 2014). More so, PA 400mg/kgbwt significantly increased glutathione peroxidase (GPx) activity compared to diabetic control. PA 200mg/kgbwt and PA 400mg/kgbwt caused an increase in the activity of GPx in the liver and reduced lipid peroxidation in the rats' liver, kidney, and heart in a similar manner to glibenclamide compared to the diabetic untreated rats. The action of this extract implies that the two doses were able to improve GPX activities which is one of the antioxidant defence mechanisms in the rat's liver, kidney and the heart (Gündüz et al., 2004). They normalised the activities of alkaline phosphatase (ALP) together with aspartate aminotransferase (AST), compared to the diabetic untreated rats. The normalisation suggests the hepatoprotective potential of *Phyllanthus amarus* plant extract (Putakala et al., 2017[a]), thus confirming its ethnobotanical uses. More so, the PA 200mg/kgbwt normalised the activity of gamma-glutamyl aminotransferase (GGT) versus the diabetic non-treated rats. These findings substantiate the fact that *Phyllanthus amarus* extract can protect against oxidative stress in the rat's liver,

kidneys and the heart (Pramyothin et al., 2007; Bhawna & Kumar, 2009; Karuna et al., 2009; Karuna et al., 2011; Putakala et al., 2017[b]). There was significant hypoproteinemia in the experimental groups compared to the normal rats, perhaps due to the diabetes-induced renal impairment in the rats. PA 200mg/kgbwt significantly reduced the triglyceride levels compared to the diabetic non-treated rats. Here, the hypolipidaemic effect of the extract was also demonstrated (Haidari et al., 2012) in agreement with the work of Adeneye (2012).

Phyllanthus amarus aqueous extract, 200 mg/kgbwt improved serum testosterone and serum oestradiol similarly to glibenclamide compared to their diabetic untreated control equivalents. Finally, this report revealed that *Phyllanthus amarus* at 200mg/kgbwt significantly improved sperm count when compared to glibenclamide; thus corroborating a previous study (Azubuike et al., 2018). In our histological studies, *Phyllanthus amarus* at 200mg/kgbwt demonstrated a reduction in seminiferous tubule diameter as well as an expanded interstitium. The dosage administered to this group could not cause the arrest of spermatogenesis that could have otherwise led to spermatogenic failure, disruptions and eventual azoospermia, severe oligozoospermia, asthenozoospermia or teratozoospermia as described by Jan et al. (2012).

7.2. Conclusions

- Aqueous extract of *Phyllanthus amarus* exhibited capacity as an antioxidant, as shown in the FRAP and TEAC results. Both hexane, together with aqueous extract fractions of PA possesses favourable antityrosinase activity, proposing the plant to be a suitable natural, alternative agent in the beauty, healthcare, and food processing industries.
- Aqueous extract of *Phyllanthus amarus* possesses a significant inhibition on α-glucosidase, improves serum insulin levels, as well as the activities of antioxidant enzymes, thereby modulating oxidative stress caused by diabetes.
- The findings in this study substantiated the antihyperglycaemic, antioxidant and lipid-lowering activities of *Phyllanthus amarus* extract, hence, its ethnobotanical use, which makes it a potential natural product for the development of antidiabetic drugs.
- *Phyllanthus amarus* extract could modulate diabetes and exert its antidiabetic effect via oestrogen-dependent pathways and oxidative stress.

7.3. Recommendations

- Isolation and characterisation of the crude extract to get pure compounds.
- Need for less expensive, fast, and efficient in vitro toxicity tests that reduce social pressure around ethical usage of laboratory animals. The exercise could involve

designing studies to include co-treatment of both crude and pure compound from *Phyllanthus amarus* to investigate its antidiabetic activities and ameliorative potentials on oxidative stress, as well as recovery of some of the dysfunctional organs affected due to diabetes.

- A study could be designed with fewer numbers of diabetic-induced animals. Further, administered with *Phyllanthus amarus* extracts for chronic treatment evaluation to see if this extract provides any potential effect on the expression and activity of caspase, glucose transporter and apoptotic proteins in diabetic and normal rats.
- Another experiment could be designed for checking the effect of *Phyllanthus amarus* alone on the parameters to be measured by administering the plant extracts, PA 200mg/kgbwt along with 400mg/kgbwt to normal rats.
- The information revealed in this study gives a unique and innovative insight into the effect of the plant extract on male reproductive function. To better understand the mechanism displayed here, a more extensive molecular mechanistic studies on endocrine, apoptotic and biochemical pathways, including gene expression analysis, is imperative.

