Antidiabetic and Antioxidant effect of *Phyllantus amarus* in TYPE 2 DIABETES in *Drosophilia melanogaster*

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ABSTRACT

Diabetes is a chronic metabolic disorder defined by increased levels of circulating blood sugar (hyperglycemia) caused by abnormal insulin secretion and/or signaling. Diabetes mellitus is divided into type 1 and type 2, a division that reflects the cause of the metabolic Dysfunction. There is increasing evidence that complications related to diabetes are associated with oxidative stress, induced by the generation of free radicals. The plant, *Phyllanthus amarus* have antidiabetic and antioxidant properties. The fruit fly, *Drosophila melanogaster* is a highly suitable system to model type 2 diabetes because mechanisms of glucose homeostasis are conserved between flies and humans, and it allows for substantial ease of experimental and genetic manipulation in comparison to rodent models. This study was done to find out the antidiabetic and antioxidant effect of P. Amarus in Type 2 diabetic *D.melanogaster* flies. Both genders of *D.melanogaster* flies (Harwich strain) of 1-3 days old were divided into four groups with each group containing 50 flies. Group 1 served as control and the flies were treated with basal diet. Group 2 flies were fed with 30% high sucrose diet. Group 3 flies were fed with 30% high sucrose diet and 40Mm *P. amarus.* Group 4 flies were fed with 30% high sucrose diet and 40mM silymarin. The flies were monitored under a natural photoperiod of about 12 hours light and 12 hours dark daily for a period of 21 days and was replicated 5 times. The flies were monitored daily and the survival was done. At the end of the

survival study, the flies were anesthetized, weighed, homogenized and centrifuged and the supernatants were used for the various biochemical analysis. The biochemical parameters include Protein, Catalase (CAT), Nitric Oxide (N.O); glutathione-s-transferase (gst), hydrogen peroxide (h2o2), glutathione (gsh) and glucose. The results were presented as mean ± standard deviation. The results showed that there was decrease in percentage survival rate in flies treated with 30% High Sucrose diet and phyllantus amarus and increase in percentage survival rate in flies treated with 30% High Sucrose diet and P.Amarus. There was a significant increase in glucose in high sucrose diet fed flies compared to control. There was a significant reduction in glucose in flies fed with 30% HSD and p.amarus when compared with the HSD fed flies ($p<0.05$). There is a significant increase ($p<0.05$) in H_2O_2 levels in flies fed with high sucrose diet when compared with the control. There was a significant improvement in catalase acivity in flies treated with both P.Amarus and high sucrose diet. There is a significant increase ($p>0.05$) in GSH in flies fed with 30% HSD and P.amarus compared with the HSD fed flies. There is a significant increase ($p>0.05$) in GST in flies fed with 30% HSD and silymarin compared with the HSD fed flies. P.amarus was able to reduce nitric oxide and its inflammatory activity. Conclusively, *P. amarus* exerts potent antidiabetic effect on diabetic *D.melanogaster* flies (Harwich strain) and there is

increased activity of ROS in High sucrose diet fruit flies and as the ROS activity increases the antioxidant enzymes activity reduces.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Diabetes is a chronic metabolic disorder defined by increased levels of circulating blood sugar (hyperglycemia) caused by abnormal insulin secretion and/or signaling (American Diabetes Association, 2014). There are a wide variety of risk factors that can fast track the development of Diabetes mellitus (DM), such as sedentary lifestyle, physical inactivity, smoking, alcohol consumption, Obstructive sleep apnea, genetic factors amongst others (Wu *et al.,* 2004).

Diabetes and related metabolic disorders are growing health problems worldwide. The WHO estimated a 4.3% prevalence of diabetes in Nigeria in 2016 and a previous study reported that about 4.7 million Nigerians had type 2 diabetes. Meanwhile in Nigeria, the current prevalence of DM among adults aged 20–69 years is reported to be 1.7% (Uloko *et al.,* 2012). Incidence has been increasing for both adults and children, with differential impact on different ethnic groups.

Diabetes is associated with a high risk of death and a lower life expectancy (Keqin *et al*., 1998). The number of adults with diabetes more than doubled in the last

three decades (Danaei, 2011). Its complications are triggered by several disturbances in homeostasis, including vascular complications that are associated with impairments of angiogenesis (Callaghan *et al.,* 2005) and poor wound healing (Gupta *et al.,* 2015), which can sometimes lead to limb amputations (Beckman, 2013).

Diabetes mellitus is divided into type 1 and type 2, a division that reflects the cause of the metabolic Dysfunction. Type I diabetes (T1D), accounts for 5–10% of all diabetes cases. Type 1 diabetes (T1D) is characterized by an immuno-mediated progressive destruction of the pancreatic b cells (Kelly et al. 2003). These diabetics represent around 10% of all diabetic patients, and in most cases, their condition is due to the death of pancreatic Langerhans islets ß-type cells, which normally secrete insulin to clear elevated glucose levels from the bloodstream, like after a meal (Kahn, 2000).,

Type 2 diabetes (T2D), originally named adult-onset diabetes, now affects both children and adults and has a more complex etiology than type 1 diabetes. It is characterized by a combination of insulin resistance and insulin secretion defects, resulting in relative insulin deficiency and hyperglycemia (Kahn, 2000). According to research outputs, the incidence of type 2 diabetes in Nigeria may be gradually increasing, although these may not be evenly distributed across the country as most studies (79%) originated form the Southern parts of the country (WHO, 2006).

Long-term complications of diabetes include neuropathy, retinopathy, heart disease, and stroke. Strong evidence points out hyperglycemia as a potent inducer of the damage or induction of oxidative stress (Hunt and Wolff, 1991).

Oxidative stress has been considered as a central mediator in the progression of diabetic complication. There is increasing evidence that complications (macro and microvascular) related to diabetes are associated with oxidative stress, induced by the generation of free radicals (Jakus, 2000). The increased ROS through pathways like protein kinase C (PKC) pathway and hexosamine pathway leads to β-cell dysfunction and insulin resistance, responsible for cell damage and death (Panigraphy *et al.,* 2016).

Reactive oxygen species (ROS) is the term collectively used to describe free radicals and other non-radical reactive derivatives known as oxidants. Biological free radicals are highly unstable molecules which are products of normal cellular metabolism (Phaniendra *et al.,* 2015). Whenever reactive oxygen species (ROS) are produced in excess of the endogenous antioxidant capacity to mop them, oxidative stress becomes inevitable. Free radicals are well recognized for playing a dual role as both deleterious and beneficial species, since they can be either harmful or beneficial to living systems (Valko *et al.,* 2007). ROS include superoxide anion ($O_2^{\cdot -}$), hydroxyl (·OH), hydrogenperoxide (H_2O_2) and hypochlorous acid (HOCl) while RNS include nitric oxide (·NO), nitrogen dioxide

($NO_2^{\cdot -}$) and peroxynitrite ($OONO^-$) (Halliwell, 2001). High concentrations of free radicals on the other hand result in deleterious processes that can damage cell structures due to oxidative stress. However, compounds with both antihyperglycemic and antioxidative properties would be useful agents. Renewed attention in recent decades to alternative medicines and natural therapies has stimulated a new way of research interest in traditional practices. The plant kingdom has become a target for the search for new drugs and biologically active compounds. Many plant extracts and plant products have been shown to possess significant antioxidant and antidiabetic activity, which may be an important property of medicinal plants (Hasani *et al.,* 2007). As it produces the significant amount of antioxidants to prevent the oxidative stress caused by photons and oxygen, they are considered as potential source of new compounds with antioxidant activity (Ali *et al.,* 2008). Among this is *Phyllanthus amarus.*

Phyllanthus amarus commonly called stone-breaker and windbreaker (Bharatiya, 1992). Belongs to the family Euphorbiaceae (the spurge family) of which the largest genus is the genus Euphorbia (Bagchi *et al.,* 1992). It was reported to have antidiabetic (Adeneye *et al*., 2006), antihypertensive, analgesic, antinociceptive, hepatoprotective, anti-plasmodial (Tran *et al.,* 2003) antiviral (Notka *et al.,* 2004) anti-bacterial (Mazumder *et al.,* 2006) and anti-diarrheal (Odetola and Akojenu, 2000) properties. The generation and subsequent

involvement of free radicals in a large number of diseases prompted the study of the antioxidant potential of *Phyllanthus amarus* extracts.

The degree to which any animal can serve as a model for human disease depends upon the degree of similarity among pathways and physiological responses.

The fruit fly, *Drosophila melanogaster* is a versatile model organism that has been used in biomedical research for over a century to study a broad range of phenomena. Drosophila melanogaster is a small, common fly found near unripe and rotted fruit. It has been established as a cornerstone for research into a wide array of subjects including diseases, development, physiology, and genetics. Their small size (2–3 mm), short generation time, the easy and inexpensive way to culture them in the laboratory, and their powerful genetic tools have established Drosophila as one of the leading animal models for education and biomedical research (Millburn *et al.,* 2016). In addition, about 75% of the genes responsible for human diseases have a homolog in flies (Pandey and Nichols, 2011). The fruit fly allows for substantial ease of experimental and genetic manipulation in comparison to rodent mode (Beckingham *et al.,* 2005). There are few or no study that has been done on the antidiabetic effect of *Phyllantus amarus* in *Drosophilia melanogaster* hence this present study was undertaken to find out the antidiabetic and antioxidant effect of P. Amarus in Type 2 diabetic *Drosophilia melanogaster* flies.

1.2 AIM

This study is aimed at finding out the the antidiabetic and antioxidant effect of P. Amarus in type 2 diabetic *Drosophilia melanogaster* flies.

1.3 JUSTIFICATION OF STUDY

Diabetes mellitus substantially leads to the generation of reactive oxygen species (ROS) triggering oxidative stress. Medicinal plants house a cocktail of phytochemicals) with vast medicinal benefits (Sandberg and Corrigan, 2001). *Phyllantus amarus* can be exploited as a viable, cheap and efficient alternative to conventional drugs currently used in the management of type 2 diabetes as *Phyllantus amarus* possess antidiabetic properties (Akah *et al.,* 2002). *Drosophila melanogaster* is a highly suitable system to model type 2 diabetes because mechanisms of glucose homeostasis are conserved between flies and humans, and the fruit fly allows for substantial ease of experimental and genetic manipulation in comparison to rodent models (Giacomotto and Segalat, 2010).

1.4 SPECIFIC OBJECTIVES

The specific objectives are as follows:

1. To determine the effect of High Sucose Diet in *Drosophila melanogaster* flies?
2. To determine the antidiabetic and antioxidant effect of *phyllantus amarus* in type 2 diabetes *Drosophila melanogaster*.

3. To determine the effect of High Sucose Diet on the survival rate and developmental time course of *Drosophila melanogaster*.

1.5. RESEARCH QUESTIONS

1. Will High sucrose diet induce type 2 diabetes in *Drosophila melanogaster*?
2. Will High sucrose diet disrupt the antioxidant defense system in *Drosophila melanogaster*?
3 Will *phyllantus amarus* have a hypoglycemic effect in Type 2 diabetic flies?
4 Will *phyllantus amarus* restore the antioxidant defense enzymes in Type 2 diabetic *Drosophila melanogaster* flies?

