# INFLUENCE OF TWO PLANT PRODUCTS (RED PALM OIL AND ROOIBOS) ON STREPTOZOTOCIN-INDUCED HYPERGLYCAEMIA AND ITS IMPLICATIONS ON ANTIOXIDANT STATUS AND OTHER BIOCHEMICAL PARAMETERS IN AN ANIMAL MODEL

**By**

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## DECLARATION

I, Ademola Olabode Ayeleso, declare that the contents of this dissertation/thesis represent my own unaided work, and that the dissertation/thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.

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# ABSTRACT

Diabetes mellitus is a major health problem not only in urban, but also in the rural areas and is diagnosed by the presence of high glucose levels in the blood. Oxidative stress is known to be actively involved in the onset and progression of diabetes and its complications. Antioxidants have important roles in biological systems by scavenging free radicals which may result in oxidative damage of biological molecules such as lipids, proteins and DNA. Red palm oil, originally from the tropical area of Africa, generally consumed as cooking oil, is known to have some beneficial health effects due to the presence of lipid soluble antioxidants such as carotenoids, tocopherols and tocotrienols. It also contains almost an equal proportion of both saturated and unsaturated fatty acids which makes it distinctive from other vegetable oils. Rooibos, on the other hand, is grown in the Cederberg area of the Western Cape in South Africa and it is commonly consumed as a beverage. It contains a complex profile of water soluble antioxidants (flavonoids) and its health promoting potentials have been reported extensively. Some of the flavonoids present in rooibos include aspalathin, nothofagin, quercetin, rutin and orientin.

The objective of this research project was to examine the potential beneficial effects of the dietary intake of red palm oil and rooibos on streptozotocin-induced hyperglycaemia and its influence on the antioxidant status and some biochemical parameters in male Wistar rats. The preliminary phase of this study was designed to investigate the biochemical effects of these two plant products at different dosages following consumption for a period of 7 weeks. The preliminary study did not reveal any adverse effects of the different dosages of red palm oil (1 ml, 2 ml and 4 ml) and rooibos (2%, 4% and 6%) on the experimental rats following dietary intake for 7 weeks. However, these natural products showed an improvement in the antioxidant status of the rats at the different doses. Using a single dose each of both plant products from the preliminary study, the main study was performed to investigate the influence of these two plant products singly and in combination on the blood and liver of streptozotocin-induced hyperglycaemic male Wistar rats.

In the main study, streptozotocin (50 mg/kg) dissolved in 0.1 M citrate buffer (pH 4.5) through intramuscular injection was used for the induction of diabetes which was confirmed by the presence of high blood glucose after 72 hours. Red palm oil or rooibos extract alone did not have any effect on the control of blood glucose in the diabetic rats. The dietary intake of the combined treatment with red palm and rooibos had more health promoting effects on the diabetic rats which included a decrease in blood glucose, glycosylated haemoglobin,

fructosamine and increased insulin levels. There was a marked increase in liver glycogen levels in all the diabetic groups. Treatment with rooibos alone showed a decrease in glycogen levels in the diabetic rats. The presence of liver enzymes in the serum, commonly used as indicators of liver damage was increased in all the diabetic rats. However, the combined treatment of diabetic rats with red palm oil and rooibos protected the liver from injury. Red palm oil improved high density lipoprotein cholesterol levels (HDL-cholesterol) in the diabetic rats. There was no effect on the activity of glucokinase, the first enzyme in the the glycolytic pathway in both the untreated and treated diabetic rats. However, the activity of pyruvate kinase, the last enzyme in the glycolytic pathway was reduced in all the diabetic groups. The combined treatment with both red palm and rooibos increased the activity of pyruvate kinase.

Oxidative stress was confirmed in the diabetic rats with an increase in the plasma thiobarbituric acid reactive substances (TBARS), an indicator of lipid peroxidation. Treatment of diabetic rats with rooibos and the combination of red palm oil and rooibos brought plasma TBARS to a level that was not significantly different from the normal control group. There was a non-significant reduction of total glutathione in the non-treated and treated diabetic groups. A non-significant increase in the activity of liver catalase was observed in all the treated diabetic groups. The activity of superoxide dismutase was significantly decreased in the liver of diabetic rats. Diabetic rats treated with red palm oil, rooibos and the combined treatment showed an increased activity of superoxide dismutase in the liver. Red palm oil and the combined treatment increased the activity of glutathione peroxidase in both the red blood cells and liver of diabetic rats. Red palm oil, rooibos and their combined treatments also improved the plasma antioxidant capacity such as ferric reducing antioxidant power (FRAP) and oxygen reducing absorbance capacity (ORAC) in the diabetic rats.

In conclusion, oxidative stress is actively involved in the progression of diabetes mellitus. Red palm oil and rooibos, most especially their combined treatment showed significant beneficial health promoting effects in the diabetic rats. The remarkable effects of the combined treatment of red palm oil and rooibos in the diabetic rats could be due to their antioxidant profiles. Based on the findings from this study, it can be adduced that these plant products could help in the management of diabetes and its complications and therefore, suggested the need for further research studies on antioxidant therapy in the management of diabetes mellitus.

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# DEDICATION

This thesis is dedicated to God,

My creator

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## GLOSSARY

| Abbreviations | Definition / Explanation |
|---|---|
| AAPH | Azobis (2-amidino-propane) dihydrochloride |
| ABTS | 2, 2’-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) |
| ACE | Angiotensin-converting enzyme |
| ADP | Adenosine diphosphate |
| AGEs | Advanced glycated endproducts |
| AIDS | Acquired immunodeficiency syndrome |
| ALT | Alanine aminotransferase |
| ANOVA | Analysis of variance |
| AOAC | Association of official analytical chemists |
| AST | Aspartate aminotransferase |
| ATP | Adenosine triphospate |
| ATP | Adenosine triophosphate |
| $Ca^{2+}$ | Calcium ion |
| CAT | Catalase |
| $CCl_4$ | Carbon tetrachloride |
| Cdkn1a | Cyclin-dependent kinase inhibitor 1A |

| | |
|---|---|
| CD | Conjugated dienes |
| Cu/Zn | copper/zinc |
| CVD | Cardiovascular diseases |
| DAG | Diacylglycerol |
| DAN | Diabetic autonomic neuropathy |
| DM | Diabetes mellitus |
| DMACA | p-Dimethylaminocinnamaldehyde |
| DME | Diabetic macular edema |
| DNA | Deoxy ribonucleic acid |
| DPN | Diabetic peripheral neuropathy |
| EDTA | Ethylene diamine tetraacetic acid |
| eNOS | Endothelial nitric oxide synthase |
| FcRn | Neonatal Fc receptor |
| $FeCl_3$ | Iron (III) chloride |
| FRAP | Ferric reducing antioxidant power |
| GGT | Gamma-glutamyl transpeptidase |
| GH | Glycogen hepatopathy |
| GK | Glucokinase |
| GLUT | Glucose transporter |

| | |
|---|---|
| GPx | Glutathione peroxidase |
| GR | Glutathione reductase |
| GSH | Reduced glutathione |
| GSHt | Total glutathione |
| GSSG | Oxidized glutathione |
| HOCl | Hydrogen oxychloride |
| $H_2O_2$ | Hydrogen peroxide |
| $H_2SO_4$ | Hydrogen tetraoxosulphate (VI) |
| HbAIc | Glycosylated haemoglobin |
| HDL | High density lipoprotein |
| HIV | Human Immunodeficiency Virus |
| $HNO_2$ | Nitrous oxide |
| HOcl | Hydrochlorous acid |
| $HRO_2^-$ | Hydroperoxyl |
| IDDM | Insulin-dependent diabetes mellitus |
| JNK | c-Jun N-terminal kinase |
| KOH | Potassium hydroxide |
| LDH | Lactate dehydrogenase |
| LDL | Low density lipoprotein |

| | |
|---|---|
| Lys | Lysine |
| MAPK | Mitogen-activated protein kinase |
| Mn | Manganese |
| MDA | Malondialdehyde |
| mRNA | Messenger ribonucleic acid |
| MUFA | Monounsaturated fatty acids |
| NAD | Nicotinamide adenine dinucleotide |
| NADPH | Nicotinamide adenine dinucleotide phosphate |
| NAFLD | Non-alcoholic fatty liver disease |
| NF-kB | Nuclear factor kappa-light-chain-enhancer of activated β- cells |
| NIDDM | Non-insulin-dependent diabetes mellitus |
| NO | Nitric oxide |
| $NO_2^-$ | Nitrogen dioxide |
| $O^{2-}$ | Superoxide anion |
| O-GlcNAc | O-glycosylation with N-acetylglucosamine |
| OGlcNAcase | O-GlcNAc-selective N-acetyl-b-d-glucosaminidase |
| OH | Hydroxyl |
| ONOO- | Peroxynitrite |
| ORAC | Oxygen radical absorbance capacity |

| | |
|---|---|
| PDR | Proliferative diabetic retinopathy |
| PCA | Perchloric acid |
| PK | Pyruvate kinase |
| PKC | Protein kinase C |
| PUFA | Polyunsaturated fatty acids |
| RAGE | Receptor for advanced glycation end product |
| RBCs | Red blood cells |
| RNS | Reactive nitrogen species |
| $RO_2$ | Peroxyl |
| RONOO | Alkyl peroxynitrates |
| ROS | Reactive nitrogen species |
| RPO | Red palm oil |
| RTE | Aqueous rooibos extract |
| SOD | Superoxide dismutase |
| SRC | Standard rat chow |
| STZ | Streptozotocin |
| TBA | 2-Thiobarbituric acid |
| TBARS | Thiobarbituric acid reactive substances |
| TC | Total cholesterol |

| | |
|---|---|
| TEAC | Trolox equivalence antioxidant capacity |
| TG | Triglycerides |
| TNFα | Tumor necrosis factor alpha |
| TPTZ | Tripyridyl triazine |
| UAE | Urinary albumin excretion |
| VLDL | Very low density lipoprotein |

# CHAPTER ONE

# INTRODUCTION

Hyperglycaemia is a condition in which a high amount of glucose circulates in the blood. Chronic hyperglycaemia is the defining characteristic of the disease known as diabetes mellitus (DM) (Conget, 2002). Uncontrolled chronic hyperglycaemia as a result of absolute insulin deficiency (type 1 diabetes) or insulin resistance with or without insulin deficiency (type 2 diabetes) is one of the primary causes of diabetic complications in a number of organs (Wang *et al.,* 2012). DM is a complex, progressive disease, which is accompanied by multiple complications. It is a metabolic disorder of the endocrine system (Li *et al.*, 2004) and among the most common disorders in both developed and developing countries (Mukund *et al.*, 2008; Zhou *et al.,* 2009). It has become a global metabolic epidemic, affecting important biochemical activities in nearly every age group (Gupta, 2008; Singh *et al.,* 2012). The number of people affected by diabetes was estimated to have risen by 50% by 2010, and will almost be doubled by 2025 (Zimmet *et al.*, 2001; Bethel *et al.*, 2007). It has been estimated that the number of people with diabetes will rise from the present 150 to 230 million in 2025 (Iraj *et al.*, 2009; Abu-Zaiton, 2010). Some of the causes of an increased risk of diabetes are due to an increase in sedentary lifestyle, consumption of an energy rich diet, obesity, higher life span (Deore *et al.,* 2012). There is also an increasing evidence for the role of genetic factors in several diabetic complications, particularly diabetic nephropathy and cardiovascular complications of diabetes (Bowden, 2002).

Prolonged hyperglycaemia results in the formation of advanced glycation end-products (AGE) in body tissues of these patients (Deepralard *et al.*, 2009). During the hyperglycaemic states, the antioxidant defence system that exists naturally in humans is altered (Jabeen *et al.,* 2012). Chronic hyperglycaemia of diabetes is linked to long term damage, dysfunction and damage to various organs (Lyra *et al.,* 2006; Murti *et al.,* 2012). Chronic hyperglycaemia leads to many long-term complications in the eyes, kidneys, nerves, heart, and blood vessels (Laakso, 2010). It has been reported that diabetes is a risk factor for cardiovascular disease (Oguntibeju *et al.*, 2009b; Laakso, 1999 & 2010) and more than 70% of type 2 diabetic patients die of cardiovascular diseases (Laakso, 2001). Oxidative stress has been suggested to be a common pathway linking diverse mechanisms for the pathogenesis of complications in diabetes (Ha and Lee, 2000; Mehrotra *et al*, 2001; Shih *et al.*, 2002). It has been reported that oxidative stress participates in the progression of insulin resistance (Evans *et al.*, 2002).

Diabetes is known to have a multifactorial pathogenicity and therefore, demands a multi-modal therapeutic approach. Great efforts have been made in the understanding and management of diabetes but serious problems like diabetic neuropathy (Shaikh and Somani, 2010), diabetic retinopathy (Schwartz and Flynn Jr., 2007), diabetic nephropathy (Djordjevi´c, 2001), hepatopathy (Levinthal and Tavill, 1999), cardiovascular diseases (Stratmann and Tschoepe, 2009) and reproductive problems (Baccetti *et al.,* 2002) continue to confront diabetic patients. The recognition of the potential role for nutraceuticals and dietary supplements in helping to reduce health risks and improve health quality is on the increase (Singh *et al.,* 2012). The control of diabetes can be attained by diet, exercise, insulin replacement therapy and by using herbal hypoglycaemic agents (Ivorra and Paya, 1989; Mallick *et al.,* 2007). Many drugs are available for use in the treatment of diabetes, but their long-term use may cause adverse side effects and hence, the increased search for natural remedies for the effective treatment of diabetes exists (Nabeel *et al*., 2010).

Plants have always been a very good source of drugs and many of the presently obtainable drugs are directly or indirectly made from them (Patel *et al.,* 2012). Plants used for medicinal purposes are frequently considered to be less toxic and induces fewer side effects than synthetic ones. Plants that are most often implicated as having anti-diabetic effects contain glycosides, alkaloids, terpenoids, flavonoids, carotenoids (Loew and Kaszkin, 2002). Anti-hyperglycaemic effects of these plants are due to their capability to improve the function of pancreatic tissues by causing an increase in insulin output or inhibit the intestinal absorption of glucose or the facilitation of metabolites in insulin dependent processes (Ngondi *et al.,* 2006). More than 800 plant species have been reported to be having hypoglycaemic activity in available literatures (Patel *et al.,* 2012).

Red palm oil (RPO) is the most broadly produced edible vegetable oil which is extracted from the tropical palm tree (*Elaeis guineensis*) fruits and has served as a nutritious source of oil for thousand of years (Chandrasekharan *et al*., 2000; Mukherjee and Mitra, 2009; Ibegbulem and Chikezie, 2012). The source of the oil palm is the tropical rain forest region of West Africa and traditionally, RPO has been an important cooking oil in the diets of people living in or near this region (Atinmo and Babre, 2003; Rice and Burns, 2010). Red palm oil has been reported to help in the improvement of human health (Oguntibeju *et al.,* 2009a). It contains lipid-soluble antioxidants such as carotenoids (α- and β- carotene, lycopenes), vitamin E (in the form of α-, β-, δ- tocotrienols and tocopherol) and ubiquinone (Oguntibeju *et al.,* 2010). The dietary intake of foods containing carotenoids was linked with a reduced risk of some types of cancer (He *et al.,* 1997; Lu *et al*., 2005) and cardiovascular diseases (Palace *et al.,* 1999; Liu *et al.,* 2001; Voutilainen *et al.,* 2006). Vitamin E has also been reported to be beneficial in reducing type 2

diabetes (Montonen *et al.,* 2004) and its complications such as cardiovascular diseases and arteriosclerosis (Naziroglu *et al.,* 2004). Studies have shown that palm oil was able to reduce oxidative stress-induced hypertension in normal rats (Edem, 2002; Bayorh *et al.*, 2005). It has been reported that red palm oil was protective against the consequences of ischaemia /reperfusion injury (Esterhuyse *et al.*, 2005; 2006, Bester *et al.*, 2006). Another study showed that red palm oil could possibly inhibit apoptosis in rat sperm (Aboua *et al.*, 2009). Oguntibeju *et al.* (2009) reported a possible role of red palm oil in reducing oxidative stress in HIV/AIDS and tuberculosis patients.