References

Abbasi, M.A., Zafar, A., Riaz, T., Aziz-ur-Rehman, Arshad, S., Shahwar, D., Jahangir, M., Siddiqui, S.Z., Shahzadi, T. & Ajaib, M. 2010. Evaluation of the comparative antioxidant potential of aqueous and organic fractions of Ipomoea carnea. *Journal of Medicinal Plants Research*, 4(18): 1883–1887.

Adedapo, A., Ofuegbe, S. & Oguntibeju, O. 2014. The antidiabetic activities of the aqueous leaf extract of Phyllanthus amarus in some laboratory animals. In *Antioxidant-Antidiabetic Agents and Human Health*. 115–137.

Adedapo, A.A., Ofuegbe, S.O. & Adeyemi, A.A. 2013. The anti-diabetic activities of the methanol leaf extract of Phyllanthus amarus in some laboratory animals. *Asian Journal of Medical Sciences*, 4(3): 223–34.

Adeneye, A.A. 2012. The leaf and seed aqueous extract of Phyllanthus amarus improves insulin resistance diabetes in experimental animal studies. *Journal of Ethnopharmacology*, 144(3): 705–711.

American Diabetes Association. 2013. *Diagnosis and classification of diabetes mellitus*. *Diabetes care*, **36** (Supplement 1), S67-S74.

Azubuike, N.C., Okwuosa, C.N., Onwukwe, O.S., Onyemelukwe, A.O., Ikele, I. & Achukwu, P.U. 2018. Effects of Phyllanthus amarus on epididymal sperm characteristics, testosterone levels and histology of reproductive organs of male rats. *Pharmacologyonline*, 3: 57–67.

Bene, J., Hadzsiev, K. & Melegh, B. 2018. Role of carnitine and its derivatives in the development and management of type 2 diabetes. *Nutrition and Diabetes*, 8(8): 1–10. http://dx.doi.org/10.1038/s41387-018-0017-1.

Bhawna, S. & Kumar, S.U. 2009. Hepatoprotective activity of some indigenous plants. *International Journal of PharmTech Research*, 4(1): 1330–1334.

Catala, A. 2007. The ability of melatonin to counteract lipid peroxidation in biological membranes. *Current Molecular Medicine*, 7(7): 638–649.

Cersosimo, E. & DeFronzo, R.A. 2006. Insulin resistance and endothelial dysfunction: The road map to cardiovascular diseases. *Diabetes/Metabolism Research and Reviews*, 22(6): 423–436.

Chang, V.S. & Teo, S.S. 2016. Evaluation of heavy metal, antioxidant and anti-tyrosinase activities of red seaweed (Eucheuma cottonii). *International Food Research Journal*, 23(6): 2370–2374.

Chen, S.J., Duan, Y.G., Haidl, G. & Allam, J.P. 2016. Predomination of IL-17-producing tryptase-positive/chymase-positive mast cells in azoospermic chronic testicular inflammation. *Andrologia*, *48*(6), pp.617-625

DeFronzo, R.A., Ferrannini, E., Groop, L., Henry, R., Herman, W., Holst, J., Hu, F., Kahn, C., Raz, I., Shulman, G. & Simonson, D. 2015. Type 2 diabetes mellitus. *Nature Reviews Disease Primers*, 1(1): 1–22.

Gezginci-Oktayoglu, S., Basaraner, H., Yanardag, R. & Bolkent, S. 2009. The effects of combined treatment of antioxidants on liver injury in STZ diabetic rats. *Digestive Diseases and Sciences*, 54(3): 538–546.

Giribabu, N., Rao, P.V., Kumar, K.P., Muniandy, S., Swapna Rekha, S. & Salleh, N. 2014. Aqueous extract of Phyllanthus niruri leaves displays in vitro antioxidant activity and prevents the elevation of oxidative stress in the kidney of streptozotocin-induced diabetic male rats. *Evidence-Based Complementary and Alternative Medicine*, 2014: 1–10.

Gouti, A. & Dekaken, A., 2013. Epigenetic pathways in type 2 diabetes and its complications. *Medical Publication J*ournal *1*(11), pp.1-5.