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Diabetes Mellitus

Diabetes mellitus is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both. The chronic hyperglycemia of diabetes is associated with long-term damage, dysfunction, and failure of various organs, especially the eyes, kidneys, nerves, heart, and blood vessels.

Diabetes mellitus (DM) is probably one of the oldest diseases known to man. It was first reported in Egyptian manuscript about 3000 years ago (Ahmed, 2002).

Several pathogenic processes are involved in the development of diabetes. These range from autoimmune destruction of the β-cells of the pancreas with consequent insulin deficiency to abnormalities that result in resistance to insulin action. The basis of the abnormalities in carbohydrate, fat, and protein metabolism in diabetes

is deficient action of insulin on target tissues. Deficient insulin action results from inadequate insulin secretion and/or diminished tissue responses to insulin at one or more points in the complex pathways of hormone action. Impairment of insulin secretion and defects in insulin action frequently coexist in the same patient, and it is often unclear which abnormality, if either alone, is the primary cause of the hyperglycemia.

Symptoms of marked hyperglycemia include polyuria, polydipsia, weight loss, sometimes with polyphagia, and blurred vision. Impairment of growth and susceptibility to certain infections may also accompany chronic hyperglycemia. Acute, life-threatening consequences of uncontrolled diabetes are hyperglycemia with ketoacidosis or the nonketotic hyperosmolar syndrome.

Long-term complications of diabetes include retinopathy with potential loss of vision; nephropathy leading to renal failure; peripheral neuropathy with risk of foot ulcers, amputations, and Charcot joints; and autonomic neuropathy causing gastrointestinal, genitourinary, and cardiovascular symptoms and sexual dysfunction. Patients with diabetes have an increased incidence of atherosclerotic cardiovascular, peripheral arterial, and cerebrovascular disease. Hypertension and abnormalities of lipoprotein metabolism are often found in people with diabetes.

Assigning a type of diabetes to an individual often depends on the circumstances present at the time of diagnosis, and many diabetic individuals do not easily fit into a single class.

TYPE 1 DIABETES

Type 1 diabetes (T1D) is characterized by an immuno-mediated progressive destruction of the pancreatic b cells (Kelly et al. 2003). The majority of type 1 diabetes is of an immune-mediated nature, in which a T cell-mediated autoimmune attack leads to the loss of beta cells and thus insulin (Rother, 2007).

Immune-mediated diabetes.

This form of diabetes, which accounts for only 5–10% of those with diabetes, previously encompassed by the terms insulin-dependent diabetes, type I diabetes, or juvenile-onset diabetes, results from a cellular-mediated autoimmune destruction of the β-cells of the pancreas. Markers of the immune destruction of the β-cell include islet cell autoantibodies, autoantibodies to insulin, autoantibodies to glutamic acid decarboxylase (GAD_{65}), and autoantibodies to the tyrosine

phosphatases IA-2 and IA-2β. One and usually more of these autoantibodies are present in 85–90% of individuals when fasting hyperglycemia is initially detected. Some patients, particularly children and adolescents, may present with ketoacidosis as the first manifestation of the disease. Others have modest fasting hyperglycemia that can rapidly change to severe hyperglycemia and/or ketoacidosis in the presence of infection or other stress. Still others, particularly adults, may retain residual β-cell function sufficient to prevent ketoacidosis for many years; such individuals eventually become dependent on insulin for survival and are at risk for ketoacidosis. At this latter stage of the disease, there is little or no insulin secretion, as manifested by low or undetectable levels of plasma C-peptide.

Idiopathic diabetes.

Some forms of type 1 diabetes have no known etiologies. Some of these patients have permanent insulinopenia and are prone to ketoacidosis, but have no evidence of autoimmunity. Although only a minority of patients with type 1 diabetes fall into this category, of those who do, most are of African or Asian ancestry. Individuals with this form of diabetes suffer from episodic ketoacidosis and exhibit varying degrees of insulin deficiency between episodes. This form of diabetes is strongly inherited, lacks immunological evidence for β-cell autoimmunity, and is not HLA associated.

TYPE 2 DIABETES

Type 2 Diabetes Mellitus (formerly known as non-insulin dependent DM) is the most common form of Diabetes Mellitus characterized by hyperglycemia, insulin resistance, and relative insulin deficiency (Maitras and Abbas, 2005).

Type 2 DM is characterized by insulin insensitivity as a result of insulin resistance, declining insulin production, and eventual pancreatic beta-cell failure (Robertson, 1995). This leads to a decrease in glucose transport into the liver, muscle cells, and fat cells. There is an increase in the breakdown of fat with hyperglycemia. The involvement of impaired alpha-cell function has recently been recognized in the pathophysiology of type 2 DM (Fujioka, 2007). As a result of this dysfunction, glucagon and hepatic glucose levels that rise during fasting are not suppressed with a meal. Type 2 DM results from interaction between genetic, environmental and behavioral risk factors (Chen *et al.,* 2011). It is important to point out that this form of diabetes frequently goes undiagnosed for many years because the hyperglycemia develops gradually and at earlier stages is often not severe enough for the patient to notice any of the classic symptoms of diabetes. Nevertheless, such patients are at increased risk of developing macrovascular and microvascular complications. There is a strong inheritable genetic connection in type 2 Diabetes Mellitus, having relatives (especially first degree) with type 2 Diabetes Mellitus

increases the risks of developing type 2 Diabetes Mellitus substantially. A number of lifestyle factors are known to be important to the development of type 2 Diabetes Mellitus. These are physical inactivity, sedentary lifestyle, cigarette smoking and generous consumption of alcohol (Hu *et al.,* 2001). Obesity has been found to contribute to approximately 55% of cases of type 2 Diabetes Mellitus (Centers for Disease Control and Prevention, 2004).

There are many medical conditions which can potentially give rise to, or exacerbate type 2 Diabetes Mellitus. These include obesity, hypertension, elevated cholesterol (combined hyperlipidemia), and with the condition often termed metabolic syndrome (it is also known as Syndrome X, Reaven's syndrome) (Alberti *et al.,* 2005). Other causes include acromegaly, Cushing's syndrome, thyrotoxicosis, pheochromocytoma, chronic pancreatitis, cancer, and drugs. Additional factors found to increase the risk of type 2 Diabetes Mellitus include aging (Jack *et al.,* 2004) high-fat diets, and a less active lifestyle (Lovejoy, 2002).

In 1936, the distinction between type 1 and type 2 Diabetes Mellitus was clearly made. Type 2 DM was first described as a component of metabolic syndrome in 1988 (Patlak, 2002). People living with type 2 Diabetes Mellitus are more vulnerable to various forms of both short- and long-term complications, which often lead to their premature death. This tendency of increased morbidity and

mortality is seen in patients with type 2 Diabetes Mellitus because of the commonness of this type of Diabetes Mellitus, its insidious onset and late recognition. Most patients with this form of diabetes are obese, and obesity itself causes some degree of insulin resistance. Patients who are not obese by traditional weight criteria may have an increased percentage of body fat distributed predominantly in the abdominal region.. The increased rate of childhood obesity between the 1960s and 2000s is believed to have led to the increase in type 2 Diabetes Mellitus in children and adolescents (Barlow, 2007). Environmental toxins may contribute to the recent increases in the rate of type 2 Diabetes Mellitus. Ketoacidosis seldom occurs spontaneously in this type of diabetes; when seen, it usually arises in Association with the Stress of another Illness Such As Infection.

Through lifestyle and diet modification. Studies have shown that there was significant reduction in the incidence of type 2 DM with a combination of maintenance of body mass index of 25 kg/m^2, eating high fibre and unsaturated fat and diet low in saturated and trans-fats and glycemic index, regular exercise, abstinence from smoking and moderate consumption of alcohol (Willi *et al.,* 2007). Suggesting that majority of type 2 DM can be prevented by lifestyle modification. Patients with type 2 DM should receive a medical nutrition evaluation; lifestyle recommendations should be tailored according to physical and functional ability (Chiniwala and Jabbour, 2011).

2.2 Reactive Oxygen Species

Oxygen has a unique molecular structure and is abundant within cells. It readily accepts free electrons generated by normal oxidative metabolism within the cell, producing Reactive oxygen species.

Reactive oxygen species (ROS) are defined as chemically reactive oxygen radicals as well as non-radical derivatives of oxygen (Halliwell, 2006). The production of oxygen based radicals is the bane to all aerobic species. These molecules, produced as byproducts during the mitochondrial electron transport of aerobic respiration or by oxidoreductase enzymes and metal catalyzed oxidation, have the potential to cause a number of deleterious events.

Processes causing uncoupling of electron transport can enhance the production of ROS, with mitochondria being a major source (Gruber *et al*., 2008). However, other cellular components, such as endoplasmic reticulum-bound enzymes, cytoplasmic enzyme systems, and the surface of the plasma membrane, also contribute (Sumimoto, 2008). The varying range of reactivity each reactive oxygen

species exhibits is crucial to its impact at the molecular level. Recent work has demonstrated that ROS have a role in cell signaling, including; apoptosis; gene expression; and the activation of cell signaling cascades (Hancock, 2001). It should be noted that ROS can serve as both intra- and intercellular messengers. Developing a balance between the overproduction of ROS and its utilization is important in maintaining healthy redox processes within the cells.

Most reactive oxygen species are generated as by-products during mitochondrial electron transport. In addition ROS are formed as necessary intermediates of metal catalyzed oxidation reactions. Atomic oxygen has two unpaired electrons in separate orbits in its outer electron shell. This electron structure makes oxygen susceptible to radical formation. The sequential reduction of oxygen through the addition of electrons leads to the formation of a number of ROS including: superoxide; hydrogen peroxide; hydroxyl radical; hydroxyl ion; and nitric oxide.

- Superoxide (O^{-}_{2}) is generated by the reduction of molecular oxygen in the electron transport chain of mitochondria, and other enzymatic routes: monooxygenase, NADPH oxidase, xanthine oxidase (Starkov, 2004).
- Hydrogen peroxide (H_2O_2) is Converted from O^{-}_{2} by enzyme superoxide dismutase (SOD) (Pacher *et al.,* 2007).
- Hydroxyl radical ($^{\bullet}OH$) is Produced in Haber-Weiss reaction from O^{-}_{2} and H_2O_2 (Fukai and Ushio-Fukai, 2011).

- Singlet oxygen ($^1O_2$) is Produced in reaction of hypochlorous acid (HOCl) and H_2O_2 (Haber and Weiss, 1934).
- Peroxynitrite (ONOO−) is Produced in reaction of nitric oxide (NO) and (O−2) (Winterbourn *et al.,* 1985).