On the other hand, the second plant product examined in this study is rooibos (*Aspalathus linearis*) and it is grown on the Cedarberg mountain range area in the Western Cape province of South Africa. It is commonly used to make a refreshing beverage referred to as rooibos tea. Rooibos is gaining popularity, as a result of its many health properties which is marked by a rapidly growing number of rooibos consumers throughout the world (Gilani *et al.,* 2006). It is a rich source of polyphenols and used to make a mild-tasting tea with no caffeine and low in tannins compared to green or black teas (Iswaldi *et al.,* 2011). A group of antioxidants is called polyphenols because they have a phenolic ring in their chemical structure and the polyphenol group is further divided into subgroups such as the flavonoids (Erickson 2003). Flavonoids are biologically active, polyphenolic constituents of plant foods which are found in various fruits, vegetables, legumes and beverages such as tea and wine (Nettleton *et al.,* 2006) and have the ability to scavenge free radicals and chelate metals (Saija *et al.,* 1995; Wilcox *et al.,* 1999; Geckil *et al.,* 2005; Nettleton *et al.,* 2006). Polyphenolic compounds are widely known to play important roles in protecting the body against various chronic diseases, such as cardiovascular diseases (Manach *et al.,* 2005; Vita, 2005; Stangl *et al.,* 2007), diabetes mellitus (Knekt *et al.*, 2002; Jung *et al.,* 2006; Lukačínová *et al.*, 2008; Pinent *et al.*, 2008), cancer (Carroll *et al.,* 1998; Mukhtar and Ahmad, 2000; Knekt *et al.*, 2002; Le Marchand, 2002; Surh, 2003; Manson, 2003) and asthma (Knekt *et al.*, 2002). Studies have shown that rooibos has anti-ageing (Inanami *et al.*, 1995), anti-HIV (Nakano *et al.,* 1997), hepatoprotective (Ulicna *et al.,* 2003), anti-spasmodic (Gilani *et al.*, 2006), anti-oxidative (Ulicna *et al.,* 2006), anti-mutagenic (Van der Merwe *et al.,* 2006), anti-cancer (Marnewick *et al.,* 2005; 2009), anti-inflammatory (Baba *et al.*, 2009), cardio-protective (Pantsi *et al.,* 2011; Marnewick *et al.,* 2011 ) and reproductive protective (Awoniyi *et al.,* 2012) effects .

**Aims of the study**

This research study was conducted to investigate the potential health promoting effects of two antioxidant rich-plant products (red palm oil and rooibos) on diabetic rats by measuring certain biomarkers in the blood and liver of streptozotocin-induced hyperglycaemic Wistar rats. This study is novel as it is the first of its kind to investigate the possible effects of the combination of red palm oil and rooibos supplementation on diabetes.

The research study conducted was two fold:

i) A preliminary investigation was carried out using three different doses of red palm oil (1 ml/day, 2 ml/day and 4 ml/day) and rooibos extracts (2%, 4% and 6% w/v) in order to investigate their biochemical effects over a period of time in non-diabetic conditions. The rats were fed these doses for each of the plant products over a period of seven (7) weeks. Various parameters such as body weight gain, lipid profiles, antioxidant status (antioxidant enzymes and antioxidant capacity) and histopathological effects were evaluated. Assessment of possible accumulation of fatty acids in the liver of the rats fed the red palm oil supplemented diet was also performed.

ii) None of the three dosages used in the preliminary studies in (i) above showed no adverse effects and hence, a dose of each plant product (2 ml red palm oil and 2% aqueous rooibos extract) was singly and in combination used on streptozotocin-induced hyperglycaemic rats over a period of seven (7) weeks. The effects of these plant products were investigated on the blood and liver of the rats. Glycaemic and lipidaemic parameters, antioxidant status, biomarkers of liver function and some biochemical parameters were assessed.

The thesis has been written in an article based-format and consists of seven chapters. Chapter I is a brief introduction which highlights the link between hyperglycaemia and diabetes as well as the need for natural remedies in the management of diabetes. The aims of the research study are also included. Chapter II (literature review) focuses on the involvement of hyperglycaemia and oxidative stress in diabetes as well as proposing the use of rooibos and red palm oil as possible treatment strategies in the management of diabetes. Chapter III is the first article entitled “Effects of dietary intake of red palm oil on fatty acid composition and lipid profiles in male Wistar rats”. This article has been published in the African Journal of Biotechnology. Chapter IV is the second article entitled “Impact of dietary red palm oil on antioxidant status and liver histopathology in male Wistar rats” which has been submitted for publication in Physiological Research and it is currently under review. Chapter V is the third

article entitled “Assessment of lipid profiles, antioxidant status and liver histopathology in male Wistar rats following consumption of rooibos” that has been submitted for publication in BMC Alternative and Complementary Medicine and it is currently under review. Chapter VI is the fourth article entitled “Ameliorative effects of red palm oil and rooibos on hyperglycaemia, lipid parameters and liver function in streptozotocin induced-diabetic male Wistar rats” that will be submitted to Journal of Ethnopharmacology for publication. Chapter VII is the fifth article entitled “Modulatory effects of rooibos and red palm oil on antioxidant status in streptozotocin induced- hyperglycaemic male Wistar rats” that will be submitted to Phytomedicine for publication. Chapter VIII is a general discussion and conclusion of the entire study.

# CHAPTER TWO

# LITERATURE REVIEW

The fundamental mechanism underlying diabetes mellitus is the lack of biologically active insulin which leads to alterations in the uptake and storage of glucose and reduced usage of glucose for energy purposes (Saravanan and Ponmurugan, 2012). Increased oxidative stress contributes to the deterioration of pancreatic β-cells progressively more due to glucose toxicity which leads to severe impairment of glucose-stimulated insulin secretion and β-cell damage (Likidlilid *et al.,* 2010). The liver is a vital insulin-dependent tissue that plays a critical role in glucose and lipid homeostasis and is severely affected during diabetes (Seifter *et al.,* 1982; Rajasekaran *et al.,* 2006). Red blood cells are distinctive, highly specialized and the most abundant cells in humans and contain high levels of both enzymatic and non-enzymatic cytoplasmic antioxidants (Pandey and Rizvi, 2010). They are the first cells in the body to be exposed to stressful stimuli and hence, prone to oxidative stress (Pandey and Rizvi, 2010). Abnormally elevated levels of free radicals and a concurrent decrease in antioxidant defence system can lead to destruction of cellular organelles and enzymes, increased lipid peroxidation and development of insulin resistance (Maritim *et al.,* 2003). The most important defence mechanism against free radicals in the body is mediated by the actions of antioxidants (Astaneie *et al.,* 2005). Much interest in the role and usage of natural antioxidants as a means to prevent oxidative damage in diabetes with high oxidative stress has developed (Babujanarthanam *et al.,* 2011).

## 2.1 MECHANISM OF INDUCTION OF DIABETES BY STREPTOZOTOCIN

Streptozotocin (STZ), originally identified in the late 1950`s as an antibiotic is a naturally occurring compound that is produced by the bacterium *Streptomyces achromogenes* and shows broad spectrum antibacterial properties (Vavra *et al.,* 1959; Sharma, 2010). STZ was later discovered to be particularly toxic to pancreatic β-cells that secrete insulin and has since been used extensively to create animal models of type I diabetes (Mansford and Opie, 1968; Pathak *et al.,* 2008). It induces diabetes which resembles human hyperglycaemic non-ketotic diabetes mellitus in animal models (Weir *et al*., 1981). STZ selectively destroys the insulin producing β-cells by inducing necrosis and hence, it is diabetogenic (Sharma, 2010). Its action on β-cells is accompanied by characteristic alterations in blood insulin and glucose concentrations (Szkudelski, 2001). The glucose moiety in the structure of STZ enables it to be

transported through GLUT 2 (Elsner *et al.,* 2000) and thus, insulin-producing cells that do not express this glucose transporter are resistant to STZ (Lenzen, 2007).

STZ is able to produce nitric oxide (NO), a bioregulatory and cytotoxic molecule and it has been indicated that direct NO-generation may be a mechanism of STZ toxicity in diabetogenesis (Kwon *et al.,* 1994). Wada and Yagihashi (2004) also reported that nucleic acid alkylation or excessive nitric oxide (NO) generation has been proposed to contribute to STZ-induced beta-cell damage. The production of NO by STZ could damage genomic DNA and may cause beta-cell dysfunction by inhibiting mitochondrial enzymes (Wada and Yagihashi, 2004). The DNA damage caused by alkylation that is mediated by STZ is being repaired by an excision repair process and requires the activation of the NAD dependent enzyme poly (ADP-ribose) synthetase (Wilson and Leiter, 1990; Sharma, 2010). This process leads to depletion of cellular NAD and ATP and the increased ATP dephosphorylation provides substrate for xanthine oxidase which leads to generation of superoxide radicals and consequently leads to the formation of hydrogen peroxide and hydroxyl radicals (Szkudelski, 2001).

The link between STZ and a cytosolic protein post-translational modification through O-glycosylation with N-acetylglucosamine (O-GlcNAc) has recently been proposed to be a mechanism of STZ toxicity effects and it is referred to as O-GlcNAc-dependent model of STZ toxicity (Pathak *et al.* 2008). Streptozotocin is proposed to induce apoptosis by inhibiting O-GlcNAcase, the enzyme that, together with O-GlcNAc transferase, is responsible for the reversible intracellular OGlcNAc post-translational modification (Pathak *et al.* 2008; He *et al.,* 2009). *O*-GlcNAc-selective *N*-acetyl-b-d-glucosaminidase (OGlcNAcase) removes *O*-GlcNAc from protein and is the final enzyme in the pathway of O-glycosylation in the β-cells (Konrad *et al.,* 2001). Streptozotocin elevated *O*-GlcNAc levels in pancreatic islets and contributed to the destruction of β-cells (Liu *et al.,* 2000). Evidence has also been shown that protein modification may be specifically important in the β-cells because *O*-GlcNAc transferase (OGT) is very much enriched in β-cells than any other cell (Liu *et al.,* 2000).

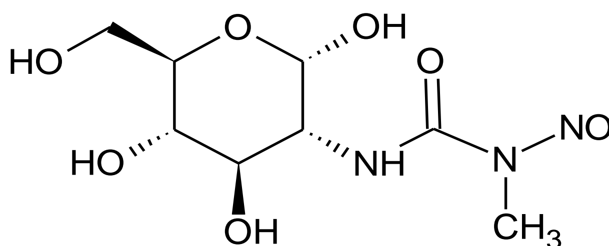



**Figure 1: Structure of Streptozotocin**

## 2.2 HYPERGLYCAEMIA AND DIABETES MELLITUS

Glucose is the major fuel for most body tissues and it is largely derived from the ingestion of carbohydrates into the body. Glucose in solution is a ring structure, in equilibrium with an open-chain aldehyde form in small amount (Monnier, 1990; Mohora *et al.,* 2007). Hyperglycaemia (high glucose level) is the net result of higher glucose influx than glucose outflow from the plasma compartment and it is directly linked to increased hepatic glucose production in the fasting state (Inzucchi *et al.,* 2012). Diabetes mellitus is a complex metabolic disorder in the endocrine system characterized by abnormalities in insulin secretion and/or insulin action that leads to the progressive deterioration of glucose tolerance which causes hyperglycaemia. Symptoms of the endocrine disorder include glucosuria, ketoacidosis, hypercholesterolaemia and hypertriglyceridaemia with loss of weight and caloric deficits (Granner, 2000; Eteng *et al.,* 2008). There are two main categories of the disease, type 1 diabetes mellitus also called insulin-dependent diabetes mellitus (IDDM) and type 2, the non-insulin dependent diabetes mellitus (NIDDM) (Raubenheimer, 2010). The most prevalent form of diabetes mellitus is type 2 diabetes and it typically makes its appearance at the later stage of life (Grundy *et al.*, 1999). The cause of type 2 diabetes may be due to the combined effects of impairment in the insulin-mediated glucose disposal and defective secretion of insulin by the β-cells of the pancreas (Grundy *et al.*, 1999). Diabetes affects numerous organs and persistent hyperglycaemia can lead to destruction of non-insulin sensitive organs where there are no “gate keepers” in the form of insulin receptors that restrict the entry of glucose into the cell (Albright and Bell, 2003). Hyperglycaemia has tissue-damaging effects on a subset of cell types such as capillary endothelial cells of the retina, mesangial cells in the renal glomerulus, and neurons in the peripheral nerves (Brownlee, 2005).

## 2.3 OXIDATIVE STRESS IN DIABETES

Oxidative stress, an imbalance between the generation of reactive oxygen species/ reactive nitrogen species and antioxidant defence capacity of the body, is actively involved in the pathogenesis of diabetes and its complications (Ha and Lee, 2000; Bonnefont-Rousselot, 2002; Johansen *et al.*, 2005; Oguntibeju *et al.,* 2010). Reactive oxygen species/ reactive nitrogen species include free radicals such as superoxide ($^{\cdot}O_2^-$), hydroxyl ($^{\cdot}OH$), peroxyl ($^{\cdot}RO_2$), hydroperoxyl ($^{\cdot}HRO_2^-$), nitric oxide (NO) and nitrogen dioxide ($^{\cdot}NO_2^-$) and non-free radical such as hydrogen peroxide ($H_2O_2$), hydrochlorous acid (HOCl), peroxynitrite (ONOO), nitrous oxide ($HNO_2$) and alkyl peroxynitrates (RONOO) (Johansen *et al.*, 2005; Higashi *et al.*, 2006; Oguntibeju *et al.*, 2010). Diabetes has been associated with increased oxidative stress, which

may contribute to microvascular and macrovascular complications (Giugliano *et al.*, 1996). Hyperglycaemia is mediated in large part, by a state of enhanced oxidative stress which results in the excessive production of reactive oxygen species which can cause adverse structural and functional changes in tissues (Mehta *et al.*, 2006; Robertson and Harmon, 2006).

Several mechanisms appear to be involved in hyperglycaemia such as glucose autoxidation, stimulation of the polyol pathway, activation of the reduced form of nicotinamide adenine dinucleotide phosphate oxidase, and production of advanced glycation end-products (AGEs) which leads to increased generation of reactive oxygen species (Bonnefont-Rousselot *et al.,* 2000; Bonnefont-Rousselot, 2002). Figure 2 illustrates the link between hyperglycaemia, mitochondria ROS generation, oxidative stress, activation of stress-sensitive pathways insulin resistance, β-cells dysfunction and diabetic complications. Glycation is a major source of reactive oxygen species and reactive carbonyl species that are caused by both oxidative (glycoxidative) and non-oxidative pathways (Rahbar and Figarola, 2003). Elevated non-enzymatic glycation of proteins, lipids and nucleic acids due to the formation of advanced AGEs is accompanied by oxidative, radical-generating reactions and therefore represents a major source for oxygen free radicals under hyperglycaemic conditions (Mohamed *et al.*, 1999). It has been reported that glycation may result in the production of superoxide (Jones *et al.*, 1987; Sakurai and Tsuchiya, 1988). The formation of AGEs is also accompanied by an increased oxidation of low density lipoprotein (LDL) and an increase in atherogenic oxidized LDL occurs in diabetes (Bucala, 1997). The non-enzymatic glycation of haemoglobin has been established and shown to be significantly increased in diabetes (Goldstein, 1995).

Protein glycation alters protein and cellular function, and binding of AGEs to their receptors can lead to modification in cell signalling and further production of free radicals (Penckofer *et al.*, 2002). Glycoxidation of collagens contributes to development of vascular complications in diabetes (Urios *et al.,* 2007). In an *in vitro* study on HIT-T15 cells, induced glycation suppressed insulin gene promoter activity and its mRNA levels by provoking oxidative stress through glycation reaction (Matsuoka *et al.*, 1997). Reactive oxygen species, particularly superoxide anions could inactivate endothelium-derived NO to form potent oxidant $ONOO^-$ which contributes to the development of endothelial dysfunction in diabetes (Kodja and Harrison, 1999; Laight *et al.*, 2000; Johansen *et al.*, 2005). $O_2^-$ can also activate several damaging pathways in diabetes including accelerated formation of AGEs, polyol pathway, hexosamine pathway and protein kinase C, all of which have been proven to be involved in micro and macro vascular complications (Johansen *et al.*, 2005).