Gündüz, F., Şentürk, Ü.K., Kuru, O., Aktekin, B. & Aktekin, M.R., 2004. The effect of one

year's swimming exercise on oxidant stress and antioxidant capacity in aged rats. *Physiological Research*, 53(2): 171–176.

Haidari, F., Shahi, M.M., Zarei, M., Rafiei, H. & Omidian, K. 2012. Effect of green tea extract on body weight, serum glucose and lipid profile in streptozotocin-induced diabetic rats: A dose-response study. *Saudi Medical Journal*, 33(2): 128–133.

International Diabetes Federation. 2019. IDF Diabetes Atlas 9th edition. *Ninth edition 2019*. https://www.idf.org/e-library/epidemiology-research/diabetes-atlas/159-idf-diabetes-atlas-ninth-edition-2019.html. (Accessed: 05/03/2020).

Iranloye, B., Oyeusi, K. & Alada, A. 2010. Effect of aqueous extract of Phyllanthus amarus leaves on implantation and pregnancy in rats. *Nigerian Journal of Physiological Sciences*, 25(1): 63–66.

Islam, A., Naskar, S., Mazumder, U.K., Gupta, M. & Ghosal, S. 2008. Estrogenic properties of Phyllanthin and hypophyllanthin from Phyllanthus amarus against carbofuran induced toxicity in female rats. *Pharmacologyonline*, 3: 1006–1016.

Jalal Hosseinimehr, S., Pourmorad, F., Shahabimajd, N., Shahrbandy, K. & Hosseinzadeh, R. 2007. In vitro Antioxidant Activity of Polygonium hyrcanicum, Centaurea depressa, Sambucus ebulus, Mentha spicata and Phytolacca americana. *Pakistan Journal of Biological Sciences*, 10(4): 637–640.

Jan, S.Z., Hamer, G., Repping, S., de Rooij, D.G., Van Pelt, A.M.M. & Vormer, T.L. 2012. Molecular control of rodent spermatogenesis. *Biochimica et Biophysica Acta*, 1822(12): 1838–1850. http://dx.doi.org/10.1016/j.bbadis.2012.02.008.

Janus, A., Szahidewicz-Krupska, E., Mazur, G. & Doroszko, A. 2016. Insulin resistance and endothelial dysfunction constitute a common therapeutic target in cardiometabolic disorders. *Mediators of Inflammation*, 2016: 1–10.

Kahn, C.R. 1985. The molecular mechanism of insulin action. *Annual Review of Medicine*, 36(1): 429–451.

Karuna, R., Bharathi, V.G., Reddy, S.S., Ramesh, B. & Saralakumari, D. 2011. Protective effects of Phyllanthus amarus aqueous extract against renal oxidative stress in Streptozotocin-induced diabetic rats. *Indian Journal of Pharmacology*, 43(4): 414–418.

Karuna, R., Reddy, S., Baskar, R. & Saralakumari, D. 2009. Antioxidant potential of aqueous extract of Phyllanthus amarus in rats. *Indian Journal of Pharmacology*, 41(2): 64.

Ko, H.-H., Chang, W.-L. & Lu, T.-M. 2008. Antityrosinase and antioxidant effects of ent -Kaurane diterpenes from leaves of Broussonetia papyrifera. *Journal of Natural Products*, 71(11): 1930–1933.

Kumar, H., Choudhary, N., Kumar, N. & Seth, R. 2014. Phenolic compounds and their health benefits : A review. *Journal of Food Research and Technology*, 2(2): 46–59.

Lawson-Evi, P., Eklu-Gadeg, K., Agbonon, A., Aklikokou, K., Creppy, E. & Gbeassor, M. 2011. Antidiabetic activity of Phyllanthus amarus schum and Thonn (Euphorbiaceae) on alloxan-induced diabetes in male Wistar rats. *Journal of Applied Sciences*, 11(16): 2968–2973.

Lucas, T.F.G., Pimenta, M.T., Pisolato, R., Lazari, M.F.M. & Porto, C.S. 2011. 17β-estradiol signaling and regulation of Sertoli cell function. *Spermatogenesis*, 1(4): 318–324.

Mazlan, N.A., Mediani, A., Abas, F., Ahmad, S., Shaari, K., Khamis, S. & Lajis, N.H. 2013. Antioxidant, antityrosinase, anticholinesterase, and nitric oxide inhibition activities of three Malaysian Macaranga species. *The Scientific World Journal*, 2013: 1–8.