Superoxide is produced by the one-electron reduction of molecular oxygen. Superoxide is then converted to hydrogen peroxide via the mitochondrial enzyme superoxide dismutase (MnSOD), or into diatomic oxygen (Fukai and Ushio-Fukai, 2011). Hydrogen peroxide itself is fairly unreactive, but plays a role in the Fenton reaction to generate hydroxyl radicals that can be damaging to cellular structures and molecules. Haber and Weiss demonstrated in 1934 that a superoxide molecule and a hydrogen peroxide molecule could interact with each other to produce these reactive hydroxyl radicals in the following net reaction:

$$\bullet O2^- + H_2O_2 \rightarrow {}^\bullet OH + OH^- + O_2$$

Hydroxyl radicals can also be formed from reactions with hypochlorous acid, which is produced by the enzyme myeloperoxidase (Arnhold, 2004). The production of hydroxyl radicals in tissue is significant for its contribution to a variety of pathologies, but also for the fact that it cannot be removed enzymatically in the same way that superoxide is converted back to oxygen via SOD.

There are many enzymatic pathways by which ROS can be generated in the cell. Most pathways involve the initial production of superoxide, which, as previously indicated, lead to the production of even more reactive compounds. Although some enzymatic systems "intentionally" generate superoxide, e.g., NADPH oxidases, this ROS is also a consequence of metabolism. Specifically, in aerobic cellular respiration, superoxide is a byproduct of oxygen utilization. The electron transport chain (ETC) of the mitochondria is a major source its generation.

The ETC is made up of three complexes and an ATP synthase enzyme; it functions by transferring electrons through a series of electron carriers. The transfer of electrons is coupled with the release of protons into the intermembrane space of the mitochondria, creating an electrochemical potential, Δp, across the inner membrane, which drives the production of ATP (Liu *et al.,* 2002). However, when electrons are leaked from the complexes, instead of being transferred, these leaked species are those that reduce oxygen to form superoxide.

The first complex in the ETC is composed of a flavin mononucleotide group and is a significant site of production of superoxides. The donation of electrons is initially provided to the chain by NADH to the FMN, which subsequently passes them along a chain of FeS to the reduction site CoQ (Murphy, 2009). Superoxide is produced when FMN is fully reduced; its degree of reduction has been shown to be dependent on the ratio of NADH/NAD+, with the proportion of FMNs reduced

correlating to a higher ratio (Hirst *et al.,* 2008). This has been confirmed with the experimental addition of rotenone, a complex I inhibitor; its function is to limit the transfer of electrons away from complex I, creating a condition where they are "backed up" onto the NADH and are available for superoxide generation (Hirst *et al.,* 2008). The third complex in the ETC is also a significant site of superoxide generation. Complex III is where electrons are transferred from CoQ to the cytochrome c. Changes in the Δp or in the reduction state of CoQ are contributors to the production of superoxide at Complex III, as well as the addition of the inhibitor antimycin (Liu *et al.,* 2002). Compared to complex I, the superoxide generation at this site is less significant. Outside of the ETC, ROS can be generated in the mitochondria by different means. In the event that complex I is inhibited, and 2-oxoglutarate is added as a substrate, there is still a high level of superoxide generated by the enzyme α-ketoglutarate dehydrogenase, which also contains a flavin subunit and utilizes the reduced NADH pool of electrons that complex I is unable to use (Starkov *et al.,* 2004). Cytochrome P450 is another enzyme within the mitochondria that has been implicated in ROS production. Its regular function involves complex reactions converting cholesterols and other steps in steroid biosynthesis; it catalyzes monooxygenase reactions that require electrons from NADPH, which can "leak" and interact with diatomic oxygen to produce superoxides (Hanukoglu, 2006). These are just two examples of a variety of

mitochondrial reactions involving the utilization of electrons that produces ROS at this organelle.

Mitochondrial respiration is not the only source of ROS generation. Other sources of ROS production include the processes of the enzymes nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase), xanthine oxidoreductase, and myeloperoxidase (Bayir, 2005). NADPH oxidase functions as a multi-subunit enzyme which, via electrons donated by NADPH, can reduce oxygen to superoxide. NADPH oxidase is well known in leukocytes, but also exists in other tissues in different forms, such as in vascular smooth muscle. The leukocyte NADPH oxidase is primarily found in polymorph-nuclear neutrophils, or PMNs, and its function is the generation and subsequent release of superoxide as a mechanism for combating bacterial infection. The vascular NADPH oxidase is mainly activated by angiotensin

II, but also thrombin, platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), tumor necrosis factor-alpha (TNF-alpha), and other mechanical stimuli such as shear stress and strain (Brandes and Kreuzer, 2005). Its function in these cells correlates to the development of cardiovascular disease, but also the dismuatation of superoxide results in the production of the vasodilator, H_2O_2 (Fujiki *et al.,* 2005).

Xanthine oxidoreductase has two interconvertible forms as an enzyme, both of which are essential to purine catabolism by oxidizing hypoxanthine to xanthine to uric acid. Its dehydrogenase oxidizes NADH to NAD, while in its oxidase form, the enzyme is capable of producing both superoxide and hydrogen peroxide from diatomic oxygen (McNally *et al.,* 2003).

Myeloperoxidase is highly significant not for the production of superoxides but because hypochlorous acid is its main product (Zgliczynski *et al.,* 1977). HOCl is a key intermediate for the generation of many different ROS and is, in itself, highly reactive—it can react with superoxides to produce hydroxyl radicals, or with hydrogen peroxide to produce a singlet oxygen (Arnhold, 2004).

Reactive oxygen species (ROS) have been given a considerable amount of scrutiny due to the disease states that they have been linked to, such as aging, cancer, and atherosclerosis (Alfadda and Sallam, 2012). However, ROS is imperative for redox homeostasis, as well as proper function in the cardiovascular system, and immune system. The body requires a balance in its ROS levels for homeostasis. If the level of ROS exceeds that which the body can handle, then oxidative stress occurs (Liemburg-Apers *et al.,* 2015). On the other hand, if the level is too low, reductive stresses occur and can also cause pathologies ranging from cancer to cardiomyopathy (Liou and Storz, 2010). Redox regulation is imperative for the body to maintain proper signaling processes. These redox reactions usually entail

ROS interacting with the amino acid cysteine on proteins. ROS modulates cell proliferation and apoptotic pathways to ensure proper regulation of the cell cycle and programmed cell death. There are multiple kinases in these pathways that interact with ROS. The mitogen- activated protein kinase (MAPK) has a MAPKKK upstream called apoptosis signal regulated kinase 1 (ASK1). ASK1 regulates transcription factors JNK and p38, which can trigger apoptosis by phosphorylating MAPKK4,3, and cGMP dependent protein kinase (PKG) and protein kinase A (PKA) are both activated by ROS as well and are involved in the MAPK signaling process. ROS can also inhibit protein phosphatases through cysteine oxidation that prevents the inhibitory actions of the phosphatase on MAPK signaling.

Consequently, transcription factors such as p38 can be regulated this way as well. Protein tyrosine phosphatase (PTP) is oxidized and inhibited by ROS and helps maintain appropriate levels of growth factor signals.

Vascular smooth muscle cells require ROS for appropriate cell growth (Taniyama and Griendling, 2003). ROS also plays an important role in the expression of transcription nuclear factor-kB, which helps the body's inflammatory process by activating the monocyte chemotactic protein-1 (MCP-1) and interleukin-6 (Kohchi *et al.,* 2009). Many reactive oxygen species, such as H2O2, play a big part in

vasomotor tone such as vasorelaxation in the pulmonary, coronary and mesenteric systems (Li and Verma, 2002).

Reactive oxygen species has an important role in the immune system. A lack of ROS in the immune system can cause disease states that impair an individual's ability to fight against foreign invasion. The innate immunity that utilizes macrophages, neutrophils, and dendritic cells are key. These cells use toll like receptors to determine a cell that is foreign to the body, such as a bacterium. As a part of the innate immune system, macrophages, neutrophils and dendritic cells can phagocytose foreign material and then express it to the acquired immune system. The phagocytosis process is made possible by the use of reactive oxygen species. The immune system ensures the production of ROS when a foreign substance is detected due to the toll like receptor-4 binding to NADPH oxidase. Such makes certain that when a foreign substance is detected in the body and it binds to the toll like receptor-4, the NADPH oxidase is consequently triggered to make sure there is ROS production to breakdown the foreign entity (Kohchi *et al.,* 2009).

When ROS production increases above basal level, however, the excessive amounts of ROS can lead to pathologies ranging from autoimmune diseases to cardiomyopathies. As mentioned previously, there are numerous sources of ROS in a cell that may occur in cytosolic, extracellular, and mitochondrial domains. The relative amounts of mitochondrial ROS produced are indicative of the metabolic

needs of the cell by acting as a mode of cell signaling (Li *et al.,* 2013). At lower levels of production, the presence of ROS may be beneficially used as a metabolic response to hypoxia by regulating the stability of HIF-1a.

Excessive levels of ROS production, however, become pathological, and may lead to mitochondrial and cell apoptosis through activation of the apoptosome protein complex. Interaction of apoptosis activating factor (APAF-1) with mitochondrial cytochrome c plays in integral role in activation of the apoptosome, which will then lead to the activation of a chain of apoptotic caspases. The decision of whether the cell enters a state of inflammation or apoptosis, dictated by relative amount of mitochondrial ROS found within the cell, highlights the importance of ROS in choosing which cell signaling pathway will proceed. Overproduction of ROS is observed to be the cause of inflammatory diseases such as rheumatoid arthritis, multiple sclerosis, and atherosclerosis by over-activating MAPKs (Li *et al.,* 2013). Excessive production of HOCl, however, may cause oxidative damage, apoptosis and inflammatory disease. The clinical significance of excessive ROS production through MPO can be seen in its role in the formation of nitrotyrosine in endothelial regions of inflammation, impairment of NO-dependent relaxation of blood vessels, and inactivation of select neutrophil granule contents during inflammation, which may then to a prolonged respiratory burst. These detriments are apparent in pathologies associated with MPO defects, such as atherosclerosis

and plaque formation, multiple sclerosis and Alzheimer's disease (Arnhold, 2004). Overproduction or incomplete metabolism of these superoxide anions can cause oxidative damage. In pathological conditions, due to excessive ROS production, NO is altered to become ONOO─, preventing endothelial-dependent relaxation, and causing endothelial dysfunction. The initial adverse event due to the decrease in NO bioavailability is impaired endothelium-dependent vasodilation, which may spiral into long-term cardiovascular complications due to the decreased vasorelaxation. This dysfunction in vascular endothelium is then associated with pathologies ranging from hypertension, preeclampsia, and atherosclerosis to coronary artery disease (Szocs, 2004). The impact of the presence of excessive ROS may also be found in its role in protein post-translational modification, in both irreversible and reversible protein oxidative modifications. The interaction between ROS, reactive nitrogen species (RNS), and amino acid residues has been observed to lead to aging and protein dysfunction.