Oxidative stress can alter insulin action through a change in the physical state of the plasma membrane of target cells, increased intracellular calcium content and reduction in NO availability (Paolisso and Giugliano, 1996). Mitochondrial overproduction of free radicals is possibly a potential mechanism causing impaired first phase of glucose-induced insulin secretion (Knight, 1998; Sakai *et al.*, 2003) and this process has been associated with the onset of type 1 diabetes via apoptosis of pancreatic β-cells, and the onset of type 2 diabetes via insulin resistance (Bonnefont-Rousselot *et al.*, 2000). Oxidative stress caused by short exposure of β- cell preparations to $H_2O_2$ has been shown to increase the production of p21 and decreases insulin mRNA, cytosolic ATP, and calcium flux in the cytosol and mitochondria (Maechler *et al.*, 1999). β-cells are particularly sensitive to reactive oxygen species due to their low free-radical quenching (antioxidant) enzymes such as catalase, glutathione peroxidase, and superoxide dismutase (Tiedge *et al.*, 1997).

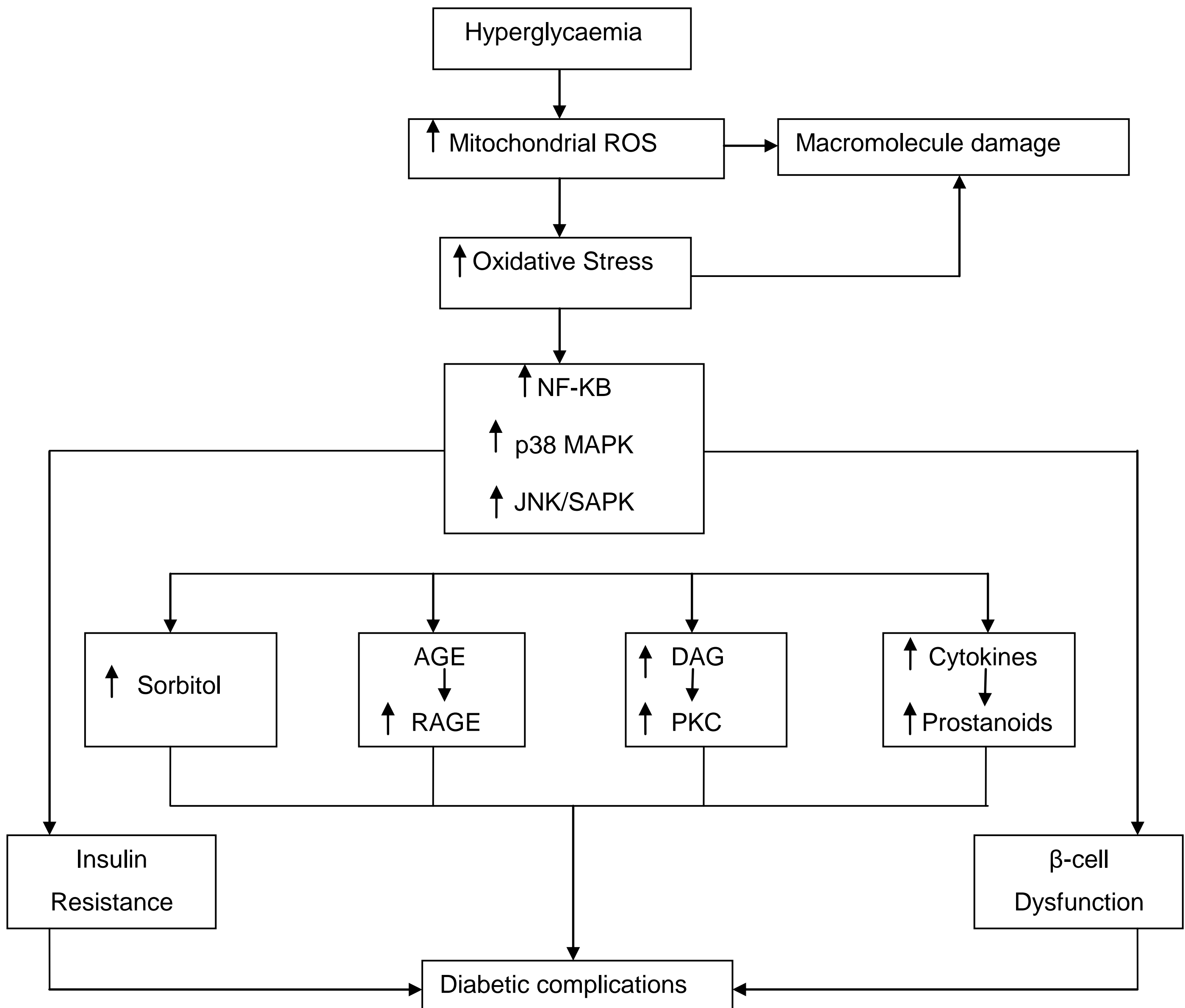



**Figure 2: The diagram describes the link between hyperglycaemia, mitochondrial ROS generation, oxidative stress, activation of stress-sensitive pathways (NF-kB, p38 MAPK, JNK/SAPK, and others), insulin resistance, B-cell dysfunction, and diabetic complications. Increased production of sorbitol (formed as a consequence of the hyperglycaemia-mediated increase in aldose reductase activity), AGE, cytokines, prostanoids, along with PKC activation could function as positive regulatory feedback loops to chronically stimulate stress-sensitive pathways. ROS (and RNS) can also cause oxidative damage directly upon cellular macromolecules and result in oxidative stress (Evans *et al.,* 2002).**

Signal transduction pathways such as c-Jun N-terminal kinase (JNK) (also known as stress-activated protein kinase), p38 mitogen-activated protein kinase (p38 MAPK), and protein kinase C (PKC) are activated by oxidative stress in several cell types including pancreatic β-cells. Kaneto *et al.* (2002) reported that activation of the JNK pathway is involved in the reduction of insulin gene expression by oxidative stress and that suppression of the JNK pathway protects β-cells from oxidative stress. The JNK pathway is reported to be activated under diabetic conditions and is possibly involved in the progression of insulin resistance (Evans *et al.*, 2002). The modulation of the JNK pathway in the liver on insulin resistance and glucose tolerance showed that, suppression of the JNK pathway in the liver produced highly beneficial effects on the insulin resistance status and glucose tolerance in both genetic and dietary models of diabetes (Nakatani *et al.*, 2004).

One major intracellular target of hyperglycaemia, ROS and oxidative stress is the transcription factor NF-kB (Barnes and Karin, 1997; Mohamed *et al*, 1999; Bierhaus *et al.*, 2001). NF-κB belongs to the Rel-family of pluriprotein transcription activators. It is a regulatory protein that controls the expression of numerous inducible and tissue-specific NF-κB responsible genes (Ghosh *et al.*, 1998). NF-κB is usually known to be a central regulator of stress responses, because it can be activated by hundreds of different stimuli, which include lipopolysaccharide, tumor necrosis factor alpha (TNFα), other pro-inflammatory cytokines and environmental stress (Wu *et al.*, 2009). Kabe *et al.* (2005) reported that reactive oxygen species could enhance the signal transduction pathways for NF-kB activation in the cytoplasm and translocation into the nucleus. Reactive oxygen species appeared to serve as common secondary messengers of many different stimuli that activate NF-κB (Shreck *et al.*, 1991).

Elevated levels of AGEs are produced under hyperglycaemic conditions and the interaction of AGEs with specific cellular receptors called AGE receptors (RAGE) is an important factor responsible for increased diabetes (Rahimi *et al.,* 2005). It has been shown that binding of AGEs (and other ligands) to RAGE results in the generation of intra-cellular oxidative stress and subsequent activation of the redox-sensitive transcription factor NF-kB *in vitro and in vivo* (Mohamed *et al.*, 1999). Modification of plasma proteins by AGEs precursors creates ligands that bind to AGE receptors, inducing changes in gene expression in endothelial cells, mesangial cells and macrophages (Brownlee, 2001).

Activation of protein kinase C (PKC) occurs in response to an increase in diacylglycerol (DAG) in various tissues in diabetes and hence, it is involved in the pathological events that cause diabetic complications (Tomkin, 2001). DAG can be generated from the hydrolysis of phosphatidylinositides or the metabolism of phosphatidylcholine by phospholipase C or

phospholipase D and also, by *de novo* synthesis from glycolytic intermediates (Park *et al,* 1999). High blood glucose level appears to stimulate messengial cell proliferation through PKC/NF-kB pathways (Park *et al.*, 2000). Hyperglycaemia-induced oxidative stress may mediate the adverse effects of PKC-beta isoforms by the activation of the DAG-PKC pathway (Koya and King, 1998). PKC can be activated by peroxynitrite, superoxide dismutase and high amount of nitric oxide (Abou-Mohammed *et al.,* 2004). The activation of PKC by intracellular hyperglycaemia has a variety of effects on gene expression (Brownlee, 2005).

The polyol pathway is based on a family of aldo-keto reductase enzymes that can use a wide variety of carbonyl compounds as substrates and reduce to their respective sugar alcohols (polyols) by NADPH (Giacco and Brownlee, 2010). In this pathway, high concentrations of glucose in the cell are reduced to sorbitol by aldose reductase which is later oxidized to fructose. In the process of reducing high intracellular glucose to sorbitol, the aldose reductase consumes the co-factor NADPH, an essential co-factor for regenerating a critical intracellular antioxidant, reduced glutathione. By a reduction in the amount of reduced glutathione, the polyol pathway increases susceptibility to intracellular oxidative stress (Brownlee, 2005). Glucose metabolism through the hexosamine pathway has been implicated in many of the adverse effects of chronic hyperglycaemia. Activation of the hexosamine pathway contributes to the β-cell dysfunction of diabetes through the induction of oxidative stress than O-linked glycosylation (Kaneto *et al.,* 2001). The elevated intracellular *O*-GlcNAc-mediated modification of certain kinds of proteins may suppress the process of glucose transport, thus causing insulin resistance (Akimoto *et al.,* 2005).

## 2.4 DIABETES AND BODY ANTIOXIDANT DEFENCE SYSTEM

Aerobic metabolism is always accompanied by the production of reactive oxygen species and all living organisms have developed antioxidant defence systems against injury as a result of oxidative stress. Free radicals that are formed are rapidly scavenged by natural cellular defence mechanisms which include enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR), catalase (CAT) (Kumawat *et al.,* 2009). Endogenous antioxidant enzymes (CAT, SOD, GPx and GR) and endogenous stimuli leading to ROS generation are illustrated in Figure 3.

### 2.4.1 Catalase (CAT)

Catalase is an antioxidant enzyme that is produced naturally in the body and found in peroxisomes in eukaryotic cells. It is particularly important in conditions where glutathione

(GSH) is limited or the activity of GPx is diminished (Caldwell *et al.,* 2008). CAT degrades Hydrogen peroxide ($H_2O_2$) to water and oxygen and hence, finishes the detoxification reaction started by SOD. Each catalase molecule can convert millions of $H_2O_2$ molecules every second. $H_2O_2$ is a powerful oxidizing agent and is potentially damaging to cells. CAT allows for important cellular processes which produce $H_2O_2$ as a by-product to occur by preventing excessive build up of hydrogen peroxide and also protect against hydrogen peroxide mediated oxidative damage. In the small intestine, CAT activity was significantly increased in the diabetic rats (Bohr *et al.,* 2004). CAT activity has been shown to be significantly high in diabetic patients (Kumawat *et al.,* 2005) and found to be significantly decreased in the liver of diabetic rats (Genet *et al.,* 2002: Sathishsekar and Subramanian, 2005; Jeyashanthi and Ashok, 2010; Meenakshi *et al.,* 2010; Pari *et al.,* 2010; Sancheti *et al.,* 2010; Babujanarthanam *et al.,* 2011; Makni *et al.,* 2011a, 2011b; Atangwho *et al.,* 2012). Decrease in the activity of catalase in the plasma (Jeyashanthi and Ashok, 2010; Makni *et al.,* 2011a), pancreas (Abdelmoaty *et al.,* 2010; Babujanarthanam *et al.,* 2011), kidney (Jeyashanthi and Ashok, 2010; Pari *et al.,* 2010; Sancheti *et al.,* 2010), brain and sciatic nerve tissues (Uzar *et al.,* 2012) of diabetic rats have been shown. Increased activities of catalase in the heart (Genet *et al.,* 2002) and hippocampus (Ceretta *et al.,* 2012) have been reported in diabetic animals. No significant effects in the activities of catalase in the kidney (Sadi *et al.,* 2012) and liver (Ugochukwu *et al.,* 2003) have been shown in diabetic rats. The uncontrolled generation of $H_2O_2$ as a result of the auto-oxidation of glucose, protein glycation and lipid oxidation in diabetes is markedly responsible for the decline in catalase activity (Saravanan and Ponmurugan, 2012).

### 2.4.2 Superoxide dismutase (SOD)

SOD is an antioxidant enzyme that catalyzes the conversion of two superoxides into $H_2O_2$ and oxygen. It acts as a major defence system against the cytotoxic effects of superoxide radicals (Caidwell *et al.,* 2008). SOD is metal-containing enzyme that depends on bound trace metals for antioxidant activity. They are of two types: copper/zinc (Cu/Zn) SOD and manganese (Mn) SOD and each type of SOD plays a different role in keeping cells healthy. Different isoforms of SOD are located at different sites within the cells (Caldwell *et al.,* 2008). Cu/Zn SOD protects the cell`s cytoplasm, and Mn SOD protects the mitochondria from free radical damage. A non-significant effect was observed in the kidney SOD of diabetic rats (Sadi *et al.,* 2012). In another study, Bohr *et al.* (2004) showed a significant increase in small intestine SOD activity of diabetic rats. A decrease in the activity of SOD in the liver of diabetic rats has been shown by several studies (Genet *et al.,* 2002; Ugochukwu *et al.,* 2003; Sathishsekar and Subramanian, 2005; Jeyashanthi and Ashok, 2010; Meenakshi *et al.,* 2010; Pari *et al.,* 2010;

Sancheti *et al.,* 2010; Babujanarthanam *et al.,* 2011; Makni *et al.,* 2011a, 2011b). A similar decrease in the activity of SOD in the plasma (Jeyashanthi and Ashok, 2010; Makni *et al.,* 2011a), pancreas (Abdelmoaty *et al.,* 2010; Babujanarthanam *et al.,* 2011), kidney (Jeyashanthi *et al.,* 2010; Pari *et al.,* 2010; Sancheti *et al.,* 2010) and striatum and amygdala (Ceretta *et al.* 2012) of diabetic rats have been shown. Increased activity of SOD in the brain of diabetic rats has also been reported (Genet *et al.,* 2002). Decrease in the activity of SOD in diabetes could possibly be a response to increased generation of $H_2O_2$ and $O_2$ by the autoxidation of glucose and non-enzymatic glycation (Pari and Latha, 2004). Kumawat *et al.* (2005) has also reported that the reduced activity of SOD in the erythrocytes of diabetic rats could be due to ageing or an increase in the glycation of SOD (Kumawat *et al.,* 2005).