Mestry, S.N., Dhodi, J.B., Kumbhar, S.B. & Juvekar, A.R. 2017. Attenuation of diabetic nephropathy in streptozotocin-induced diabetic rats by Punica granatum Linn. leaves extract. *Journal of Traditional and Complementary Medicine*, 7(3): 273–280.

Nimse, S.B. & Pal, D. 2015. Free radicals, natural antioxidants, and their reaction mechanisms. *Royal Society of Chemistry Advances*, 5(35): 27986–28006.

Ngo, H.T., Hwang, E., Seo, S.A., Park, B., Sun, Z.W., Zhang, M., Shin, Y.K. & Yi, T.H. 2017. Topical application of neem leaves prevents wrinkles formation in UVB-exposed hairless mice. *Journal of Photochemistry and Photobiology B: Biology*, *169*, pp.161-170

Okon, U., Owo, D.U., Udokang, N.E., Udobang, J.A. & Ekpenyong, C.E. 2012. Oral administration of aqueous leaf extract of Ocimum gratissimum ameliorates polyphagia, polydipsia and weight loss in streptozotocin-induced diabetic rats. *American Journal of Medicine and Medical Sciences*, 2(3): 45–49.

Pramyothin, P., Ngamtin, C., Poungshompoo, S. & Chaichantipyuth, C. 2007. Hepatoprotective activity of Phyllanthus amarus Schum. et. Thonn. extract in ethanol-treated rats: In vitro and in vivo studies. *Journal of Ethnopharmacology*, 114(2): 169–173.

Putakala, Mallaiah., Gujjala, S., Nukala, S., Bongu, S.B.R., Chintakunta, N. & Desireddy, S. 2017[b]. Cardioprotective effect of Phyllanthus amarus against high fructose diet-induced myocardial and aortic stress in a rat model. *Biomedicine and Pharmacotherapy*, 95: 1359–1368.

Putakala, Mallaiah, Gujjala, S., Nukala, S. & Desireddy, S. 2017[a]. Beneficial effects of Phyllanthus amarus against high fructose diet-induced insulin resistance and hepatic oxidative stress in male Wistar rats. *Applied Biochemistry and Biotechnology*, 183(3):

744–764.

Ren, B., Qin, W., Wu, F., Wang, S., Pan, C., Wang, L., Zeng, B., Ma, S. & Liang, J. 2016. Apigenin and naringenin regulate glucose and lipid metabolism and ameliorate vascular dysfunction in type 2 diabetic rats. *European Journal of Pharmacology*, 773: 13–23.

Robertson, R.P. 2006. Oxidative stress and impaired insulin secretion in type 2 diabetes. *Current Opinion in Pharmacology*, 6(6): 615–619.

Russo, A., Acquaviva, R., Campisi, A., Sorrenti, V., Di Giacomo, C., Virgata, G., Barcellona, M.L. & Vanella, A. 2000. Bioflavonoids as antiradicals, antioxidants and DNA cleavage protectors. *Cell Biology and Toxicology*, 16(2): 91–98.

Scheen, A.J. 2003. Pathophysiology of type 2 diabetes. *Acta Clinica Belgica*, 58(6): 335–341.

Van Den Oever, I.A.M., Raterman, H.G., Nurmohamed, M.T. & Simsek, S. 2010. Endothelial dysfunction, inflammation, and apoptosis in diabetes mellitus. *Mediators of Inflammation*, 2010: 1–15.

Young, J., Wahle, K.W.J. & Boyle, S.P. 2008. Cytoprotective effects of phenolic antioxidants and essential fatty acids in human blood monocyte and neuroblastoma cell lines: Surrogates for neurological damage in vivo. *Prostaglandins, Leukotrienes and Essential Fatty Acids*, 78(1): 45–59.