2.3 *Phyllanthus amarus*

Phyllanthus amarus is a plant of the family Euphorbiaceae and has about approximately 800 species which are found in tropical and subtropical countries of the world (Tahseen and Mishra, 2013). The name 'Phyllanthus' means "leaf and flower" and named so because of its appearance where flower, fruit and leaf

appears fused (Kuman *et al.,* 2011). *Phyllanthus amarus* is a branching annual glabrous herb which is 30-60 cm high and have slender, leaf-bearing branchlets, distichous leaves which are subsessile elliptic-oblong, obtuse, rounded base. Flowers are yellowish, whitish or greenish, auxillary, male flowers in groups of 1-3 whereas females are solitary. Fruits are depressed-globose like smooth capsules present underneath the branches and seeds are trigonous, pale brown with longitudinal parallel ribs on the back (Itoro *et al.,* 2013).

Phyllanthus amarus is widely distributed as a weed in cultivated and waste lands (Joseph and Raj, 2011). *Phyllanthus amarus* has received global recognition and appraisal (Srividiya and Perival, 1995). It is called "Oyomokeisoamankedem" in Efik, "Ebebenizo" in Bini, and "Iyin Olobe" in Yoruba (Etta, 2008). *Phyllanthus amarus* herb has found its traditional usefulness in several health problems such as diarrhoea, dysentery, dropsy, jaundice, intermittent fevers, urinogenital disorders, scabies and wounds. It can be used topically as a poultice for skin ulcers, sores, swelling and itchiness. The plant is bitter, astringent, cooling, diuretic, stomachic, febrifuge and antiseptic. It is useful in dropsy, jaundice, diarrhoea, dysentery, intermittent fevers, diseases of urino-genital system, scabies ulcers and wounds. The young shoots of the plant are administered in the form of an infusion for the treatment of chronic dysentery. Its efficacy in the field of gastro intestinal disorders like dyspepsia, colic,

diarrhoea, constipation and dysentery is undisputed. In females it is used as a galactogogue, in leucorrhoea, menorrhagia and mammary abscess. In skin conditions, especially scabby or crusty lesions, bruises, wounds, scabies, offensive ulcers and sores, oedematous swellings, tubercular ulcers and ringworm, it has been utilized with good effect since many years. It is applied effectively in intermittent fevers and gonorrhoea as well as in ophthalmia and conjunctivitis. It has a urolithic property, dissolving renal calculi. Also, used in cough, asthma and other bronchial affections. It's antifungal, antiviral and anticancerous properties have also been demonstrated in experimental animals (Khatoon *et al.,* 2004). The powdered leaves of *Phyllanthus amarus* were used in clinical studies evaluating its usefulness in patients suffering from chronic damage to the liver due to the protracted hepatitis B virus infection. This type of infection results in inability of the body's immune system to eliminate the virus from the liver cells (Sen and Batra, 2013). Its effect in excretory system is due to its antiurolithic property and is used in the treatment of kidney/gallstones, other kidney related problems, appendix inflammation and prostate problems (Ushie *et al.,* 2013). The young shoots of plant are administered in the form of an infusion for the treatment of chronic dysentery. Fresh leaf paste has wound healing capacity and used to cure white spots on skin & jaundice. The stem juice is also used as wound healers. The

whole plant extract is used in urinary problems & swelling of liver. The root extract is used to cure stomach pain. The flower paste of plant is applied externally as antidote against snake bite (Zingare, 2013).

Phyllanthus amarus have numerous phytocompounds such as alkaloids, flavonoids, tannins, lignins, polyphenolic compounds and tetracyclic triterpenoids (Kassuya *et al.,* 2006; Srivastava *et al.,* 2008).

Phyllanthus amarus possess various pharmacological properties like Anti-Inflammatory activity, Antioxidant activity, Antinociceptive activity, Anti hepatotoxic activity, Immunostimulant activity, Antitumour and anticarcinogenic activity, Anti-viral activity, Antibacterial activity, Anti hyper glycemic activity, Hepatoprotective, nephroprotective and cardio protective activity (Akinjogunla *et al.,* 2010).

Source: natureloveyou.sg

2.4 *Drosophila melanogaster*

Drosophila melanogaster belong to the Droso–philidae family. *Drosophila melanogaster* is a fruit fly, of the kind that accumulates around spoiled fruit. It is also one of the most valuable organisms in biological research, particularly in genetics and developmental biology. D. melanogaster is typically used in research because it can be readily reared in the laboratory, has only four pairs of chromosomes, breeds quickly, and lays many eggs (Sang et al 2001). It is a little insect about 3mm long.

The fruit fly is simple to work with, with a relatively short lifecycle/generation time of 12 days and its small size allows it to be produced in large numbers. These

practical considerations make it suitable for many studies. The fruit fly is well understood at the phenotype level and has a simple genome, enabling molecular genetics studies. Despite this, it still has ~60% of the genes involved in human genetic diseases and some cancers.

The duration of the stages of development varies with the temperature. At 20^{o}C, the average length of the egg-larval period is 8 days; at 25^{o}C it is reduced to 5 days. The pupal life at 20^{o}C is about 6.3 days, whereas at 25^{o}C is about 4.2 days. Thus at 25^{o} c the life cycle may be completed in about 10 days, but at 20^{o}C about 15 days are required. A single fertile female can lay hundreds of eggs and *Drosophila* embryogenesis lasts approximately 24 h. Drosophila cultures ought to be kept in room temperature where the temperature does not range below 20^{o}C or above 25^{o}C. Continued exposure to temperatures above 30° C may result in sterilization or death and at low temperatures the viability of flies is impaired and life cycle prolonged (Demerec and Kaufman, 1996). Sexual dimorphism is characteristic of *Drosophila* spp. Therefore, males can be easily differentiated from females having differences in size and color. The gender of *Drosophila* can be differentiated by the structure of the external genitalia and their color. Furthermore, the abdomen is pale and relatively smooth in mature female, in comparison with dark genitalia that are found in mature male. Additionally, a secondary sexual character is also present in the male flies, which is called sex comb, a structure that consists of a minor cluster

of about 10 black hairs in front of the last large segment (third segment counting from the end of the body). The same is also present even in immature males. Likewise, another secondary sexual character in male, the presence of a cluster of spiky hairs (claspers) surrounding the reproducing parts used to attach to the female during mating (Chiang and Hodson, 1950). However, the length of female is 2.5 mm, moreover, male is somewhat smaller than female with dorsal sites of male's body being darker due to a distinct black patch at the abdomen. Furthermore, in newly emerged flies and sex comb, sexual dimorphism is less noticeable (Chiang and Hodson, 1950).

Drosophila melanogaster breeds in bulky and comparatively scarce substrates. About 10–20 eggs are matured at the same time; therefore, female lays them together in one place. In eggs of *D. melanogaster*, a biological clock has been observed and their maggots greatly adapt to their ecological cycles, therefore, their survival becomes easy and they gain the highest benefits in their environment (Patterson, 1999).

Fig 1; Diagrammatical representation of Drosophila melanogaster

Source; [*https://andor.oxinst.com*](https://andor.oxinst.com)

According to Bohdana et al (2015), high sucrose consumption promotes obesity whereas its low consumption induces oxidative stress in Drosophila *melanogaster* and meanwhile in a report made by Manasa and Ashadevi (2015), the *Phyllanthus amarus* extract increases the resistance ability against oxidative stress and prolongs the longetivity in *Drosophila melanogaster.* This study was done to find out the antidiabetic and antioxidant effect of P. Amarus in Type 2 diabetic *Drosophilia melanogaster* flies.

CHAPTER THREE

3.0 MATERIALS AND METHOD

3.1 Collection of plant material:

Phyllanthus amarus leaves were obtained from various sites at Uroho Community, Ikpoba-Okha Local Government Area, Edo State, Nigeria and botanically identified and authenticated at the Central Research Laboratory, University Of Benin, Edo State. Its leaves were shade dried on a laboratory table for 14 days and reduced to powder by using dry grinder. They were pulverized, weighed and stored in an airtight container for further extraction.

3.2 Extraction of plant material

The shade dried leaf powder of *Phyllantus amarus* was subjected to the extraction process as follows. The powdered leaves was extracted in 1000ml of water and stirred for hours to mix them thoroughly. The mixture was then filtered on cheese cloth to get the supernatant which was kept in an airtight container and residue was discarded. The crude extract was obtained from the filtrate by evaporating in a laboratory incubator at a temperature of 60^{o}C. Subsequently, the crude extract was transferred to a refrigerator for storage before use.

3.3 *D. Melanogaster* culture

Both genders (1-3 days old) of Wild-type fruit fly (Harwich strain) stock culture (originally from the National Species Stock Centre, Bowling Green, OH, USA) was obtained from Drosophila Laboratory, Department of Biochemistry, University of Ibadan (U.I), Oyo State, Nigeria.

The flies were allowed to mate in vials monitored under a regulated temperature until the eggs metamorphosed into young adult fruit flies. The flies were maintained, and reared on normal diet made up of corn meal medium containing 1% w/v brewer's yeast and 0.08% v/w nirpagin at room temperature under a natural photoperiod of about 12 hours light and 12 hours dark daily in the Central Research Laboratory, University of Benin, Edo state, Nigeria. All the experiments were carried out with the same *Drosophila melanogaster* strain.

3.4 Drosophila Melanogaster basal diet Formulation and its Handling

Materials for preparation of standard basal diet:

I. Corn meal: source of carbohydrate
II. Yeast: source of protein
III. Glucose
IV. Agar: thickening agent
V. Nipargin (methyl paradym)
VI. Gas cooker
VII. Distilled Water
VIII. Cooking pot and spoon
IX. Ethanol

Note: The corn meal must be fine, smooth and without shaft.

Methods for preparation of basal meal

- For preparation of standard meal, the following amount of materials were weighed with a scale and kept in small tubes: corn meal (52g), agar (7.9g), glucose (3.5g), brewer's yeast (5g), and Nipargin (1g), and ethanol (2 ml).
- 850ml of distilled water was measured with a measuring cylinder; 150ml was taken out of this to mix the corn meal.

- The Remaining water (700ml) was turned into a pot on the gas cooker.
- Little quantity of water was taken from the already warm water to dissolve the yeast.
- The 7.9g of agar was turned into the boiling water to melt and stirred for 10 minutes.
- Corn meal was added to the boiling water containing cornmeal and stirring continues.
- Glucose was added and then stired for 5 minutes until the dissolved yeast was added.
- The gas cooker was put off and allowed to cool off until the 1g nipargin dissolved in 2ml ethanol was added.
- The meal was then allowed to cool off before they can be used for transfer of flies.

The flies are sensitive to:

- Humidity.
- Improper preparation of meal.
- Handling

  And preparation must be aseptic as 100% ethanol must be used to clean the glass bottles before transferring.

3.5 Experimental Design

3.5.1 Experiment 1: Survival Assay

For the survival assay, flies (both genders) of 1-3 days old were divided into four groups, with each group having 5 vials each. Each vial contained 30 flies each with varied concentrations of sucrose, silymarin and *Phyllantus amarus*.

GROUP I: Flies fed on high 30% Sucrose diet

GROUP II: Flies fed on 30% high Sucrose diet and *Phyllantus amarus*.

GROUP III: Flies fed on high 30% Sucrose diet and Silymarin drug.

GROUP IV: Control flies fed on diet mixed with distilled water.