### 2.4.3 Glutathione peroxidase (GPx)

Glutathione peroxidase is a group of enzymes of which most contain selenium. It helps to protect the cell from damage due to free radicals like hydrogen and lipid peroxides and its actions take place in the presence of glutathione, the master antioxidant. They act like catalase by degrading hydrogen peroxide. GPx metabolizes hydrogen peroxide to water with the usage of reduced glutathione as a hydrogen donor (Maritim *et al.,* 2003; Caldwell *et al.,* 2008). They also reduce organic peroxides to alcohols, providing another way for the removal of toxic oxidants. Decreased activity of GPx in the liver of diabetic rats has been reported (Genet *et al.,* 2002; Sathishsekar and Subramanian, 2005; Lapshina *et al.,* 2006; Jeyashanthi and Ashok, 2010; Meenakshi *et al.,* 2010; Pari *et al.,* 2010; Atangwho *et al.,* 2012) while its activity was significantly high in diabetic patients (Kumawat *et al.,* 2005). Increase in the activities of GPx in the kidney (Genet *et al.,* 2002; Sadi *et al*., 2012) and muscle tissues (Kurt *et al.,* 2011) of diabetic rats has been documented. A decline in GPx activity of the small intestine (Bohr *et al*., 2004), kidney (Jeyashanthi and Ashok, 2010; Pari *et al.,* 2010), lens (Preet *et al.,* 2006), brain (Nakhaee *et al.,* 2010) and plasma (Jeyashanthi and Ashok, 2010) have been shown in diabetic rats. A decrease in the activity of GPx in the pancreas of diabetic rats has also been reported (Ugochukwu *et al.,* 2003; Abdelmoaty *et al.,* 2010; Babujanarthanam *et al.,* 2011). Reduced activity of GPx could be due to low content of glutathione in diabetic state, since glutathione serves as a substrate and cofactor of GPx (Saravanan and Ponmurugan, 2012). Decrease in GPx activity could be a result of a number of deleterious effects due to the accumulation of toxic products (Saravanan and Ponmurugan 2011; 2012).

### 2.4.4 Glutathione reductase (GR)

Glutathione reductase is a cellular antioxidant enzyme that regenerates glutathione from glutathione disulfide by recycling using NADPH (Maritim *et al.,* 2003). For every mole of oxidized glutathione (GSSG), one mole of NADPH is needed to reduce GSSG to reduced glutathione (GSH). GR maintains the levels of glutathione in the cells (Blakytny and Harding, 1992). GR plays an essential function in the protection of haemoglobin, red cell enzymes and biological cell membranes against oxidative damage (Waggiallah and Alzohairy, 2011). Bohr *et al.* (2004) showed no significant difference in the activity of GR in the small intestine of diabetic rats. Similarly, no significant difference in the activity of GR in the cardiomyocyte has also been shown in diabetic rats (Ghosh *et al.,* 2004). An increase in the activity of GR in muscle tissues of diabetic rats has been shown (Kurt *et al.,* 2011). In diabetic patients, the level of GR in the erythrocytes was significantly decreased (Kumawat *et al.,* 2005). A decrease in the activity of GR in the pancreas (Babujanarthanam *et al.,* 2011), brain (Nakhaee *et al.,* 2010) and lens (Preet *et al.,* 2006) of diabetic rats has been reported. Decrease in GR is due to reduced GSH concentration which results in increased free radicals and leads to oxidative stress in diabetes (Waggiallah and Alzohairy, 2011).

### 2.4.5 Glutathione (GSH)

Glutathione is classified as a tripeptide because it is made up of three amino acids: cysteine, glutamic acid, and glycine. It can be found in every part of the body, particularly the lungs, intestinal tract, and liver. It can be found in large concentrations in the liver where it is used to detoxify harmful compounds so that they can be removed from the body through the bile. Reduced GSH is a major component of the intracellular defence system (Caldwell *et al.,* 2008). GSH functions as a direct free-radical scavenger, co-substrate for glutathione peroxidase activity, co-factor for many enzymes and also forms conjugate in endo- and xenobiotic reactions (Maritim *et al.,* 2003). Not only does it protect the body against free radical attacks, it is also helpful in a well functioning immune system. Sakamaki *et al.* (1999) reported a decrease in GSH of embryonic tissues of diabetic pregnant rats. The level of GSH is significantly decreased in kidneys of diabetic rats (de Cavanagh *et al.,* 2001; Sathishsekar and Subramanian, 2005; Sancheti *et al.,* 2010; Sadi *et al.,* 2012). A decrease in the level of GSH in the liver of diabetic rats has been reported (de Cavanagh *et al.,* 2001; Ozsoy-Sacan *et al.,* 2004; Patriarca *et al.,* 2005; Sathishsekar and Subramanian, 2005; Sancheti *et al.,* 2010; Babujanarthanam *et al.,* 2011; Makni *et al.,* 2011a, 2011b). Similar reduction of GSH in the blood of diabetic rats has also been shown (Ozsoy-Sacan *et al.,* 2004). In another study, the GSH level in the livers of diabetic rats did not change (Lapshina *et al.,* 2006). A decrease in the levels of GSH in the plasma (Makni *et al.,* 2011a), cardiomyocyte (Ghosh *et al.,* 2004) and

pancreas (Babujanarthanam *et al.,* 2011) has been reported in diabetic rats. In diabetes, decrease in GSH levels could probably be due to its increased use by the hepatic cells as a result of decreased synthesis or increased degradation of GSH by oxidative stress (Saravanan and Ponmurugan, 2012).

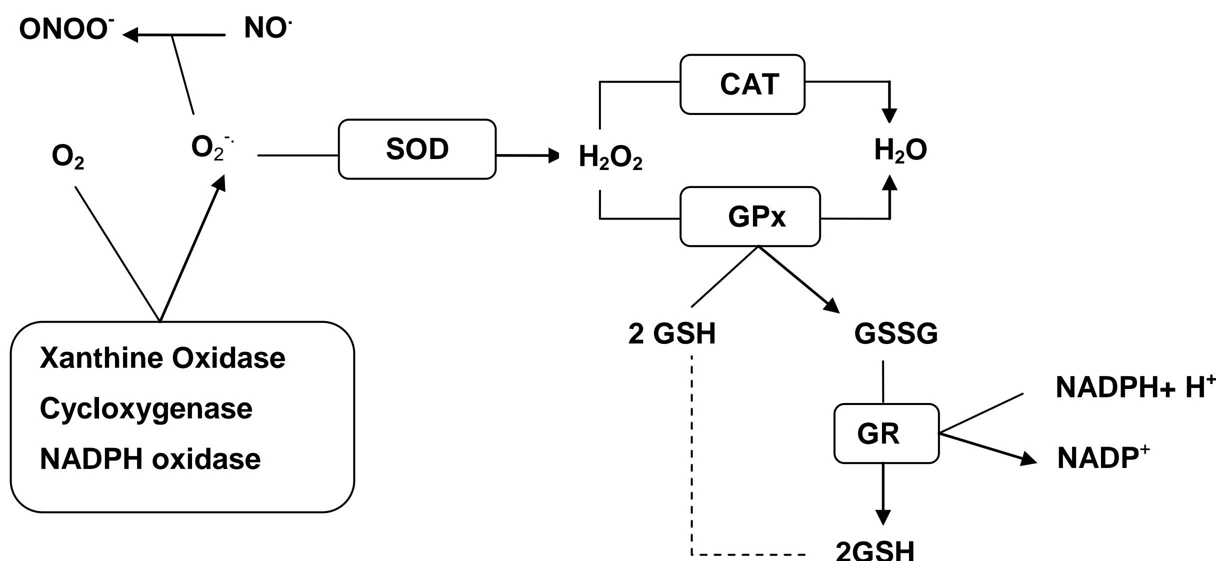



**Figure 3: Production of one ROS may lead to the generation of others through radical chain reactions. Superoxide anion is produced by one electron reduction of oxygen by several different oxidases which includes NAD(P) oxidase, xanthine oxidase, cyclooxygenase and endothelial nitic oxide synthase (Enos) under certain conditions. Nitric oxide ($NO^{\cdot}$) reacts with superoxide anion ($O2^{-}$) and generates the highly reactive molecule peroxynitrite ($ONOO^{-}$). It also illustrates the endogenous antioxidant enzymes (SOD, CAT, GPx, GR) which function to maintain redox equilibrium (Adapted from Mohora *et al.,* 2007).**

## 2.5 DIABETES AND ITS COMPLICATIONS

Hyperglycaemia is the initiating cause of diabetic tissue damage and the process is modified by both genetic determinants of individual susceptibility and by independent accelerating factors such as hypertension and hyperlipidaemia (Brownlee, 2005).

### 2.5.1 Neuropathy

Neuropathy (disease or abnormality of the nervous system) is a microvascular complication of diabetes mellitus which results in considerable morbidity and a decreased quality of life (Van Acker *et al.,* 2009; Mijnhout *et al.,* 2010; Bertolotto and Massone, 2012). It is characterized by a slowly progressive, length-dependent loss of sensation that correlates with duration of

diabetes and glycaemic control (Kern *et al,* 2009). Neuropathy is the most common complication of diabetes mellitus and occurs in 60% of the patients and affects their quality of life (Shaikh and Somani, 2010). Diabetic neuropathy causes foot ulceration, may lead to amputation and chronic pain with reduced quality of life and the most common among the diabetic neuropathies are diabetic peripheral neuropathy (DPN) and diabetic autonomic neuropathy (DAN) (Sasase and Ohta, 2011). Shaikh and Somani (2010) reported that many abnormalities that are found in diabetic patients with neuropathy, including hyperalgesia (extreme sensitivity to pain), allodynia (pain that results from a non-injurious stimulus to the skin), slow nerve conduction velocity and progressive sensory and sensory motor deficit are seen in diabetic rodents.

### 2.5.2 Retinopathy

Diabetic retinopathy (disorder of retinal blood vessels) is often the cause of new cases of blindness among adults aged 20–74 years and the duration of diabetes is probably the strongest predictor for development and progression of retinopathy (Fong *et al.,* 2004). Diabetic retinopathy is duration-dependent which develops in stages and it is often not detected in the first few years of diabetes, but increases to 50% by 10 years and to 90% by 25 years of diabetes (Kowluru and Chan, 2007). It is regarded as a disease of the retinal microvasculature and has been divided into an early, non-proliferative (or background) stage, and a later, proliferative stage (Kern, 2007). The two most vital visual complications of diabetic retinopathy are diabetic macular edema (DME) and proliferative diabetic retinopathy (PDR) (Schwartz and Flynn Jr., 2007). Retinopathy is characterized by a spectrum of retinal lesions and abnormalities that show vascular damage and death or dysfunction of the neural retina (Kern *et al*, 2009). At all stages of retinopathy, macular edema, characterized by retinal thickening from leaky blood vessels, can develop (Fong *et al.,* 2004). In this diabetic complication, the microvasculature of the retina is damaged, the blood vessels swell and seep out fluid and if not prevented, new vessels start to grow, which eventually lead to the detachment of the retina (Frank, 2004; Aylward, 2005; Kowluru and Chan, 2006).

### 2.5.3 Nephropathy

Diabetic nephropathy (diabetic kidney disease) is a major microvascular complication (Anjaneyulu and Chopra, 2004). It is categorized into stages: microalbuminuria, the presence of small amounts of albumin in the urine (UAE > 20 μg/min and ≤ 199 μg/min) and macroalbuminuria, the presence of high amounts of albumin in the urine (UAE ≥ 200 μg/min) (Gross *et al.,* 2005). Diabetic nephropathy occurs in ~ 30% of people with type 1 diabetes and 25-40% of people with type 2 diabetes, often irrespective of glycaemic control (Hall, 2006).

The presence of microalbuminuria is considered to be a manifestation of renal and generalized endothelial injury and strongly predicts progressive diabetic nephropathy and cardiovascular risk and hence, microalbuminuria appearance is used as an important indicator of effective treatment intervention (Hall, 2006). It is the chief cause of chronic kidney disease in patients starting renal replacement therapy and is associated with increased cardiovascular mortality (Gross *et al.,* 2005). Pathophysiological changes associated with diabetic nephropathy include renal and glomerular hypertrophy, mesangial cell hypertrophy and matrix accretion, glomerular basal membrane thickening and functional alterations in glomerular filtration barriers (Djordjevi´c, 2001).

### 2.5.4 Hepatopathy

Diabetic hepatopathy (disease of the liver) causes lesions to develop in the liver. Diabetic patients have a high prevalence of liver disease and it is an important cause of death in type 2 diabetes (Abolfathi *et al.,* 2011). Increased occurrence of liver disease arises in both type 1 and type 2 diabetic patients, resulting in an increased prevalence of hepatic complications (Albright and Bell*,* 2003). Liver disease such as abnormal liver enzymes, non-alcoholic fatty liver disease (NAFLD), cirrhosis, hepatocellular carcinoma, and acute liver failure are seen in patients with type 2 diabetes (Abolfathi *et al,* 2011). There has been a reported increase in the incidence of cirrhosis and cholelithiasis (presence of stones in the gall bladder) in diabetes mellitus and conversely, at least 80% of patients with cirrhosis have glucose intolerance (Levinthal and Tavill, 1999). Excess hepatic glycogen and fat accumulation are also reported to be seen in diabetic complications (Levinthal and Tavill, 1999). In poorly controlled diabetes, glycogen hepatopathy (GH) has been characterized as a pathologic overloading of hepatocytes with glycogen which leads to clinical signs and symptoms such as abdominal discomfort, tender hepatomegaly and elevated transaminases (Fridell *et al.,* 2007). It is also the rare cause of elevated serum transaminases, mostly confined to type 1 diabetics (van den Brand *et al.,* 2009).

### 2.5.5 Cardiovascular diseases

Cardiovascular disease (CVD) is a class of diseases which affect the heart and/or blood vessels and it is frequently linked with any disease that affects the cardiovascular system such as atherosclerosis (Oguntibeju *et al.*, 2009b). Diabetic patients have an increased risk of cardiovascular disease (Desouza *et al.,* 2010). There is an increase in the correlation of type 2 diabetes and the death rate from cardiovascular disease which is two-fold to eight-fold higher in diabetics than people without diabetes (Grundy *et al.,* 2002; Lago *et al.,* 2007). The leading cause of death in diabetes mellitus (DM) is cardiovascular disease and it has been implicated

in more than 80% of the cases of the diabetes disease (Selvaraju *et al.,* 2012). About 80% of all patients with CVD may have diabetes or impaired glucose tolerance (Giugliano *et al.,* 2009). Diabetes mellitus is a major cause of cardiovascular morbidity and mortality in developed countries and atherothrombosis (a condition in which a thrombus originates in an atheromatous blood vessel) is the cause of most deaths among diabetic patients (Stratmann and Tschoepe, 2009). Atherothrombosis comes up as a result of atherosclerosis progression with clinical manifestations such as sudden cardiac death, myocardial infarction (MI), ischaemic stroke, and peripheral arterial ischaemia (Stratmann and Tschoepe, 2009). In diabetic patients, the interaction of auto-antibodies with AGEs is capable of forming AGE-immune complexes which may play a role in atherogenesis (Turk *et al*, 2001). Atherogenesis involves endothelial dysfunction, activation and injury, inflammation, and smooth muscle cell migration and proliferation (Mehta *et al*., 2006). One mechanism by which diabetes promotes atherosclerosis is through abnormal lipid metabolism (Dokken, 2008). Insulin deficiency and insulin resistance promote dyslipidaemia with an increased oxidation, glycosylation, and triglyceride enrichment of lipoproteins (Dokken, 2008).

### 2.5.6 Reproductive damage

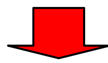
Erectile dysfunction is a common complication of diabetes (Agostini *et al.,* 2006). Diabetes has been linked with reproductive impairment in both men and women (Baccetti *et al.,* 2002). The occurrence of sexual dysfunction in diabetic men approaches 50% while diabetic women seem to be slightly lower (Amaral *et al.,* 2008). This deleterious effect on male reproductive function is possibly through an increased production of reactive oxygen species and imbalance between antioxidants and oxidants (Amaral *et al.,* 2006). Thakur and Dixit (2008) also reported that oxidative stress is increased in diabetes resulting in impaired sexual dysfunction and impotence in the modern world. Diabetes causes damage to nerves throughout the body which includes the penis (Agostini *et al.,* 2006). In diabetic men, poor semen quality which includes decreased sperm motility and concentration, abnormal morphology, increased seminal plasma abnormalities as well as decreased serum testosterone due to impaired Leydig cell function have been reported (Amaral *et al.,* 2008). The sexual problems in diabetic women include decreased sexual arousal with slow and/or inadequate lubrication and sexual desire (Enzlin *et al.,* 2002) and good glycaemic control would be essential to restore a normal sexual activity in diabetic women (Bultirini *et al.,* 2004). Streptozotocin caused testicular dysfunction and degeneration under situations of experimentally induced diabetes in animals (Shrilatha and Muralidhara, 2007) and alloxan-induced diabetes in male rats was reported to reduce semen parameters and impair distinct phases of spermatogenesis (Arikawe *et al*., 2006).