ADDENDUM

RESEARCH OUTPUT EMANATED FROM THE STUDY

PUBLISHED MANUSCRIPTS, BOOK CHAPTERS, AND CONFERENCE PAPERS

1. Folorunso Adewale Olabiyi, Yapo Guillaume Aboua, Olugbenga Kayode Popoola, Thomas Klaus Monsees, Oluwafemi Omoniyi Oguntibeju. Evaluation of Antioxidant, Antityrosinase Activities and Cytotoxic effects of *Phyllanthus amarus* Extracts. *The Natural Products Journal*. 2020, 10(2), 130-138. https://doi.org/10.2174/2210315509666190405100745
2. Folorunso Adewale Olabiyi, Yapo Guillaume Aboua, Oluwafemi Omoniyi Oguntibeju. Therapeutic potentials of selected medicinal plants for the management of diabetes mellitus: A Review. In: Innovation in Plant Science for Better Health: From soil to fork. Megh R. Goyah & Ademola O. Ayeleso (Eds). *Bioactive compounds of Medicinal Plants: Properties and Potentials for Human Health.* Apple Academic Press, Inc. USA. 2019, Pp 187-218.
3. Olabiyi FA, Aboua YG, Monsees TK, Oguntibeju OO. Antioxidative, Skin Depigmentation Potentials and Cytotoxicity studies on *Phyllanthus amarus* extracts. Being paper presented at the 6th U6 Consortium International Conference, Research, Innovation and Technology for African Development and published in the abstract book. 4-6 September 2018.
4. Olabiyi FA, Aboua YG, Popoola OK, Oguntibeju OO. Evaluation of Antioxidant and Antityrosinase activities of *Phyllanthus amarus* extracts. Being paper presented at the

23rd International Congress of Clinical Chemistry and Laboratory Medicine (IFCC WORLDLAB, DURBAN, 2017) and published in the abstract book 22-25 October 2017, Durban, KwaZulu-Natal, South Africa.

5. Olabiyi, FA, AA Oyagbemi, TO Omobowale, YG Aboua, AA Adedapo, OO Oguntibeju. *Phyllanthus amarus* inhibits the activity of α-glucosidase and modulates glycaemic indices and pancreatic oxidative stress in streptozotocin-induced diabetic male Wistar rats. Being paper presented at the 4th International Conference of the Society for Medicinal Plants and Economic Developments (SOMPED) and published in the abstract book 26-29 August 2019 held at the National Open University of Nigeria (Headquarters), Abuja, Nigeria.

Prepared Manuscripts for Publication

1. **FA Olabiyi, AA Oyagbemi, M Tijani, OT Omobowale, YG Aboua, AA Adedapo, OO Oguntibeju (2020).** *Phyllanthus amarus* inhibits the activity of α-glucosidase and modulates glycaemic indices, and pancreatic oxidative stressed streptozotocin-diabetic male Wistar rats (LIFE SCIENCES).
2. **FA Olabiyi, AA Oyagbemi, M Tijani, YG Aboua, TK Monsees, AA Adedapo, OO** Oguntibeju **(2020*).*** *Phyllanthus amarus* ameliorates sperm abnormality, and testicular oxidative stressed diabetic rats *via* phytoestrogenic as well as radical-scavenging actions (ANDROLOGIA).
3. **FA Olabiyi, AA Oyagbemi, YG Aboua, AA Adedapo, OO Oguntibeju (2020).** *Phyllanthus amarus* attenuates hepatic damage and associated biochemical derangements in a diabetic animal model (BIOMEDICINES).

SUPPLEMENTARY FILE

Mean and Standard Deviation of Diameter of Seminiferous Tubules (mm)

Group	Mean	N	Std. Deviation
NC	.3003	100	.05394
DC	.2484	100	.03145
PA 200 mg	.2549	100	.02167
PA 400 mg	.1811	100	.03071
Glibenclamide	.3253	100	.04184

Mean and Standard Deviation of Height of seminiferous Tubular Epithelium (mm)

Group	Mean	N	Std. Deviation
NC	.1029	100	.03095

NC	.0682	100	.01452
PA 200Mg	.0779	100	.00988
PA 400 Mg	.0508	100	.01228
Glibenclamide	.1156	100	.02380
			.

ANOVA

Diameter of Seminiferous Tubules

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.225	4	.306	216.879	.000
Within Groups	.699	495	.001		
Total	1.925	499			

ANOVA

Height of seminiferous Tubular Epithelium

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.274	4	.069	172.690	.000
Within Groups	.196	495	.000		
Total	.470	499			

One-way ANOVA to compare the means between the different groups at 95% confidence interval and significance level of $p < 0.05$ was done. From the result, the changes observed in the means of the diameters of seminiferous tubules and height of seminiferous tubular epithelium between the groups was significant as P in both cases was 0.000 (encircled) which is far less than 0.05.

DATASET:

(**Cite this dataset**)
Olabiyi, Folorunso (2021), “OLABIYI_FOLORUNSO ADEWALE_213065401_DATASET”,

https://data.mendeley.com/drafts/w6yw9cwdry