Group 1	Basal diet + sucrose
Group 2	Basal diet + sucrose + *phyllantus amarus*
Group 3	Basal diet + sucrose +silymarin
Group 4	Basal diet only + distilled water

The survival assay was carried out in five replicates of each concentration. The diet was changed every seven days, during the period of this experiment. The survival rate was determined with all the groups, and both the live and dead flies were recorded daily. By the end of this experiment (14 days), the data obtained were accumulated and plotted as percentage of live and dead flies. The result was then compared with that of the control.

3.5.2 Developmental Assay

Here, flies were treated as reported above but for 24 hours. The empty treated diet is kept at the adequate temperature needed for the flies to develop. The number of newly emerged flies are counted and compared. This assay was used to investigate the effect of sucrose, silymarin and *Phyllantus amarus* on the emergence rate of the flies.

3.5.3 Preparation of Samples for Biochemical Assay

HOMOGENIZATION

For the determination of biochemical assays, a second group experiment was carried out. In this experiment, flies (both genders) of 1-3 days old were divided into four groups, with each group having 3 vials each. Each vial contained 50 flies with vary concentrations of sucrose, *Phyllantus amarus,* silymarin in each treatment vial relatively for a period of five (5) days.

GROUP I: Control flies fed on diet mixed with distilled water.

GROUP II: Flies fed on high 30% Sucrose diet only

GROUP III: Flies fed on high 30% Sucrose diet and *Phyllantus amarus*

GROUP IV: Flies fed on high 30% Sucrose diet and Silymarin drug.

The flies were collected after anesthetizing on ice, weighed, homogenized in 0.1 M phosphate buffer, pH 7.4 (ratio of 1 mg:10 μL), and centrifuged at 4000 rpm for 10 mins at 4°C (Allegra X-15R Centrifuge, Beckman Coulter, USA). Subsequently, the supernatant was separated from the pellet into labelled Eppendorf tubes and used for the various biochemical assays.

Caution was taken when counting the flies and an appropriate brush with soft ends was used. Much care was taken in handling the flies as to prevent “handling stress”.

Homogenizing Buffer (0.1 M Phosphate Buffer, pH 7.4).

10.677 g of K2HPO4 and 5.267 g of KH2PO4 were dissolved in 800 ml of distilled water and made up to 1 litre and then stored at 40°C.

3.6 Biochemical Assays

Biochemical parameters that were assessed to understand the effect of *Phyllantus amarus* on R.O.S and antioxidant enzymes of type 2 diabetes fruit flies includes:

3.6.1 **Protein Determination**

The protein concentrations of the various samples were determined using Lowry method (Lowry *et al.,* 1951) with some few modifications.

Principle

Under alkaline conditions, the divalent copper ions forms a complex with peptide bonds in which it is reduced to a monovalent ion. Monovalent copper ion and the radical groups of tyrosine, tryptophan and cysteine react with Folin reagent to produce an unstable product that becomes reduced to molybdenum/tungsten blue. The principle of the assay is based on the reaction of Cu+, produced by the oxidation of peptide bonds, with Folin-Ciocalteu reagent (a mixture of Phosphotungstic acid and phosphomlybdic acid in the Folin-Ciocalteu reaction). The reaction mechanism involves reduction of the Folin- Ciocalteu reagent and oxidation of aromatic residues (mainly Tryptophan, also tyrosine). Also cysteine residues in protein probably also contribute to the absorbance seen in Lowry assay (Everette *et al.,* 2010). The concentration of the reduced Folin reagent is measured by absorbance at 750nm (Lowry *et al*., 1951).

METHODOLOGY

Reagents

A. Reagent A consist of 0.1M sodium Hydroxide and sodium bicarbonate (2%)

B. Reagent B consist of 1% copper sulphate (CuSO4.5H2O) and 2% sodium potassium tartarate.

C. Make up solution A 9.8ml and solution B 200 µL to make solution C.

Procedure

(1) Make up a set of standard using Bovine albumin serum or other source in the

Concentration 0.5 mg/ml. In the Lowry procedure you will use 25 µl of each sample.

(2) To 25 µl of your unknown samples and dilutions of your samples and to different

Volumes of the standard add 400 µl of solution C and 40 µl of folin. Mix well and let them stand.

(3) Measure the absorbance at650 nm against a blank consisting of 60 µl of sample buffer or water and solution C & folin.

(4) Determine your protein concentration from a standard curve

3.6.2 Determination Of Catalase Activity

Catalase activity was determined according to the method of Claiborne (1985).

Principle. The method is based on the loss of absorbance observed at 240 nm as catalase splits hydrogen peroxide. Despite the fact that hydrogen peroxide has no absorbance maximum at this wave length, its absorbance correlates well enough with concentration to allow its use for a quantitative assay. An extinction coefficient of 0.0436 mM-1cm-1 (Noble and Gibson, 1970) was used.

Reagents

1. Phosphate buffer (0.05 M, pH 7.4) Dipotassium hydrogen phosphate trihydrate (0.696 g) and potassium dihydrogen phosphate (0.265 g) were dissolved in 90 ml

of distilled water, the pH adjusted to 7.4 and the volume made up to 100 ml with distilled water.

2. Hydrogen peroxide (19 mM) 194 µl of 30% H2O2 was added to 50 ml of 0.05 M phosphate buffer, pH 7.4 and the volume made up to 100 ml with the same.

Procedure: Hydrogen peroxide (590 µl of 19 mM solution) was pipetted into a 1 cm quartz cuvette and 10 µl of sample added. The mixture was rapidly inverted to mix and placed in a spectrophotometer. Change in absorbance was read at 240 nm 10 seconds for 2 min.

3.6.3 Determination Of Nitrite (Nitric Oxide) Level

DETERMINATION OF NITRITE (NITRIC OXIDE) LEVEL

Principle

As NO rapidly recombines into its stable oxidative metabolites (NO3- and NO2-) in aqueous solution (Palmer *et al.*, 1987), serum concentrations of NO3 - and NO2- were estimated as an index of N.O**.** production. The NO**.** radical plays an important role as a physiological messenger (Moncada *et al.*, 1991). NO is formed from L-arginine (Palmer *et al.*, 1988) by NO synthase, which exists in several isoforms (Griffith and Stuehr, 1995). Constitutive calcium-dependent isoforms (cNOS) modulate the control of vascular tone in endothelial cells or the neurotransmission in neurons, whereas inducible calcium-independent isoforms (iNOS) are located in macrophages, chondrocytes and hepatocytes and are induced by cytokines and endotoxin (Bredt and Snyder, 1994; Nathan, 1992). Pathological conditions associated with increased release of cytokines and endotoxin, e.g. inflammation or sepsis (Curzen *et al.*, 1994) can therefore increase NO production. Upon coming into the blood stream, nitrite reacts immediately with oxyhaemoglobin to form methaemoglobin.

Procedure

The amounts of nitrite in supernatants or in serum were measured following the Griess reaction (Green *et al*., 1982) by incubating a 250uL of sample with 250uL of Griess reagent [0.1% N-(1-naphthyl) ethylenediamine dihydrochloride; 1% sulfanilamide in 5% phosphoric acid; 1:1 purched a] at room temperature for 20 min. The absorbance at 550 nm (OD 550) was measured spectrophotometrically. Nitrite concentration was calculated by comparison with the OD 550 of a standard solution of known sodium nitrite concentrations.

Calibration curve

Calibrator at various concentrations was prepared by diluting stock 20 mmol/L solutions of $NaNO_2$ with distilled water. The nitrate calibrator was diluted with glycine buffer just as the serum samples were. Calibration curve was made over a linear range of nitrate between 0 and 100 μmol/L.

3.6.4 ESTIMATION OF GLUTATHIONE-S-TRANSFERASE ACTIVITY

Glutathione-S-transferase (GST) activity was determined according to Habig and Jakoby (1981).

Principle

The principle is based on the fact that all known glutathione-S-transferase demonstrate a relatively high activity with 1-chloro-2, 4, - dinitrobenzene as the second substrate, consequently, the conventional assay for glutathione-S-transferase activity utilizes 1-chloro-2, 4, - dinitrobenzene as substrate. When this substance is conjugated with reduced glutathione, its absorption maximum shifts to a longer wavelength. The absorption increase at the new wavelength of 340nm provides a direct measurement of the enzymatic reaction.

Reagents

1. 1-Chloro- 2, 4, - dinitrobenzene (25mM) 1ml of CDNB was prepared by dissolving 0.0198grams in 1ml ethanol

2. Reduced Glutathione (0.1M) 0.003073grams of GSH was dissolved in 10ml of distilled water

3. 2.5mM EDTA (0.073g) was dissolved in 50ml of 0.25M potassium phosphate buffer (pH 7.0).

4. 0.25M Phosphate buffer at pH 7.0 Weigh 3.4g of KH2PO4 and dissolve in 100ml distilled water. Also weigh 5.7g of K2HPO4 and dissolve in 100ml distilled water.

Take 19.5ml of KH2PO4 and 30.5ml of K2HPO4 in a 100ml flask and add EDTA.

Make up to mark with distilled water and confirm pH with a pH meter Sample Dilution (1:5)

Procedure

1. 20ml of 0.25M phosphate buffer (containing 2.5mM EDTA) was added to 10.5ml of distilled water which is added to 500uL of GSH to make solution A

2. 20uL of sample was added to solution A

3. 10uL of CDNB was then added

The reaction mixture was shaken and read the absorbance at 340nm for 5 minutes (10 sec interval).

3.6.5 HYDROGEN PEROXIDE GENERATION

Hydrogen peroxide generation was determined according to the method of Wolff, 1994.

Principle

Reagents

1. 100mol/l xylenol orange (molecular weight: 760.6)

7.6mg of xylenol orange was dissolved in 10ml of distilled water.

2. 250mol/l ammonium ferrous sulfate (MW: 392.14)

9.8mg of ferrous sulfate was dissolved in 10ml of distilled water.

3. 100mmol/l Sorbitol (MW: 182.2)

1.822g of sorbitol was dissolved in 10ml of distilled water.

4. 25mmol/L H_2SO_4

140µl of 1M H_2SO_4 was made up to 50mls with distilled water

5. Phosphate buffer, 0.1M. pH7.4

4.96g of dipotassium hydrogen orthophosphate (K_2HPO_4 Mol. Wt. = 174.18)(Hopkins and Williams, Ltd) and 9.73g of potassium dihydrogen orthophosphate (KH_2PO_4Mol. Wt. = 136.09) (Hopkins and Williams, Ltd) were dissolved in 900ml of distilled water. The Ph was adjusted to 7.4 and the made up to a 1000ml with distilled water.

Procedure

Table 2.10: The assay medium for Hydrogen peroxide

FOX1 = 10ml X0 + 10ml sorbitol + 50ml AFS + 30ml distilled water

590µl of FOX1 + 10µl of sample

The assay mixture is thoroughly mixed by vortexing till it foamed. A pale pink color complex is generated after incubation for 30 minutes at room temperature. The absorbance was read against blank (distilled water) at 560 nm wavelength. The concentration of the hydrogen peroxide generated was extrapolated from the standard curve.