## 2.6 RED PALM OIL, A NATURAL PLANT PRODUCT

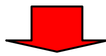
Red palm oil (RPO) is a natural oil obtained from oil palm fruit (*Elaeis guineensis*). *Elaies guineensis* originated from West Africa and was first introduced to Brazil and other tropical countries in the 15th Century by the Portuguese (Corley *et al.,* 1976). The oil palm is a perennial tree that provides fruit year-round and the palm fruit can be harvested three years after planting. The tree has an economic life span of 25 to 30 years and can grow to a height of 20 to 30 meters (Edem, 2002). The female bunch produced by the oil palm can weigh as much as 30-40 kg which has up to 2000 fruitlets that are black in colour when young and turn to orange-red when it is ripe (Edem, 2002). It is a plant food that naturally overcomes the problem of poor bioavailability (Rice and Burns, 2010). Red palm oil is extracted from the fleshy mesocarp of the fruit which is 45-55% oil and the colour varies from light yellow to orange-red and melts at 25$^{o}$C (Ekwenye and Ijeomah, 2005). The pictures of oil palm trees, oil palm fruits and red palm oil are shown in Figure 4 and the picture of the Malaysian palm fruit oil (Carotino) used in this study is shown in Figure 5. Red palm oil contains lipid-soluble antioxidants such as carotenoids (α- and β- carotenes, lycopenes), vitamin E (in the form of α-, β-, δ- tocotrienols and tocopherol) and ubiquinone (Oguntibeju *et al.,* 2010). It derives its red colour from the high content of α- and β- carotenes which can make up 0.08% (w/w) of the crude oil (Monica *et al*., 2006; Dauqan *et al*., 2011).

Red palm oil is known to be the richest natural plant source of carotenoids in terms of provitamin A equivalents (Sundram *et al*., 2003; Yoshida *et al*., 2003) and in general, contains a total of 500-800 mg of provitamin A carotenoids /kg oil, which is 15 times higher than the carotenoid content of carrots on a weight-by-weight basis (Rice and Burns, 2010). Red palm oil also contains carotenoids of which 80-90% is present as α-carotene and β-carotene in ratio 2:1 respectively (Tan and Chu, 1991; Farombi, 2003). The structures of α- and β- carotenes are illustrated in Figures 6 and 7 respectiviely. The vitamin E content in RPO consist mainly of tocotrienols (70%) and tocopherols (30%) (Al-Saqer *et al.,* 2004). The structures of tocopherols and tocotrienols are illustrated in Figures 8 and 9 respectively. Red palm oil contains 50% saturated fatty acids, 40% unsaturated fatty acids, and 10% polyunsaturated fatty acids and this makes it distinctive from other plant and animal oils (Atawodi *et al.,* 2011). The major fatty acids in palm oil are myristic, palmitic, stearic, oleic and linoleic (Siew, 2000). It is the only vegetable oil with a balanced composition of saturated and unsaturated fatty acids both in processed and unprocessed forms (Aboua *et al.,* 2009).

Oil palm trees



Oil palm fruits



Red palm oil

**Figure 4: The pictures of oil palm trees, oil palm fruits and red palm oil.**

**Figure 5: Malaysian palm fruit oil (Carotino) used in this study.**

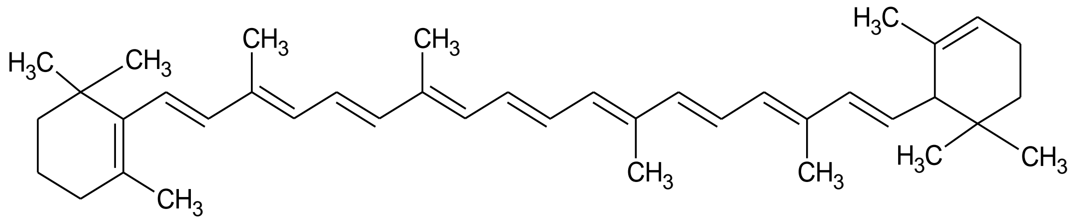



**Figure 6: Structure of α-carotene (adapted from Farombi *et al.,* 2003).**

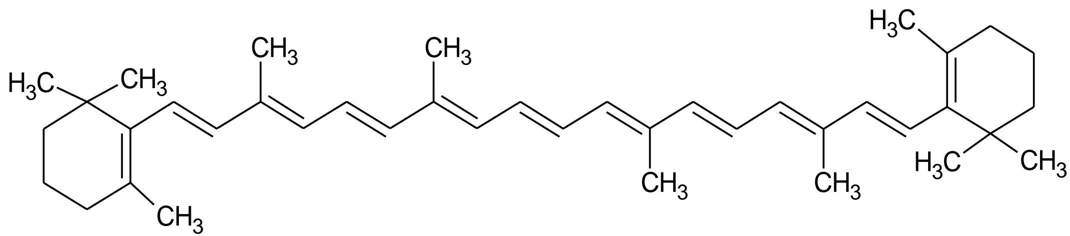



**Figure 7: Structure of β-carotene (adapted from Farombi *et al.,* 2003).**

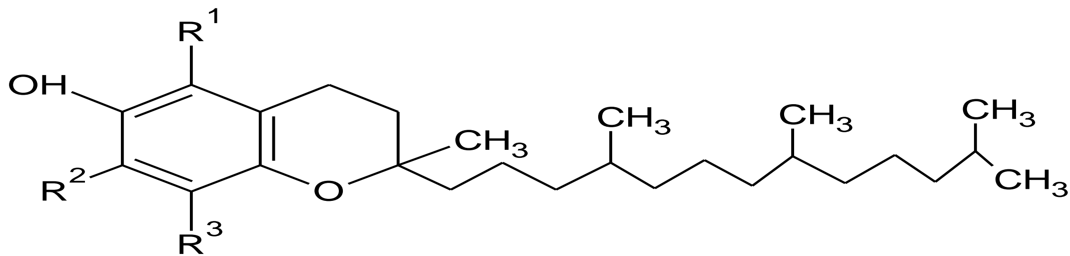



**Figure 8: Structure of tocopherols (adapted from Sen *et al.,* 2006).**

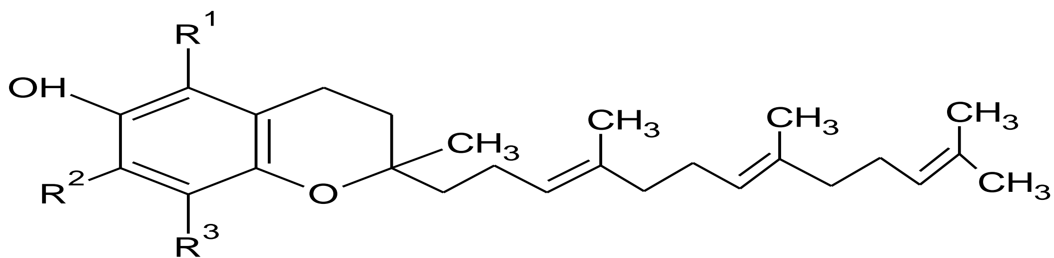



**Figure 9: Structure of tocotrienols (adapted from Sen *et al.,* 2006).**

### 2.6.1 Red palm oil and its health benefits

Red palm oil has been reported to have favourable effects on arterial thrombosis and hypertension due to induced oxidative stress (Edem, 2002; Ganafa *et al.*, 2002; Narang *et al.,* 2004). It has been shown that RPO provided protection against the consequences of ischemia /reperfusion injury (Esterhuyse *et al.*, 2005, 2006; Bester *et al.*, 2006). A long term oral supplementation of palm olein oil, a liquid fraction obtained from the refining of palm oil was shown to augment endogenous antioxidants of heart and hence, protected the heart against oxidative stress following ischemia-reperfusion (Narang *et al.,* 2004). Oguntibeju *et al.* (2010) reported the beneficial role of RPO in reducing oxidative stress in HIV/AIDS and tuberculosis patients. Prasad *et al.* (1999) reported that antioxidants such as carotenoids (primary polar carotenoids) and Vitamin E (primarily α- tocopheryl succinate) induced cell differentiation and growth inhibition to various degrees in rodent and human cancer cells by complex mechanisms.

Another study showed that RPO could possibly inhibit apoptosis in rat sperm (Aboua *et al.*, 2009). RPO could likely provide Vitamin A which is known to play a part in reproduction through the synthesis of sex steroids in embryogenesis and spermatogenesis (Edem, 2002). It has been suggested that the effects of RPO on reproductive capacity is due to improving the efficiency of protein biosynthesis or utilization in a way that was favourable to sex hormone function (Edem, 2002). It has been reported that chronic feeding of fresh RPO does not raise the tissue levels of phospholipids in various organs and cerebroside in the brain and similarly, does not increase free fatty acid contents of some organs such as the brain and testes in experimental animals (Ebong *et al.,* 1999). Budin *et al.* (2009) showed that tocotrienol-rich fractions of RPO reduced oxidative stress biomarkers, blood glucose level and improved dyslipidaemia. Similarly, RPO was able to attenuate oxidative stress produced in diabetic condition hence, it was suggested that palm oil supplementation may be helpful in the management of diabetes mellitus (Ogugua and Ikejiaku, 2005). Furthermore, studies on the various vitamins that are present in the red palm oil on diabetes have been investigated as discussed below.

### 2.6.2 Vitamins present in red palm oil and their beneficial effects on diabetes

Vitamin E, a lipid-soluble vitamin is a generic term which includes four tocopherols (α, β, δ, γ) and four tocotrienols (α, β, δ, γ) (Brigelius-Flohe´ and Traber, 1999). It is essential for the inhibition of oxidation in body tissues, formation of red blood cells and prevention of the

breakdown of body tissues. It efficiently scavenges peroxyl radicals in cell membranes to inhibit lipid peroxidation (Duo *et al.,* 2009). In the diabetic state, Vitamin E reduced systolic and diastolic pressure probably by interfering with several harmful pathways that contributes to the occurrence of hypertension in diabetic conditions (Haidara *et al.,* 2009). Vitamin E has been reported to improve beta cell function and insulin resistance in tissues as well as reducing blood glucose and glycated haemoglobin levels (Naziroglu *et al.,* 2004). Apart from the reduction of blood glucose and glycated haemoglobin, tocotrienols also reduced plasma LDL-cholesterol and triglycerides and increased HDL-cholesterol (Aggarwal *et al.,* 2010). The long term administration of vitamin E has been reported to improve insulin sensitivity and may improve endothelial function (Paolisso *et al.,* 1993; Skyrme-Jones *et al.,* 2000). Vitamin E supplementation has also been shown to provide significant cardioprotective effects against cardiac dysfunction and concomitant myocardial oxidative stress induced by type 1 diabetes (Hamblin *et al.,* 2007).

Vitamin E has been documented to reduce ROS generation and damaging oxidative substances and maintain membrane fluidity in the brain of diabetic rats (Hong *et al.,* 2004). Another study by Tiwari *et al.* (2009) has suggested that the antioxidant potentials of both the isomers of vitamin E may be responsible for the protection against intra-cerebroventricular STZ induced oxidative stress by possibly increasing the endogenous defensive capacity of the brain. Tocotrienols exhibit antioxidant activities and its activities are mediated through the induction of antioxidant enzymes such as SOD, NADPH: quinine oxidoreductase and glutathione peroxidase which quench free radicals such as superoxide ions (Aggarwal *et al.*, 2010). Vitamin E supplementation was shown to prevent glucose-induced lipid peroxidation in rat mesangial cells and hence, could limit the development of glomerulosclerosis in diabetic nephropathy (Trachtman, 1994). The favourable effect of vitamin E on oxidative stress in the renal cortex of diabetic rats has been shown (Jachec *et al.,* 2002). Vitamin E supplementation has also been shown to significantly lower lipid peroxidation and lipid levels in the blood of diabetic patients (Jain *et al.,* 1996). Niedowicz *et al.* (2005) reported that the preventive effect of vitamin E supplementation in diabetic complications is possibly through a decrease in lipid peroxidation. Vitamin E supplementation reduced glycaemia and glycated haemoglobin levels significantly and had a neuroprotective effect on the total myenteric population, without affecting intestinal area or thickness of the intestinal wall or muscular tunic (Roldi *et al.*, 2009).

Vitamin A, a lipid-soluble vitamin is an isoprenoid compound with a 6-membered ring and an 11-carbon side chain and is found in plants as a provitamin called β-carotene (the most abundant carotenoid which can be converted to vitamin A by an oxygenase present in the intestine (Edem, 2009). The basic molecule of vitamin A is retinol which is the most

biologically active and commonest form in mammalian tissues (Edem, 2009). It is easily destroyed by ultraviolet light, acids, oxygen and heat (Anosike, 1994; Edem, 2009). It is an essential nutrient needed for normal growth, reproduction, embryonic development, vision and immune function (Purev *et al.,* 2004). The combination of Vitamin A and insulin could protect the heart against the damaging effects of diabetic-induced pre-oxidative stress (Zobali *et al.*, 2002). It has been shown that vitamin A supplementation could improve wound healing even in the absence of insulin and this suggested that vitamin A may be useful in wound management of insulin-resistant diabetic patients (Seifter *et al.,* 1981).

Lycopene, another major powerful lipid-soluble vitamin found in RPO, is a major carotenoid with powerful antioxidant properties that may offer protection against the development of type 2 diabetes mellitus (Wang *et al.,* 2006). Diabetic rats treated with lycopene significantly reduced sensitivity to pain, probably by inhibiting the release of nitric oxide and tumour necrosis factor-alpha (Kuhad *et al.,* 2008). It attenuated cold allodynia (pain that results from a non-injurious stimulus to the skin) and thermal hyperalgesia (extreme sensitivity to pain) and hence, shows the role of lycopene as an adjuvant therapy in the treatment of diabetic neuropathy (Kuhad and Chopra, 2008). A study conducted by Gao *et al.* (2012) showed that chronic lycopene administration significantly and dose dependently restored erectile dysfunction in diabetic rats by lowering blood glucose, reducing oxidative stress and up-regulating eNOS expression. Lycopene was able to attenuate endothelial dysfunctions by reducing oxidative stress in STZ-induced diabetic rats and hence, useful in preventing diabetic vascular complications associated with endothelial dysfunction (Zhu *et al.,* 2011). The administration of graded doses of lycopene resulted in a decrease in glucose levels, an increase in insulin concentration and antioxidant status in diabetic rats (Ali and Agha, 2009).

## 2.7 ROOIBOS, A NATURAL PLANT PRODUCT

Rooibos (*Aspalathus linearis*) is a popular indigenous herbal tea grown in the Cederberg mountain range area of the Western Cape, Republic of South Africa. The genus *Aspalathus* (Fabaceae, Tribe Crotalarieae) has more than 270 species of which most are widespread in the Cape Floristic Region (Dahlgren 1968; Joubert and De Beer, 2011). The characteristics of the cultivated type of rooibos are bright green, needle-like leaves on straight, slender branches with relatively short internodes and the leaves should turn red brown when bruised (Dahlgren 1968; Joubert and De Beer, 2011). Rooibos, as a shrubby legume has nodules of nitrogen-fixing bacteria on its roots and this enables the plant to survive in the natural setting as a result of the relatively high amounts of nitrogen fixed, despite the poor Clanwilliam soil

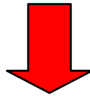
(Muofhe and Dakora, 1999). The soils that sustain the growth of rooibos and its microsymbionts are not only highly acidic, but also very nutrient-poor to support legume growth (Muofhe and Dakora, 1999). Rooibos plants were first reported by botanists in 1772 when they were introduced to the tea by Khoi people (WESGRO, 2001; Erickson, 2003). The indigenous people of the mountainous region of Western Cape in South Africa were the first to collect wild rooibos and use it as tea more than 300 years ago and it became a cultivated crop in the early 1930s (WESGRO, 2001; Erickson, 2003). At the beginning of the 20th century, rooibos tea, produced from *A. linearis* (Burm.f.) Dahlg had no commercial value but it is a well known herbal tea today which is enjoyed in more than 37 countries (Joubert and De Beer, 2011). It is now exported worldwide to countries such the Netherlands, England, Malaysia, South Korea, Poland, China, and the United States (WESGRO, 2001; Erickson, 2003).

Green rooibos, the unfermented product, is processed in such a way as to minimise the oxidation of its polyphenols (Schulz *et al.,* 2003; Joubert and De Beer, 2011). During fermentation, the colour of the unfermented rooibos product changes from green to red with oxidation of the constituent polyphenols and this is referred to as fermented or 'red' rooibos (Schulz *et al.,* 2003; Mckay and Blumberg, 2007; Awoniyi *et al.,* 2012). The pictures of the rooibos plant, fermented rooibos and rooibos tea are shown in Figure 10. Rooibos has been consumed as a healthy beverage for more than a century in the Republic of South Africa and Europe (Baba *et al.,* 2009). It is drunk for enjoyment, as an alternative to oriental tea, but also for its potential medicinal properties (Sinisalo *et al.,* 2010). It has become an acceptable alternative to conventional tea and coffee and it is gaining recognition as a result of low tannin content, no caffeine and high ascorbic acid (Morton, 1993; Baba *et al*., 2009). It has also been reported that rooibos contain some minerals which include iron, potassium, calcium, copper, zinc, magnesium, fluoride, manganese and sodium (Kamen, 2000; Gilani *et al.,* 2006).

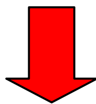
Wide spectrums of polyphenolic constituents present in rooibos are effective as antioxidants (Awoniyi *et al.,* 2012). Antioxidants are substances that act by protecting cells from the damage caused by unstable molecules known as free radicals. Rooibos contains abundant flavonoids particularly, aspalathin, isoorientin, and nothofagin (Kazuno *et al*, 2005). Other flavonoids reported to be present in rooibos include luteolin, chrysoeriol, quercetin, isoquercetin, hyperoside, orientin and rutin (Bramati *et al.,* 2002; Joubert, 2008). Flavonoids are a class of secondary plant phenolic compounds that are well distributed in the plant kingdom. Flavonoids are characterized by two or more aromatic rings, each bearing at least one aromatic hydroxyl group connected with a carbon bridge (Clifford, 2001; Beecher, 2003). The basic flavonoid structure is the flavan nucleus, which consists of 15 carbon atoms

arranged in three rings (C6−C3−C6) (Pietta, 2000). The various classes of flavonoids vary in the level of oxidation and pattern of substitution of the C ring while individual compounds within a class vary in the pattern of substitution of the A and B rings (Pietta, 2000).

Rooibos plant

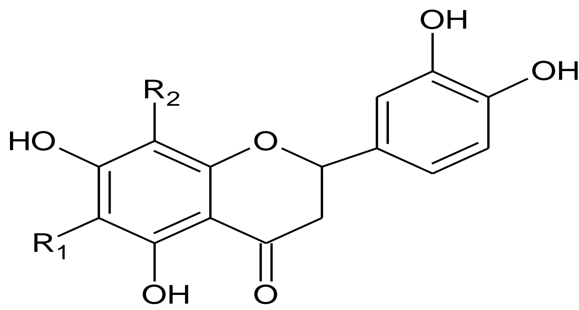


Fermented rooibos



Rooibos tea

**Figure 10: The picture of rooibos plant, fermented rooibos and rooibos tea.**



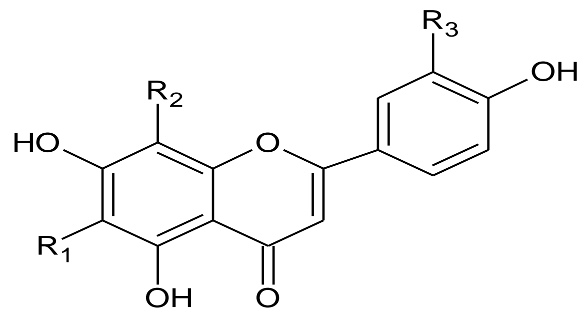

**Flavanones**

(S)/ Eriodictyol-6-C-β-D-glucopyranoside, $R_1$ = Glu, $R_2$=H

(S)/ Eriodictyol-6-C-β-D-glucopyranoside, $R_1$ = H, $R_2$=Glu

(R)- Eriodictyol-6-C-β-D-glucopyranoside, $R_1$ = Glu, $R_2$=H

(R)- Eriodictyol-6-C-β-D-glucopyranoside, $R_1$ = H, $R_2$=Glu




**Flavones**

Orientin, $R_1$ = $H_2$, $R_2$ = Glu, $R_3$ = OH

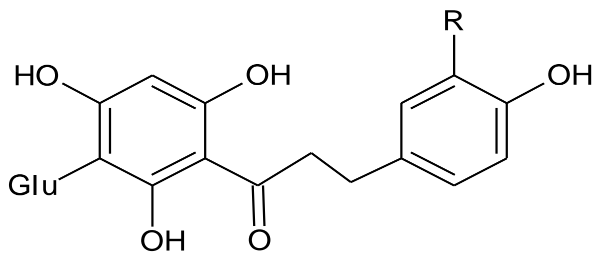
Isoorientin, $R_1$ = Glu, $R_2$ = H, $R_3$ = OH

Vitexin, $R_1$ = H, $R_2$ = Glu, $R_3$ = H

Isovitexin, $R_1$ = Glu, $R_2$ = H, $R_3$ = H

Luteolin, $R_1$ = H, $R_2$ = H, $R_3$ = OH

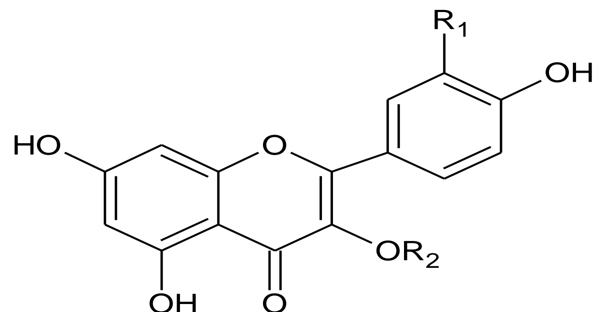
Chrysoeriol, $R_1$ = H, $R_2$ = H, $R_3$ = $OCH_3$




**Dihydrochalcones**

Aspalathin, R = OH

Nothofagin, R = H




**Flavonols**

Rutin, $R_1$ = OH, $R_2$=Rut

Hyperoside, $R_1$ = OH, $R_2$ = Gal

Isoquercitrin, $R_1$ = OH, $R_2$ = Glu

Ouercetin, $R_1$ = OH, $R_2$ = H

Rut - Rutinose, Gal - Galactose, Glu - Glucose

**Figure 11: Structures of the different classes of flavonoids present in rooibos (Adapted from Krafczyk *et al.,* 2009).**

### 2.7.1 Rooibos and its health benefits

Chronic administration of rooibos tea has been shown to prevent age-related accumulation of lipid peroxides in several regions of rat brain (Inanami *et al.*, 1995). Nakano *et al.* (1997) reported that acid polysaccharides from alkaline extracts of rooibos tea could suppress HIV infection. In another study, the anti-haemolytic effect of rooibos tea on red blood cells of Japanese quails has been reported (Simon *et al.,* 2000). Ulicna *et al.* (2003) showed the hepatoprotective effect of rooibos tea on $CCl_4$-induced liver damage in rats. Using an animal model, the chemopreventive ability of rooibos tea fractions on skin cancer has been shown to significantly suppressed tumour growth in mice with skin cancer (Marnewick *et al.,* 2005). Similarly, in an *in vitro* study, rooibos extract has been shown to have strong anti-mutagenic effect by suppressing mutation and thus avert cancer (Van der Merwe *et al.,* 2006). Rooibos has been shown to partially prevent oxidative stress in STZ-induced diabetic rats especially by protecting the ocular (eye) membrane systems against peroxidation (Ulicna *et al.,* 2006). Antispasmodic effects of rooibos in rabbit jejunum (intestine) tissue has shown that rooibos has soothing effects on the digestive system and therefore, can be used as a good treatment for stomach cramps and diarrhoea (Khan and Gilani, 2006).

Rooibos has also been shown to restore immune function in immune-suppressed rats (Ichiyama *et al.,* 2007) and also reduce inflammation in rats with colitis through improved antioxidant activity with a consequent decrease in DNA damage due to oxidation (Baba *et al.,* 2009). The chemoprotective properties of rooibos against cancer promotion in rat liver has been shown (Marnewick *et al.*, 2009). Rooibos was found to significantly inhibit the activity of angiotensin-converting enzyme (ACE), an enzyme that is known to be involved in the development of cardiovascular disease and hence, its usefulness in the treatment of hypertension and heart disease (Persson *et al.,* 2010). Marnewick *et al.* (2011) showed that humans, consuming six cups of rooibos per day for a period of six weeks had the biomarkers associated with cardiovascular disease significantly reduced, thereby protecting the body against oxidative damage of blood lipids. Pantsi *et al.* (2011) reported the ability of rooibos to protect the heart against ischemic injury. Recently, rooibos has been reported to improve sperm quality and protected sperm against oxidative damage (Awoniyi *et al.,* 2012). Even though, scanty information is available on the potential health benefits of rooibos on diabetes, several studies have shown the effects of the various flavonoids that are present in rooibos on diabetes as discussed below.

### 2.7.2 Flavonoids present in rooibos and their beneficial effects on diabetes

Flavonoids are powerful chain-breaking breaking antioxidants with a variety of biochemical and pharmacological actions (Middleton e*t al.*, 2000). Flavonoids may have the ability to reduce the occurrence of diabetes by preventing the progressive impairment of pancreatic beta-cells function (Coskun *et al.*, 2005; Song *et al.*, 2005) and thereby regenerate the damaged pancreatic cells or stimulate the secretion of insulin by *β*–cells of the pancreas (Seetharam *et al.*, 2002). Naik *et al.* (1991) had also earlier reported that flavonoids exerted their effects by either promoting the entry of glucose into cells, stimulation of glycolytic enzymes and glycogenic enzymes, depression of gluconeogenic enzymes or inhibiting the gluco-6-phosphatase in the liver and subsequently reducing the release of glucose in the blood. Flavonoids have been reported to improve hyperglycaemia in diabetes mellitus by affecting glucose transport (Ong and Khoo, 1996; Hsu *et al.*, 2003), insulin-like properties (Choi *et al.*, 1991) and insulin receptor function (Shisheva and Shechter, 1992). Jung *et al.* (2004) reported that flavonoids play important roles in preventing the progression of hyperglycaemia partly by increasing hepatic glycolysis and glycogen concentration and/or by lowering hepatic gluconeogenesis.

Flavonoids can exert their antioxidant activity by various mechanisms such as scavenging or quenching of free radicals, chelating of metal ions, or by inhibiting enzymatic systems responsible for free radical generation (Pietta, 2000; Nijveldt *et al.,* 2001). Lukačínová *et al.* (2008) studied the hypoglycaemic and antioxidant effects of quercetin in alloxan-induced diabetic rats and found that serum glucose elevation was prevented. It was suggested that the protective effect of quercetin is partly related to their antioxidative/chelatory properties and partly to the alteration of renal glucose absorption (Lukačínová *et al.,* 2008). Quercetin attenuated renal dysfunction and oxidative stress in diabetic rats and the neuropathic pain that accompanies the disease (Anjaneyulu *et al.,* 2003; Anjaneyulu and Chopra, 2004). Machha (2007) also showed that the administration of quercetin to diabetic rats restored vascular function, probably through enhancement in the bioavailability of endothelium-derived nitric oxide coupled to reduced blood glucose level and oxidative stress. The ability of quercetin to offer protection against oxidative stress-induced cellular damage is commonly associated with its anti-oxidative action as well as its metal chelatory properties (Mira *et al.*, 2002; Anjaneyulu and Chopra, 2004).

In another study, quercetin was shown to cause regeneration of pancreatic islets and increased insulin release in streptozotocin-induced diabetic rats (Vessal *et al.*, 2003). Kobori *et al.* (2009) suggested that quercetin increased pancreatic insulin production by promoting

cell proliferation through suppression of Cyclin-dependent kinase inhibitor 1A (Cdkn1a) expression induced by STZ. Quercetin is reported to have potent inhibitory effects on both glycogen phosphorylase *a* (phosphorylated, active) and *b* (unphosphorylated, inactive) in isolated muscle (Jakobs *et al*., 2006). Hif and Howell (1985) reported that quercetin was able to stimulate insulin release and enhanced $Ca^{2+}$ uptake from isolated islet cells which suggested the involvement of flavonoids in non-insulin dependent diabetes. Quercetin was also found to increase hexokinase and glucokinase activity in diabetic rats (Vessal *et al*., 2003). Increase in hepatic glucokinase, a sensitive indicator of glycolysis can improve the use of blood glucose for glycogen storage in the liver (Iynedjian *et al.,* 1988; Jung *et al.,* 2004). Quercetin was found to increase hexokinase and glucokinase activity in diabetic rats (Vessal *et al.,* 2003). Quercetin has also been reported to significantly increase sperm viability, motility and total serum testosterone levels and the degeneration and inflammation in testis cells associated with diabetes were improved (Khaki *et al.,* 2009; 2010).

Rutin has been reported to decrease blood glucose levels (Kamalakkannan and Prince, 2006b; Sattanathan *et al.,* 2011) and prevent STZ-induced oxidative stress (Kamalakkannan and Prince, 2006b). Rauter *et al.* (2010) reported that rutin significantly improved glucose tolerance in diabetic rats. In a long-term treatment of diabetic rats, a significant increase in plasma insulin levels and histopathological observations were indicative of the protective role of rutin in streptozotocin-induced diabetes mellitus (Kamalakkannan and Prince, 2006a). Rutin has also been associated with marked decreases in hepatic and cardiac levels of triacylglycerols in streptozotocin-induced diabetic rats (Fernandes *et al.,* 2010). Flavonoids have been reported to inhibit protein glycation (Wu and Yen, 2005; Urios *et al*., 2007). The inhibitory mechanism of flavonoids against glycation was, at least partly, be related to their antioxidant properties (Wu and Yen, 2005). The characteristic of AGEs has been shown to decrease in STZ-diabetic rats treated with rutin (Odetti *et al*., 1990). Asgary *et al.* (1999), through an *in vitro* study showed that flavonoids such as quercetin and rutin were able to inhibit glycation possibly due to the relation between structure activity of flavonoids and the preventive effect on haemoglobin glycosylation. Kawano *et al.* (2009) showed that aspalathin, a rooibos tea component from *Aspalathus linearis* significantly suppressed increases in fasting blood glucose levels and improved the impaired glucose tolerance in db/db mice. Through an *in vitro* study, aspalathin was able to increase both glucose uptake by muscle cells and insulin secretion from pancreatic β-cells (Kawano *et al*., 2009).

## 2.8 REFERENCES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# CHAPTER THREE

## EFFECTS OF DIETARY INTAKE OF RED PALM OIL ON FATTY ACID COMPOSITION AND LIPID PROFILES IN MALE WISTAR RATS

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## ABSTRACT

Little is known about the effects of dietary intake of red palm oil on fatty acid composition in the liver of rats. Male Wistar rats were randomly divided into four groups and were fed with different doses of red palm oil. The control group received no red palm oil; while the experimental groups were fed with 1 ml, 2 ml and 4 ml of red palm oil daily for seven weeks. In the liver of all the groups, palmitic acid (C16:0) followed by stearic acid (C18:0) were predominantly present among the saturated fatty acids. Oleic acid (C18:1c) and linoleic acid (C18:2) were largely present among the unsaturated fatty acids. There was no significant ($P>0.05$) increase in the levels of palmitic acid (C16:0) in all the groups while oleic acid (C18:1) significantly increased at 4 ml red palm oil when compared with the control ($p<0.05$). The total cholesterol (TC), triglycerides (TG) and very low density lipoprotein (VLDL)-cholesterol levels were not significantly different in all the groups ($P>0.05$) when compared with the control group. Overall, there were no significant effects of red palm oil on the levels of serum cholesterol and triglycerides as well as accumulation of saturated fatty acids in the liver of the experimental rats.