3.6.6 DETERMINATION OF THE LEVELS OF TOTAL THIOLS

The total thiols level was assayed for according to the method of Ellman (1951).

Principle

The reduced form of glutathione and other protein with sulfhydryl are available in cellular prooxidant and anti-oxidant system in tissues. This method is based on the development of a relatively stable (yellow) colour when Ellman's (5, 5'-dithiobis-(2-nitro-benzoic acid) is added to

sulfhydryl compounds. The chromophoric product resulting from the reaction of Ellman's reagent with the reduced sulfhydryl groups which is 2-nitro-5-thiobenzoic acid possesses a molar absorption at 412 nm.

Reagents

1. GSH working standard

3.073 mg of GSH was dissolved in 10 mls of 0.1 M phosphate buffer at pH 7.4

2. 0.1 M PHOSPHATE BUFFER (pH7.4)

10.677 g of K2HPO4 and 5.267 g of KH2PO4 were dissolved in 800 ml of distilled water and made up to 1 litre and then stored at 40C.

3. Ellman's reagent (5,5'-dithiobis-(2-nitro-benzoic acid)

This was prepared by dissolving 0.03964 g of Ellman's reagent in 10 ml of 0.1 M phosphate buffer (pH 7.4).

PROTOCOL FOR DETERMINATION OF TOTAL THIOLS CONTENT

Table 2. 4 : Protocol for determination of total thiol content

Standard Phosphate buffer (0.1M, pH 7.4)

Procedure

An aliquot of the sample supernatant was added to Ellman's reagent and read at absorbance of 412nm at 30 minutes incubation time

STANDARD CURVE FOR TOTAL THIOL

Figure 2 2 : Standard curve for total thiol concentration (Elman, 1951)

3.7 Statistical Analysis

The results were analyzed using the statistical package for social sciences program (SPSS) version 21.0 (Chicago IL). Values obtained in this study were presented as mean ± standard deviation. One way analysis of variance (ANOVA) was used to assess the significant differences among multiple groups under various treatments, followed by Turkey's post hoc test. In all the groups, differences with a p-values < 0.05 were considered statistically significant.

CHAPTER FOUR

RESULTS

4.1 Effect of *Phyllantus amarus* on survival rate of *Drosophilia melanogaster* exposed to high sucrose diet.

The effect of *Phyllantus amarus* on longevity and survival rate of *Drosophilia melanogaster* exposed to high sucrose diet is shown in Figure 1. The result shows an increase in survival rate in contrl flies and flies fed with 30% high sucrose diet and silymarin. There was a decrease in survival rate in flies fed with 30% sucrose diet only and flies fed with 30% high sucrose diet and *Phyllantus amarus.*

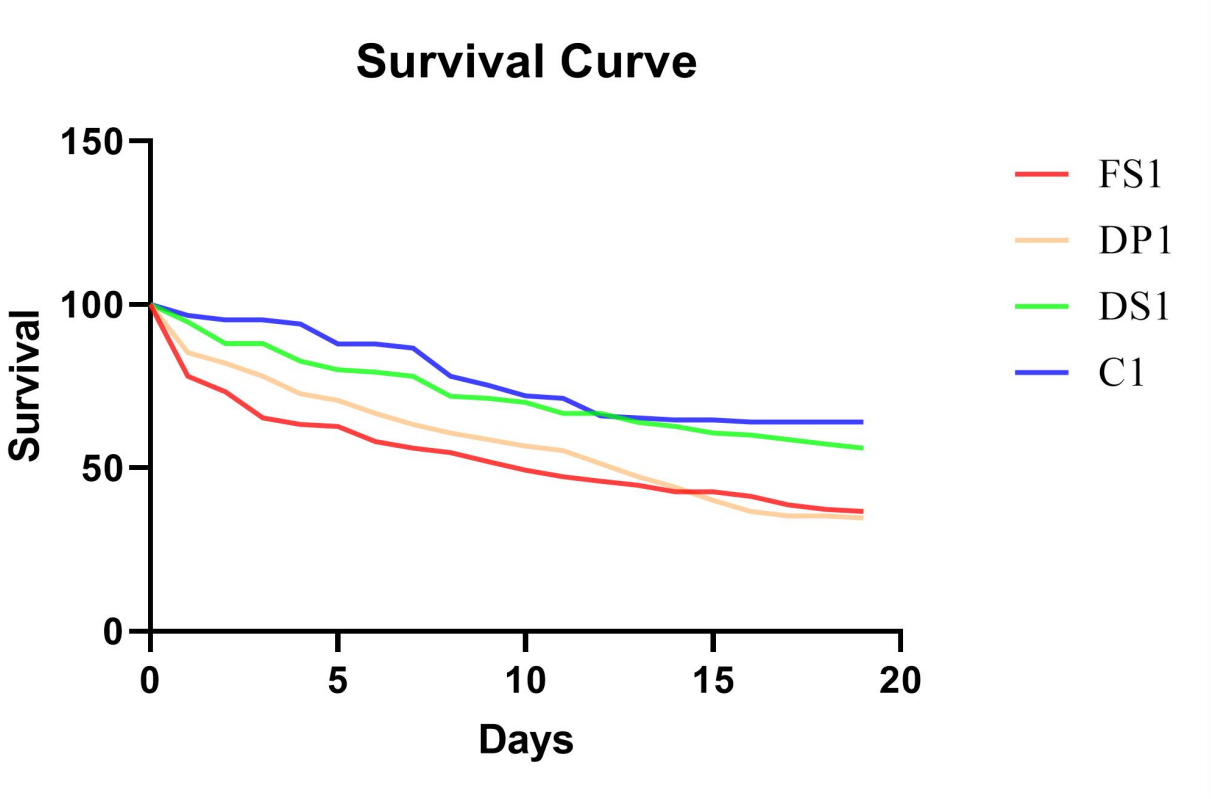


Figure 1: Effect of *Phyllantus amarus* on survival rate of diabetic *Drosophilia melanogaster* exposed to high sucrose diet.

FS - Flies and 30% sucrose

DP - 30% sucrose and *Phyllantus amarus*

DS - 30% sucrose and silymarin

C - Control flies

4.2 Effect of *Phyllantus amarus* on glucose level in *Drosophilia melanogaster*

The effect of *Phyllantus amarus* on glucose level in *Drosophilia melanogaster* is shown in Table 1. The result shows a significant increase ($p<0.05$) in glucose levels of

flies treated with diet containing 30% high sucrose diet relative to the control, while flies fed diet containing a combination of 30% sucrose and the aqueous extract of the leaves of *phyllantus amarus* increase non-significantly ($p>0.05$) compared with the control group. The glucose levels of the flies fed diet containing 30% sucrose and the drug silymarin had a non-significant decrease ($p>0.05$) compared with the control

Table 1: Effect of *Phyllantus amarus* on glucose level in *Drosophilia melanogaster*

GROUP	**Glucose (mg/dl)**
Control	70.67 ± 16.74
S diet	136.67 ± 16.17[a]
SP diet	104.00 ± 32.42
SS diet	72.00 ± 19.97

Data reported as mean ± standard deviation. n=3, Values with superscript [a] are significantly different ($p < 0.05$) from the control group.

Legend

FS diet: diabetic flies with 30% high sucrose diet only

DP diet: diabetic flies with 30% high sucrose diet + *phyllantus amarus*

DS diet: diabetic flies with 30% high sucrose diet + Silymarin

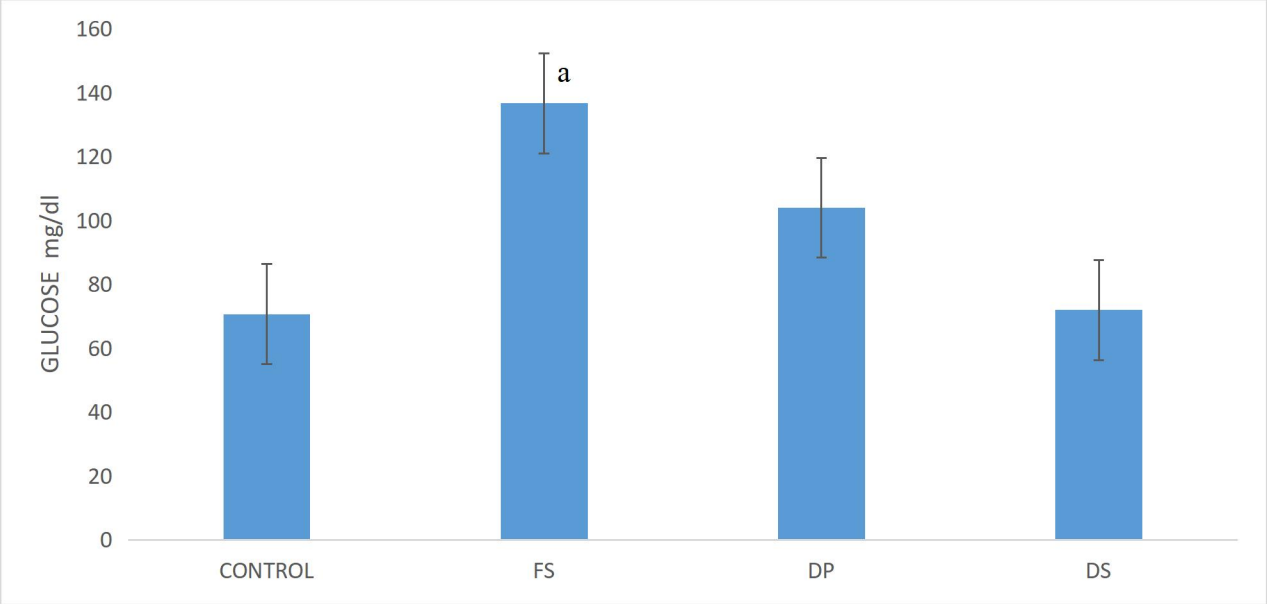


Figure 1

4.3 Effect of *Phyllantus amarus* on protein and antioxidant enzymes in diabetic *Drosophilia melanogaster*

The effect of *Phyllantus amarus* on protein, catalase, GSH and glutathione S-transferases (GST) activities **i**n diabetic *Drosophilia melanogaster* is shown in **Table 2**. The result shows a non-significant increase ($p>0.05$) in **PROTEIN** in the flies treated with diet containing 30% sucrose and that containing a combination of 30% sucrose and the aqueous extract of the leaves of *phyllantus amarus* compared with the control group, while those treated with diet containing a combination of 30% sucrose and the drug silymarin had similar protein level relative to the control group. **CATALASE** activity decrease in the flies decrease in the three (3) experimental groups but only the flies treated with diet containing 30% sucrose was significant lower ($p<0.05$) compared with the control.

The result also shows a non-significant decrease ($p>0.05$) in **GSH** in flies treated with diet containing 30% sucrose and that containing a combination of 30% sucrose and silymarin, compared to the control. However, flies treated with diet containing a combination of 30% sucrose and the aqueous extract of the leaves of *phyllantus amarus* increase non-significantly ($p>0.05$) compared with the control group.