**Key Words- Lipid Profiles, Fatty Acid, Red Palm Oil, Rats**

## INTRODUCTION

Red palm oil (RPO) has a deep orange-red colour and is extracted from the mesocarp of fruits of palm oil trees (*Elaeis guineensis*). All over the world, 90% of the RPO produced is used for edible purposes (Idris and Samsuddin, 1993; Edem, 2002). RPO contains a variety of antioxidant vitamins necessary for maintaining good health (Bayorh, 2005). It is a good source of vitamin A (carotenes) (Sundram *et al.,* 2003; Arora *et al.,* 2006; Oguntibeju *et al.,* 2010; Aboua *et al.,* 2011) and vitamin E (tocopherols and tocotrienols) (Sundram *et al.,* 2003; Arora *et al.,* 2006; Muharis *et al.,* 2010) and these are capable of scavenging free radicals thus preventing the damaging effects of oxidation in tissues. The characteristic colour of RPO is as a result of the abundance of carotenoids (500 - 700 mg/L) in the crude oil (Edem and Akpanabiatu, 2006; Edem, 2009). The combined effect of carotenoids, tocopherols, tocotrienols and 50% unsaturation of the fatty acids gives palm oil a higher oxidative stability as compared to other vegetable oils (Arora *et al.,* 2006). RPO supplies fatty acids necessary for proper growth and development. Fatty acids play a vital role in metabolism because they are the building blocks of fat in the body and in food. They are a source of energy for the cell and form the structural basis of the cell. Red palm oil contains 50% saturated, 40%

monounsaturated and 10% polyunsaturated fatty acids (Rukmini, 1994; Edem, 2002). From the nutritional point of view, the major concern for RPO has to do with their degree of saturation and the effect they have on blood lipids (Hayes and Khosla, 2007). Palmitic and stearic acids, are saturated fatty acids which account for 45% and 5% of total fatty acids in red palm oil respectively (Hayes and Khosla, 2007; Dauqan *et al.,* 2011). More than 95% of palm oil consists of mixtures of triglycerides, each esterifiied with three fatty acids (Akinola *et al.,* 2010). The various types of dietary lipids have shown to affect lipid metabolism differently (Ajayi and Ajayi, 2009). Wu *et al.* (2011) reported that dietary lipids directly affect fatty acids composition in animal tissues. The aim of this study was to investigate the levels of fatty acids and lipid profiles in rats following the dietary intake of red palm oil at different doses.

## MATERIALS AND METHODS

Experimental Animals and Management

Male Wistar rats (195-240 g) were obtained from Stellenbosch University, Tygerberg, South Africa and used throughout the study. The study was conducted after obtaining Ethical Committee Clearance from Cape Peninsula University of Technology (CPUT/HAS-REC 2010/A002). The rats were individually housed in a well controlled environment set at 22$^{o}$C ± 2 with 50% ± 5% humidity and a 12-h hour light cycle. They were randomly placed in four groups. Group 1 (*n*=5) received no supplementation and served as the control while group 2 (*n*=6), 3 (*n*=6) and 4 (n=6) received 1 ml, 2 ml and 4 ml red palm oil (RPO) respectively. Each group of rats was allowed frees access to water and standard rat chow (SRC) for seven weeks. Carotino palm fruit oil from Malaysia at different doses (1 ml, 2 ml and 4 ml) was added to the standard rat chow daily diet of the experimental animals for seven weeks. The nutritional composition of the red palm oil is shown in Table 1. At the end of the seven weeks, all the animals were sacrificed by euthanasia after overnight fasting. Blood samples were collected from the abdominal aorta and then centrifuged to obtain the serum which was used for lipid analysis while the liver was removed and processed for fatty acid determination.

**Fatty Acid Determination**

Fatty acid determination was carried out by the modified method of AOAC (2005). The liver samples were placed on the vortex to achieve homogeneity. Liver samples ranging from 0.4 to 1 g were weighed into 70 ml digestion tubes and 100 mg pyrogallic acid was added followed by 2ml of undecanoic acid (internal standard) solution, 2 ml of ethanol and 10 ml of 32% hydrochloric acid. The tubes were then placed in the water bath at 75$^{o}$C with gentle shaking for 40 mins. The fatty acids were extracted by adding 25 ml of diethyl ether and 25 ml of

petroleum ether. The organic phase was dried and the residue was derivatised using 2 ml of 2% sulphuric acid in methanol and 1 ml of toluene at 100°C for 45 min. After cooling to room temperature, 5 ml distilled water and 1 ml of hexane were added and the hexane solution was then dried with anhydrous sodium sulphate and transferred into a vial for gas chromatographic analyses.

**Lipid Profile Determination**

Triglycerides (TG), total cholesterol (TC) and high density lipoprotein (HDL)-cholesterol were evaluated with kits using a clinical chemistry analyzer (EasyRA Medical, USA) according to the manufacturer`s instructions. Very low denisity lipoprotein (VLDL) and low denisity lipoprotein (LDL)-cholesterol were calculated according to Friedewald's formula (Friedewald *et al.,* 1972). VLDL-cholesterol = TG/5 and LDL-cholesterol = TC – VLDL-cholesterol – HDL-cholesterol.

**Statistical Analysis**

Data were expressed as the means ± standard deviations. Significant differences between mean values of different groups were determined by one-way analysis of variance (ANOVA) with Turkeys test using GraphPad Prism 5. Differences were considered significant at $p<0.05$.

**Table 1**: **Nutritional Composition of Carotino Red Palm Oil.**

| Serving size: 1 tablespoon | Per 100 ml | Per 14 g serving |
|---|---|---|
| Energy | 3400 KJ | 510 KJ |
| Total Fat | 92 g | 14 g |
| Monounsaturates | 43 g | 6.5 g |
| Polyunsaturates | 12 g | 1.9 g |
| Saturates | 37 g | 5.6 g |
| Trans fat | 0 g | 0 g |
| Cholesterol, Sodium | 0 mg | 0 mg |
| Protein, Carbohydrate, Dietary Fibre | 0 g | 0 g |
| Natural Carotenes | 46 mg | 7.0 mg |
| Beta Carotene | 22 mg | 3.3 mg |
| Alpha Carotene | 17 mg | 2.6 mg |
| Other Carotenes | 7.3 mg | 1.1 mg |
| Natural Vitamin E | 74 mg | 11 mg |
| 19.5% Tocopherols | | |
| 80.5% Tocotrienols | | |
| Co- Enzyme Q10 | 4.0 mg | 0.6 mg |

**Source - Table adapted from the nutritional label of the Carotino Palm Fruit Oil from Malaysia.**

## RESULTS AND DISCUSSION

Table 2 indicates the % body weight gain in the rats fed with different doses of RPO. There were significant increases in the body weight gain in both 2 ml and 4 ml RPO fed groups when compared with the control group.

**Table 2: Body weight gain in the rats fed with different doses of RPO.**

| RPO Dosage (ml) | Initial weight (g) | Final weight (g) | Body weight gain (%) |
|---|---|---|---|
| 0 | 225 ± 11.79 | 352 ± 18.43 | 56 ± 4.02 |
| 1 | 222 ± 10.52 | 360 ± 29.93 | 62 ± 6.48 |
| 2 | 212 ± 11.31 | 359 ± 21.26 | 69 ± 4.37* |
| 4 | 214 ± 17.25 | 387 ± 26.62 | 80 ± 6.04* |

(*) Indicates significant difference from control group at $p<0.05$

Table 3 indicates the total fatty acids in the liver of rats fed with different doses of RPO. There was no significant difference in the total fatty acids in all palm oil fed groups when compared with the control group.

**Table 3: Total fatty acids (g/100g) in the liver of rats fed with different doses of RPO.**

| RPO Dosage (ml) | Total fatty acids |
|---|---|
| 0 | 1.136 ± 0.0950 |
| 1 | 1.176 ± 0.1383 |
| 2 | 1.131 ± 0.1806 |
| 4 (n=5) | 1.245 ± 0.1025 |

The levels of saturated fatty acids in the liver of rats fed with different doses of red palm oil are indicated in Table 4. The two most abundant saturated fatty acids in the liver of all the groups were palmitic acid (C16) and stearic acid (C18). The values of palmitic acid were not significantly different in all RPO fed groups when compared with the control. Stearic acid was significantly lower for the 4 ml RPO group only when compared to the control. Other saturated fatty acids present were myristic acid (C14), pentadecylic acid (C15), margaric acid (C17) and lignoceric acid (C24). No significant differences were noted for myristic acid for any of RPO fed groups. Pentadecyclic acid (C15) and margaric acid (C17) significantly decreased in all palm oil fed groups while C24 significantly decreased at 2 ml and 4 ml RPO when compared with the control group.

**Table 4: Levels of saturated fatty acids (g/100g) in the liver of rats fed with different doses of RPO.**

| RPO Dosage (ml) | C14 | C15 | C16 | C17 | C18 | C24 |
|---|---|---|---|---|---|---|
| 0 | 0.003 ± 0.0004 | 0.004 ± 0.0005 | 0.298 ± 0.0209 | 0.010 ± 0.0011 | 0.254 ± 0.0118 | 0.008 ± 0.0007 |
| 1 | 0.004 ± 0.0003 | 0.003 ± 0.0006* | 0.313 ± 0.0350 | 0.007 ± 0.0005* | 0.243 ± 0.0242 | 0.008 ± 0.0005 |
| 2 | 0.003 ± 0.0006 | 0.002 ± 0.0000* | 0.300 ± 0.0441 | 0.005 ± 0.0004* | 0.228 ± 0.0129 | 0.007 ± 0.0005* |
| 4 (n=5) | 0.003 ± 0.0005 | 0.002 ± 0.0005* | 0.330 ± 0.0219 | 0.004 ± 0.0005* | 0.222 ± 0.0175* | 0.006 ± 0.0006* |

(*) Indicates significant difference when compared with control group at $p<0.05$

The levels of unsaturated fatty acids in the liver of rats fed with different doses of red palm oil are indicated in Table 5. The two most abundant liver unsaturated fatty acids in all the groups were oleic acid (C18:1c) and linoleic acid (C18:2). There was a significant increase in C18:1c at 4 ml RPO while C18:2 at 2 ml and 4 ml RPO supplementation were significantly decreased when compared with the control. Other unsaturated fatty acids present in minute amounts were elaidic acid (C18:1t), linolenic acid (C18:3) and docosahexaenoic acid (DHA) (C22:6). There was a significant decrease in C18:1t and C18:3 levels in all experimental groups when compared with the control group. The level of C22:6 was significantly reduced for the 4 ml RPO fed group when compared with the control group.

**Table 5: Levels of unsaturated fatty acids (g/100g) in the liver of rats fed with different doses of palm oil.**

| RPO Dosage (ml) | C18:1t | C18:1c | C18:2 | C18:3 | C22:6 |
|---|---|---|---|---|---|
| 0 | 0.020 ± 0.0018 | 0.162 ± 0.0279 | 0.302 ± 0.0392 | 0.009 ± 0.0024 | 0.065 ± 0.0073 |
| 1 | 0.015 ± 0.0016* | 0.249 ± 0.0847 | 0.265 ± 0.0289 | 0.006 ± 0.0017* | 0.064 ± 0.0038 |
| 2 | 0.011 ± 0.0008* | 0.278 ± 0.0898 | 0.224 ± 0.0340* | 0.004 ± 0.0011* | 0.060 ± 0.0040 |
| 4 (n=5) | 0.009 ± 0.0005* | 0.386 ± 0.0470* | 0.229 ± 0.0193* | 0.003 ± 0.0005* | 0.052 ± 0.0043* |

(*) Indicates significant difference when compared with control group at $p<0.05$

The serum lipid profiles of rats at different doses of palm oil are indicated in Table 6. There were no significant differences in the total cholesterol (TC), triglycerides (TG) and VLDL-cholesterol when compared with the control group. There was a significant increase in the level of HDL-cholesterol in 1 ml RPO fed group and a significant decrease in LDL-cholesterol in 4 ml RPO fed group when compared with the control group.

**Table 6: The lipid profiles in the serum of the rats at different doses of palm oil.**

| RPO Dosage (ml) | TC (mmol/L) | TG (mmol/L) | HDL-Cholesterol (mmol/L) | VLDL-Cholesterol (mmol/L) | LDL-Cholesterol (mmol/L) |
|---|---|---|---|---|---|
| 0 | 1.70 ± 0.07 | 0.57 ± 0.14 | 0.48 ± 0.05 | 0.11 ± 0.03 | 1.11 ± 0.06 |
| 1 | 1.92 ± 0.26 | 1.07 ± 0.49 | 0.60 ± 0.05* | 0.21 ± 0.10 | 1.10 ± 0.15 |
| 2 | 1.65 ± 0.09 | 0.53 ± 0.12 | 0.54 ± 0.04 | 0.11 ± 0.02 | 1.01 ± 0.08 |
| 4 | 1.62 ± 0.11 | 0.79 ± 0.28 | 0.55 ± 0.07 | 0.16 ± 0.06 | 0.91 ± 0.11* |

(*) means significantly different when compared to with control at $p < 0.05$. TC, total cholesterol; TG, triglycerides; HDL, high density lipoprotein; LDL, low density lipoprotein; VLDL, very low density lipoprotein.

Total cholesterol is made up of LDL-cholesterol, HDL-cholesterol, and VLDL-cholesterol and increased levels of LDL is known to increase the risk of heart disease and stroke while high levels of HDL has been reported to reduce the risk of cardiovascular disease (Birtcher and Ballantyne, 2004). Hayes and Khosla (2007) also reported that circulating cholesterol is linked to heart disease and can serve as relevant index of our nutritional well-being that is sensitive to fat intake and composition. The vital lipids whose increase are implicated in the hindrance of blood supply to the heart, brain, liver or kidney and could cause coronary heart diseases, stroke or kidney failure are cholesterol and triacylglycerols (Owolabi *et al.,* 2010). Yuan *et al.* (2007) reported that high levels of triglycerides could contribute independently to increased risk of cardiovascular disease and severe hypertriglyceridaemia is also associated with an increased risk of acute pancreatitis. Oguntibeju *et al.* (2009) reported that the link between dietary fats and cardiovascular disease has created an increasing interest in dietary red palm oil research. The intake of saturated fatty acids increased total cholesterol, LDL and HDL while polyunsaturated fatty acids in fats decreased these values (Hayes and Khosla, 2007).

Dauqan *et al.* (2011) showed a significant decrease in cholesterol levels in animals fed with red palm olein. Red palm oil supplementation has been reported to have beneficial or neutral effects on serum total cholesterol despite its high saturated fat content (Kruger *et al.,* 2007). Our results indicate that RPO does not significantly increase cholesterol and triglycerides levels in the RPO fed rats after a seven week feeding period. Ajayi and Ajayi (2009) reported that both polyunsaturated fatty acids (PUFA) and monounsaturated fatty acids (MUFA) could have an effect on lipoprotein metabolism with a hypocholesterolaemic effect. Palm oil contains only 0.2% lauric acid (Kochikuzhyil *et al.,* 2010) and a high quantity of palmitic acid as well as considerable amounts of oleic and linoleic acids (Edem, 2002). Lauric and palmitic acids are hypercholesterolaemic when compared with oleic acid while lauric acid increased cholesterol levels more than palmitic acid (Temme *et al.,* 1996). Similarly, Sundram *et al.* (1994) reported that the dietary combination of lauric and myristic fatty acids increased serum cholesterol than palmitic acid in healthy normocholesterolaemic men fed with low cholesterol diet. Red palm oil contains equivalent amounts of saturated and unsaturated fatty acids (Oguntibeju *et al.,* 2010). Our results showed no abnormal retention of saturated fatty acids in the liver of the rats which could be damaging to liver functions. Palmitic acid which is known to be largely present among the saturated fatty acids in RPO did not increase significantly in the liver of the RPO fed groups when compared with the control group.

In conclusion, dietary intake of RPO did not result in accumulation of saturated fatty acids in the liver. Also, it did not significantly alter the serum levels of both cholesterol and triglycerides levels and it could have the potential to reduce the levels of bad cholesterols and triglycerides especially in diseased conditions. Hence, further investigations are recommended as RPO could help to lower the risk of atherosclerosis and other related diseases.