GST activity increase in the flies treated with diet containing 30% sucrose and that containing a combination of 30% sucrose and silymarin, but only those treated with diet containing a combination of 30% sucrose and silymarin were significantly higher ($p<0.05$) compared to the control, while those treated with diet containing a combination of 30% sucrose and the aqueous extract of the leaves of *phyllantus amarus* had similar GST activity relative to the control group.

Table 2: Effect of *Phyllantus amarus* on protein and antioxidant enzymes in diabetic *Drosophilia melanogaster*

GROUP	PROTEIN (Units/g)	CATALASE (Units/g)	GSH (Units/g)	GST (U/ml)
Control	0.15 ± 0.04	352.24 ± 64.22	381.39± 55.08	0.03 ± 0.01
S diet	0.16 ± 0.04	197 ± 50.68[a]	303.46± 40.45	0.06 ± 0.07
SP diet	0.17 ± 0.04	326.17 ± 35.10	414.19± 74.26	0.03 ± 0.03
SS diet	0.15 ± 0.01	220.42 ± 33.45	324.86 ± 18.28	0.15 ± 0.01[a]

Data reported as mean ± standard deviation. n=3, Values with superscript [a] are significantly different ($p < 0.05$) from the control group.

Legend

S diet: diabetic flies with 30% high sucrose diet

SP diet: diabetic flies with 30% high sucrose diet + *phyllantus amarus*

SS diet: diabetic flies with 30% high sucrose diet + Silymarin

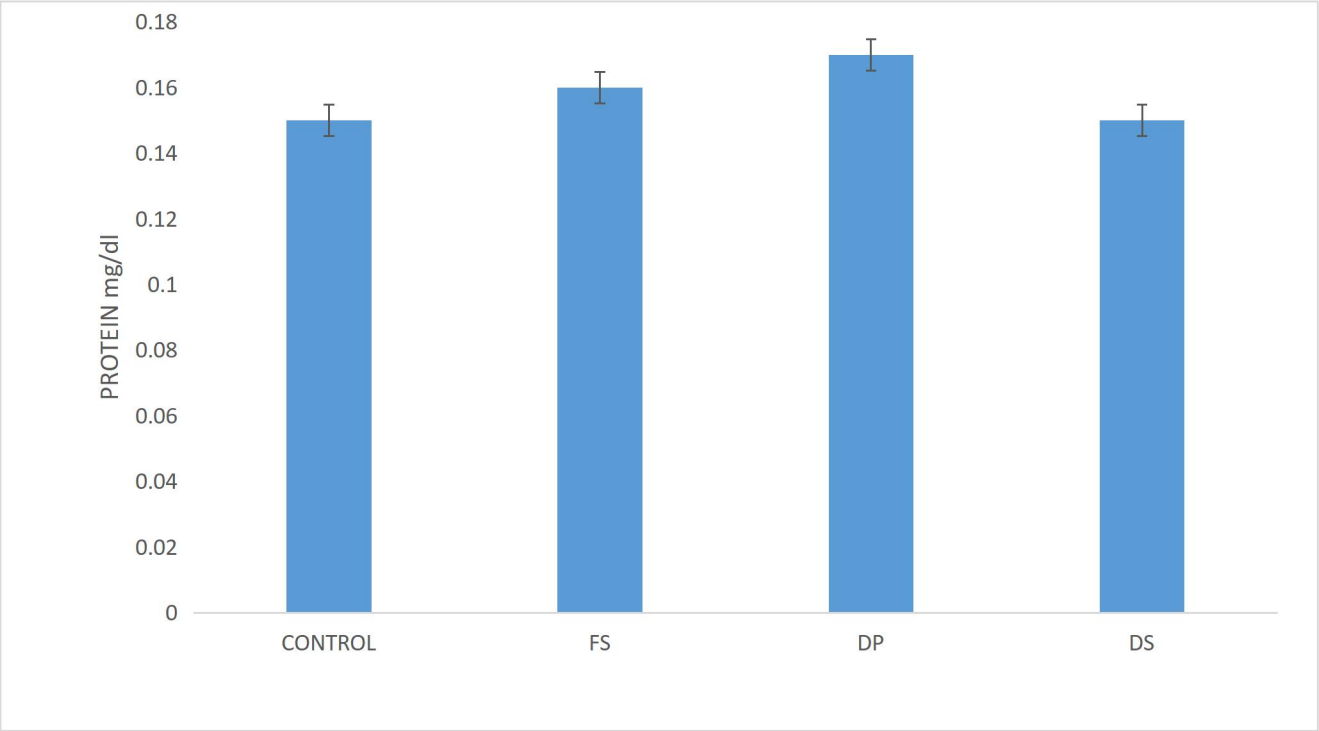


Figure 2:

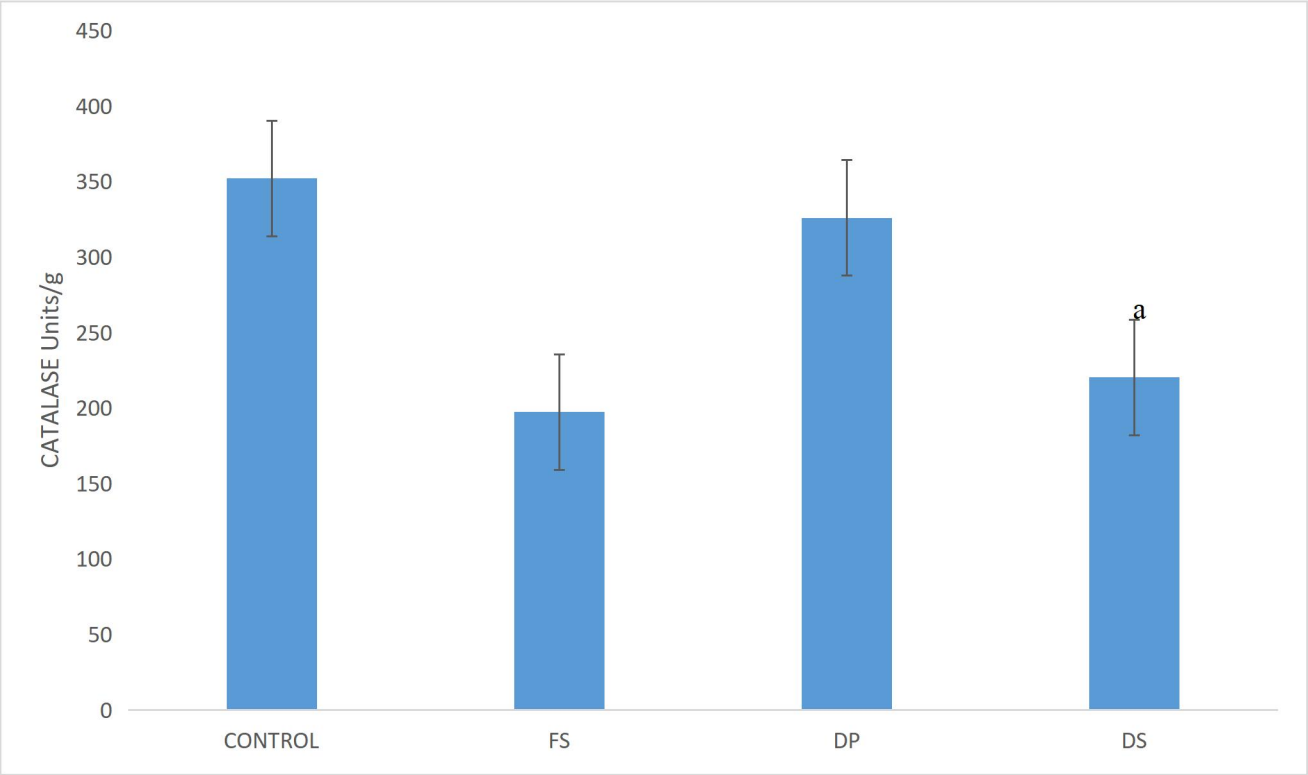


Figure 3:

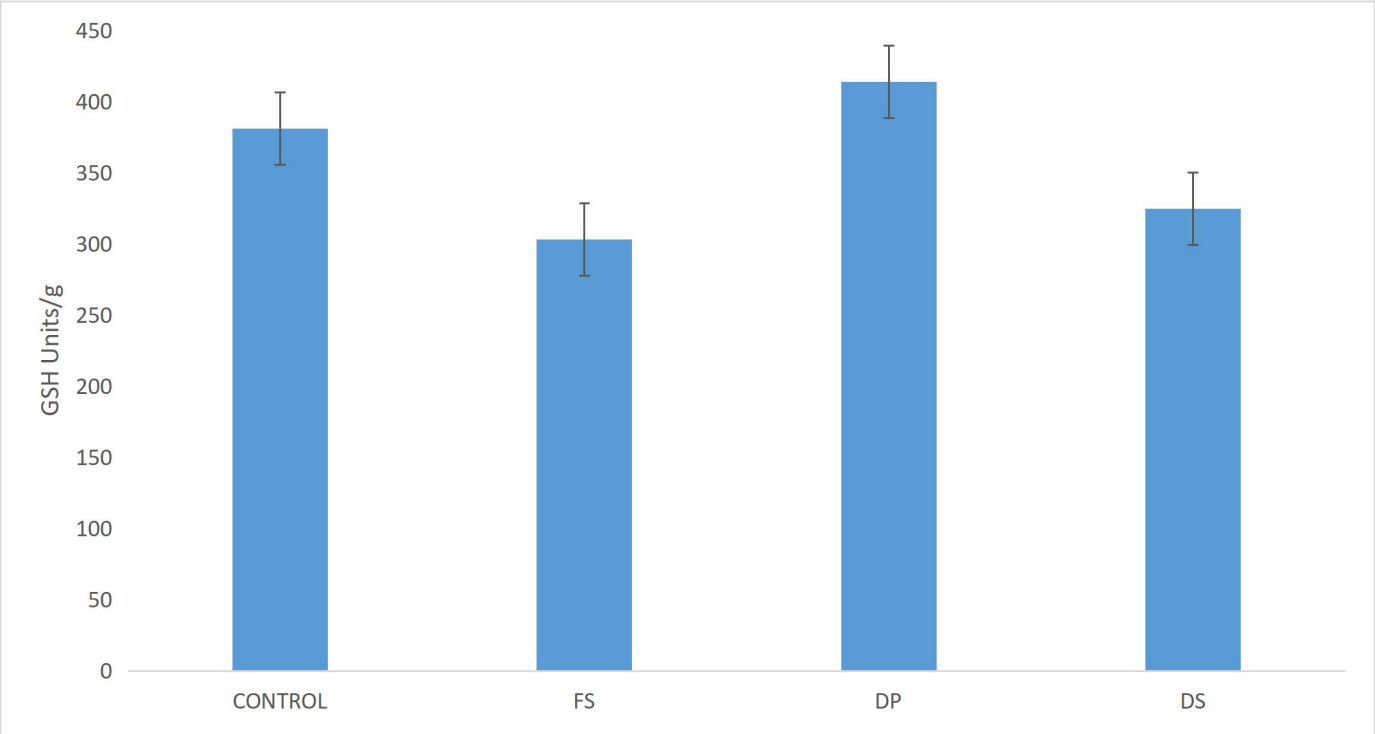


Figure 4:

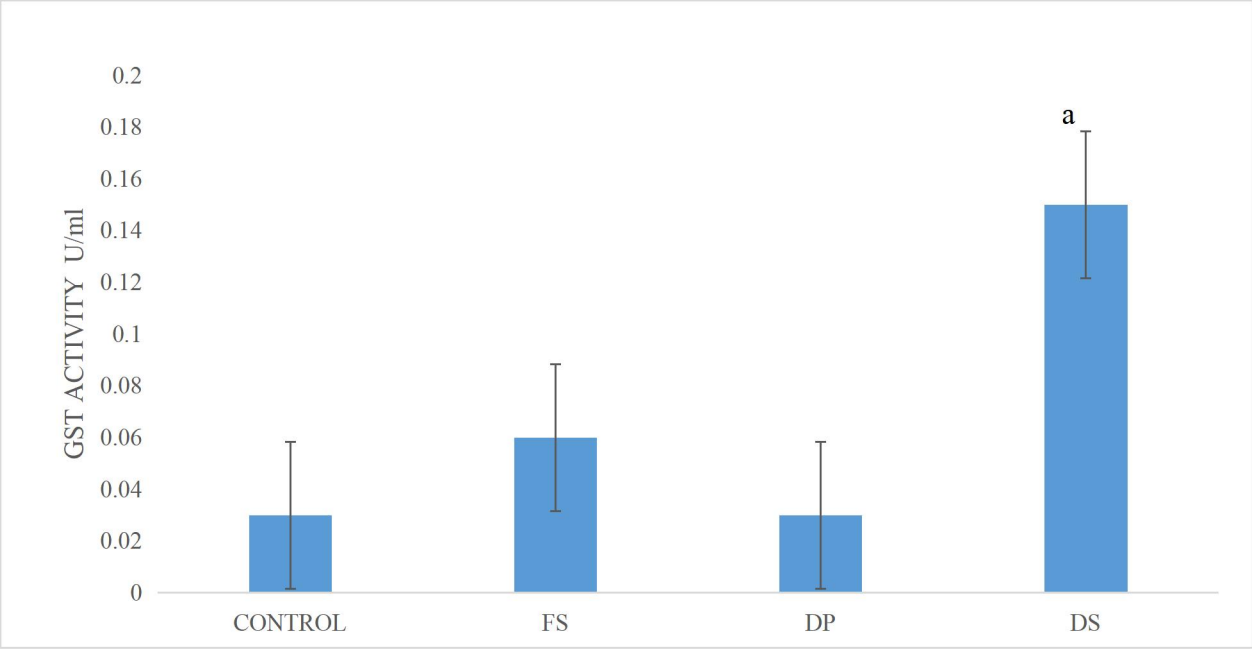


Figure 5:

4.4 Effect of *Phyllantus amarus* on reactive oxygen species in diabetic *Drosophilia melanogaster*

The effect of *Phyllantus amarus* on NO and H_20_2 levels in diabetic *Drosophilia melanogaster* is shown in Table 3. There was no significant change ($p>0.05$) in **NO** level in the experimental groups except for the flies treated with diet containing a combination of 30% sucrose and the aqueous extract of the leaves of *phyllantus amarus*, which had a significant decrease ($p<0.05$) compared to the control. The result also shows a significant increase ($p<0.05$) in **H_2O_2** levels in flies treated with diet containing 30% sucrose compared with the control. A decrease in H_2O_2 level was observed in flies treated with diet containing a combination of 30% sucrose and the aqueous extract of the leaves of *phyllantus amarus* and that containing a combination of 30%

sucrose and sillymarin, but only flies treated with diet containing a combination of 30% sucrose and sillymarin decrease significantly ($p<0.05$) compared with the control.

Table 3: Effect of *Phyllantus amarus* on reactive oxygen species in diabetic *Drosophilia melanogaster*

GROUP	NO (x10^{-2}Units/g)	H_2O_2 (Units/g)
Control	0.15 ± 0.81	2.65 ± 0.02
S diet	0.16 ± 0.01	2.84 ± 0.03[a]
SP diet	0.12 ± 0.01[a]	2.63 ± 0.11
SS diet	0.15 ± 0.01	2.56 ± 0.06[a]

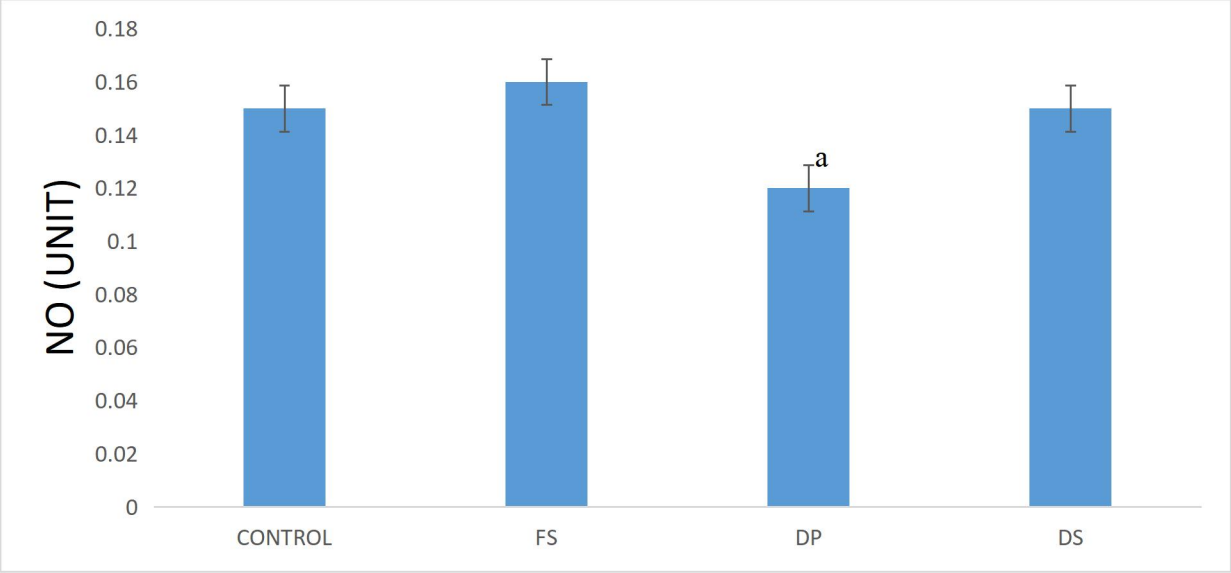
Data reported as mean ± standard deviation. n=3, Values with superscript [a] are significantly different ($p < 0.05$) from the control group.

Legend

S diet: diabetic flies with 30% high sucrose diet

SP diet: diabetic flies with 30% high sucrose diet + *phyllantus amarus*

SS diet: diabetic flies with 30% high sucrose diet + Sillymarin



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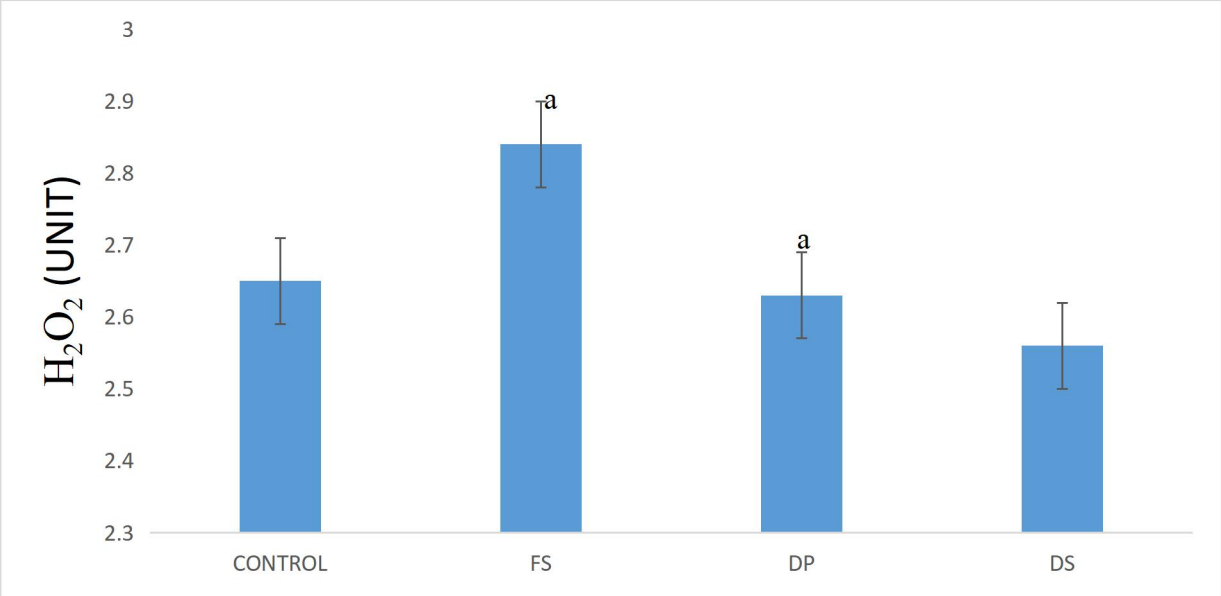

CHAPTER FIVE

DISCUSSION

This study was undertaken to find out the antidiabetic and antioxidant effect of P. Amarus in Type 2 diabetic *Drosophilia melanogaster* flies. From the results

obtained from this experiment showed that there was an increase in glucose level in the flies fed with 30% high sucrose diet. This result is somewhat similar to what morris et al., who observed that Insulin-resistant Drosophila have also been generated by rearing flies on high-sugar diet (HSD) (Morris *et al.,* 2012).

There is a significant increase ($p<0.05$) in $\mathbf{H_2O_2}$ levels in flies fed with high sucrose diet when compared with the control. This result is similar to the result obtained by Góth in 2008, which stated that type 2 diabetes can result to increase in hyprogen peroxide concentration.

There was a significant improvement in catalase activity in flies treated with both phyllantus and high sucrose diet and it is in line with the findings obtained by Manasa and Ashadevi, 2015 which stated that the catalase enzyme activity of Phyllantus Amarus Extract treated groups was gradually increased from 15 to 30 days aged flies and showed maximum activity.

There is a significant decrease ($p>0.05$) in **GST** in flies fed with 30% HSD and *phyllantus amarus* compared with the HSD fed flies which is in variance with the findings of Kumar and Kuttan, who reported P. amarus treatment increased the activity of glutathione-S-transferase (GST) (Kumar and Kitten, 2004)

In the result, there was a significant increase in GSH in flies fed with 30% high sucrose diet and phyllantus amarus compared with the 30% High sucrose fed flies. This result is in agreement with the result obtained from Kumar and Kuttan

who reported the Phyllantus amarus treatment increased the activity of GSH (Kumar and Kuttan, 2004).

CONCLUSION

Phyllanthus amarus was able to exert potent antidiabetic and antioxidant effect on diabetic Drosophila melanogaster flies (Harwich strain).

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