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# CHAPTER FOUR

## IMPACT OF DIETARY RED PALM OIL ON ANTIOXIDANT STATUS AND LIVER HISTOPATHOLOGY IN MALE WISTAR RATS

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## ABSTRACT

Antioxidant status and liver histopathology in male rats following dietary consumption of red palm oil were investigated in a rat model. Male Wistar rats were randomly divided into four groups. Group 1 (n=5) received no red palm oil supplementation and served as the control while group 1 (n=6), group 2 (n=6) and group 3 (n=6) received 1 ml, 2 ml and 4 ml red palm oil daily respectively. Liver and plasma ferric reducing antioxidant power, plasma total polyphenols, total glutathione in the red blood cells as well as catalase, glutathione peroxidase and superoxide dismutase activities in the red blood cells and liver were determined. In this study, the results showed no significant differences ($p>0.05$) in both liver and plasma ferric reducing antioxidant power, plasma polyphenols and total glutathione in the red blood cells in all palm oil fed groups when compared with the control group. Catalase activities significantly increased ($p<0.05$) at both 2 ml and 4 ml red palm oil groups in both the liver and red blood cells. There was no significant ($p>0.05$) difference in the liver glutathione peroxidase activities in palm oil fed groups while glutathione peroxidase activities in the red blood cells significantly ($p<0.05$) increased at 2 ml and 4 ml red palm oil when compared with the control group. Red palm oil did not significantly increase superoxide dismutase in the red blood cells while its activities were increased in the liver. There were no histopathological alterations in the liver of red palm oil fed groups when compared with the control rats. In conclusion, red palm oil could up-regulate the levels of antioxidant enzymes and hence, its dietary consumption could help to boost antioxidant status in the body and thus promote overall well-being.

**Keywords- Dietary, Red palm oil, Antioxidant, Histopathology, Wistar rats.**

## INTRODUCTION

Red palm oil (RPO) comes from the fruit of the oil palm (*Elaeis guineensis*), originating from the rain forest region of West Africa, used mainly for cooking and it is one of the economically viable products for export for many years (Oyewole and Amosu, 2010). Red palm oil is distinctive as compared to other dietary fats in that palm oil contains the highest known concentrations of natural antioxidants, especially provitamins A carotenes and vitamin E (Paul and Sumit, 2002). Carotenoids such as alpha and beta- carotenes are precursors of vitamin A that are converted into vitamin A *in vivo* (Oyewole and Amosu, 2010). Dietary antioxidants which include nutrient antioxidants are chain breaking antioxidants and at the same time with enzyme antioxidants, scavenge the reactive oxygen

species (ROS) and reactive nitrogen species (RNS) within physiological limits (Singh *et al.,* 2010). The carotenoid in crude palm oil is considered to be about 15 times more than in carrots and it plays an important role by acting as biological antioxidants, protecting cells and tissues from the damaging effect of free radicals (Mukherjee and Mitra, 2009). The antioxidant properties of tocotrienols in palm oil bring many benefits to the human body such as preventing skin aging, prevention of fat oxidation, reduction of blood pressure as well as having anti-cancer activities (Mukherjee and Mitra, 2009). Palm oil is beneficial by reducing blood pressure and thrombotic tendency of platelets while offering protection against oxidative damage of the liver and other organs (Edem 2002). Palm oil is oxidatively stable owing to a fatty acid composition with low polyunsaturation and high antioxidant content (Schroeder *et al.,* 2006). The bioavailability of palm oil nutrients is excellent as the fat soluble vitamins are embedded in the oil medium. Consumption of food containing phytochemicals with potential antioxidant properties could lessen the risk of human disease (Temple, 2000). Antioxidant defence mechanisms involve both enzymatic and non-enzymatic strategies which include vitamins A, C, and E, glutathione and enzymes such as superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase (Maritim *et al.,* 2003). They act synergistically with each other and against different types of free radicals (Maritim *et al.,* 2003). The antioxidant enzymes are very good biochemical markers of stress and their elevated activity may confirm a potential for remediation (Kopyra and Gwozdz, 2003; Dauqan *et al.,* 2011). The study was carried out to investigate if graded doses of red palm oil could be well-tolerated and potentially confer effective antioxidative benefits following its dietary intake in male Wistar rats.

## MATERIALS AND METHODS

### Animal care

Male Wistar rats (195-240 g) were obtained from Stellenbosch University, Tygerberg, South Africa and used throughout the study. The study was conducted after obtaining Ethical Committee Clearance from Cape Peninsula University of Technology (CPUT/HAS-REC 2010/A002). The rats were individually housed in a well controlled environment set at $22^{o}C \pm 2^{o}C$ with 50% ± 5% humidity and a 12-h hour light cycle. They were randomly placed in four groups. Group 1 (*n*=5) received no supplementation and served as the control while groups 2 (*n*=6), 3 (*n*=6) and 4 (n=6) received 1ml, 2 ml and 4 ml red palm oil (RPO) respectively. Each group of rats was allowed free access to water and standard rat chow (SRC) for seven weeks. Carotino palm fruit oil from Malaysia at different doses (1 ml, 2 ml and 4 ml) was added to the standard rat chow daily diet of the experimental animals for seven weeks. At

the end of the seven weeks, all the animals were sacrificed after overnight fasting. Blood samples were collected from the abdominal aorta into appropriate tubes and then centrifuged to obtain the serum, plasma and red blood cells for biochemical analysis. The liver was removed, frozen in liquid nitrogen and stored at -80$^{o}$C until analysis.

**Antioxidant enzymes assay**

The activities of antioxidant enzymes in the liver and red blood cells were determined. Liver homogenates (10% w/v) were prepared in a phosphate buffer, centrifuged at 10,000g (4$^{o}$C) for 10 mins and the supernatant kept at -80$^{o}$C for enzyme analyses. Catalase (CAT) activity was determined spectrophotometrically at 240 nm by monitoring the decomposition of $H_2O_2$ and expressed as µmole $H_2O_2$/min/µg protein according to the method of Aebi (1984) while superoxide dismutase (SOD) activity was determined by the method of Crosti *et al.* (1987) modified for a microplate reader at 490 nm and expressed as the amount of protein (µg) required to produce a 50% inhibition of auto-oxidation of 6-hydroxydopamine. Glutathione peroxidase (GPx) activity was measured spectrophotometrically (340 nm) by the method of Ellerby and Bredesden (2000) and expressing activity as nmoles NADPH/min/µg protein.

**Ferric reducing antioxidant power (FRAP) assay**

The ferric reducing antioxidant power was determined using the method described by Benzie and Strain (1996). Ten (10) µl of the plasma and liver homogenates was mixed with 300 µl FRAP reagent in a 96-well clear plate. The FRAP reagent was a mixture (10:1:1, v/v/v) of acetate buffer (300 mM, pH 3.6), tripyridyl triazine (TPTZ) (10 mM in 40 mM HCl) and $FeCl_3.6H_2O$ (20 mM). After incubation at room temperature for 30 min, the plate was read at a wavelength of 593 nm in a Multiskan Spectrum plate reader (Thermo Fisher Scientific). Ascorbic acid (AA) was used as the standard and the results expressed as µmol AAE/L for plasma and *µ*mol AAE/g tissue for liver homogenates.

**Total glutathione, total protein, albumin and globulin analysis**

Total glutathione level (GSHt) in the red blood cells (RBCs) was determined according to the method of Asensi *et al.* (1999). The sample was deproteinised using 5% metaphosphoric acid (MPA) solution. Briefly, 50µl of the samples was added to plate wells, 50µl of 5, 5'-dithiobis-2-nitrobenzoic acid (DTNB) was added, followed by 50µl of glutathione reductase. The reaction was initiated by addition of 50µl of nicotinamide adenine dinucleotide phosphate (NADPH) to a final volume of 200 µl. The change in absorbance was monitored at 412 nm for 5 min and levels of total glutathione (GSHt) calculated using pure glutathione as standard and expressed as µmole/mg protein. Total protein and albumin levels in the serum were measured with kits using an automated chemistry analyzer (EasyRA Medical,

USA) according to manufacturer`s instructions. Globulin level was determined by using the formula (Globulin = Total protein – Albumin).

**Plasma total polyphenols determination**

The plasma was deproteinised using 0.5 M perchloric acid (PCA) (1:1 v/v). Folin–Ciocalteu method was used to determine the total plasma polyphenol according to the method of Singleton and Rossi (1965). Briefly, the reaction was initiated by the addition of 125 µl of Folin reagent (0.2 N) and 100 µl of sodium carbonate (7.5% $Na_2CO_3$) to 25 µl of sample into a clear 96-well microplate. A blue colour was formed and measured at 765 nm after 2 hr incubation at room temperature in a Multiskan Spectrum (Thermo Electron Corporation – USA). Gallic acid as the standard and the result expressed as mg/L GA.

**Histopathological evaluations**

At the end of the treatment, animals were sacrificed to collect the liver. The organ was blotted to remove excess blood, fixed in 10% neutral formalin, trimmed and processed for paraffin embedment and 5 µm thick of tissue sections were stained with haematoxylin and eosin. Histopathological examinations of the liver were examined using light microscopy at 20x magnification.

**Statistical analysis**

Data were expressed as the means ± standard deviations. Significant differences between mean values of different groups were determined by one-way analysis of variance (ANOVA) with MedCalc software. Data not normally distributed was log transformed and analyzed using the Kruskal–Wallis one-way ANOVA on ranks hypotheses. Differences were considered significant at $p<0.05$.

## RESULTS

Figures 1-6 indicate the activities of CAT, GPx and SOD in the liver and red blood cells of rats fed with different doses of red palm oil. There was a significant ($p<0.05$) increase in the activities of CAT in both the liver and RBCs at 2 ml and 4 ml in comparison to the control group. Similarly, RPO at all the different doses used in this study significantly elevated the activity of GPx in the RBCs while GPx activity in the liver showed no significant ($p>0.05$) increase when compared with the control rats. SOD activity was also significantly ($p<0.05$) increased in the liver at 1 ml and 4 ml RPO while there was no significant ($p>0.05$) increase in the RBCs.

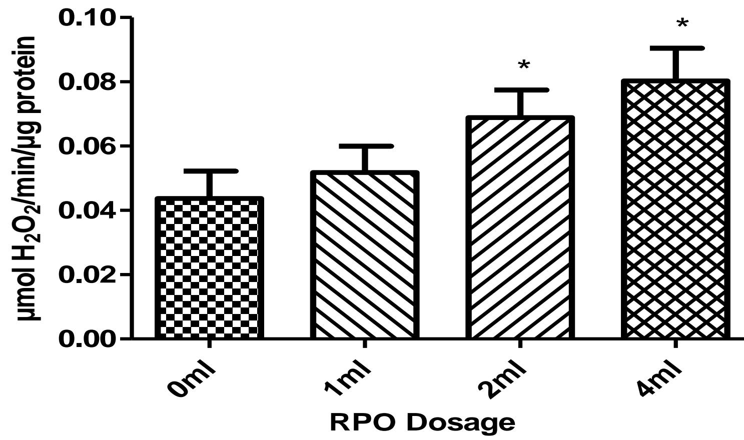


**Figure 1: Effect of dietary red palm oil on the activity of catalase (CAT) in the liver.**

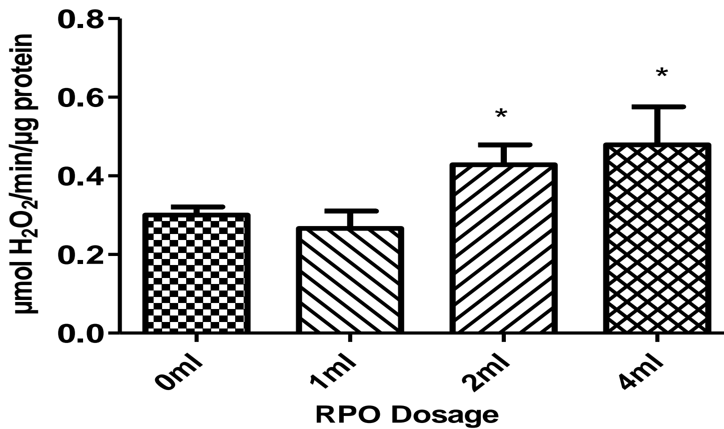


**Figure 2: Effect of dietary red palm oil on the activity of catalase (CAT) in the red blood cells.**

(*) Indicates significant difference from control group at $p<0.05$

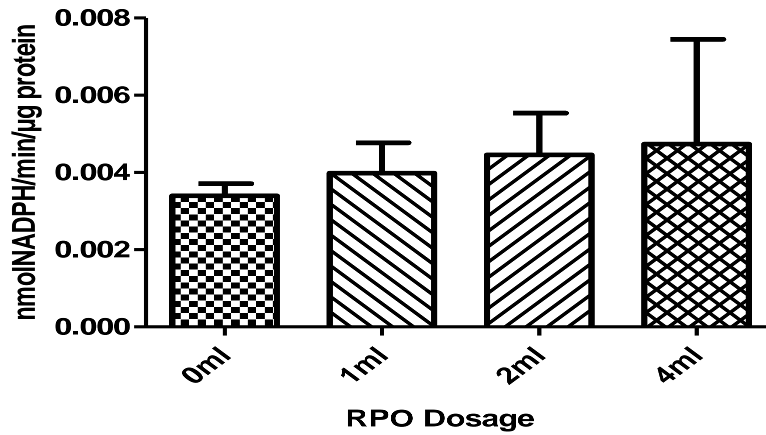


**Figure 3: Effect of dietary red palm oil on the activity of glutathione peroxidase (GPx) in the liver.**

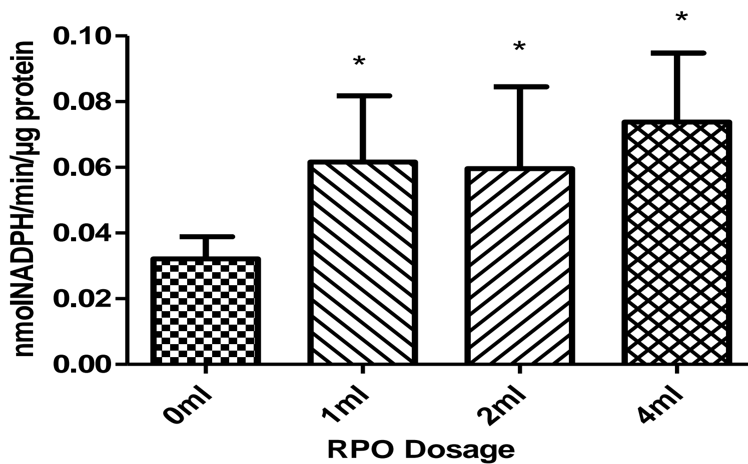


**Figure 4: Effect of dietary red palm oil on the activity of glutathione peroxidase (GPx) in the red blood cells.**

(*) Indicates significant difference from control group at $p<0.05$

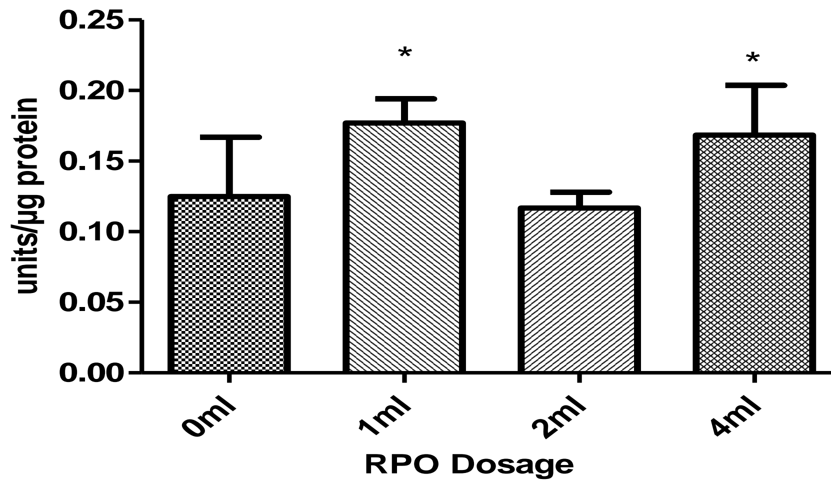


**Figure 5: Effect of dietary red palm oil on the activity of superoxide dismutase (SOD) in the liver.**

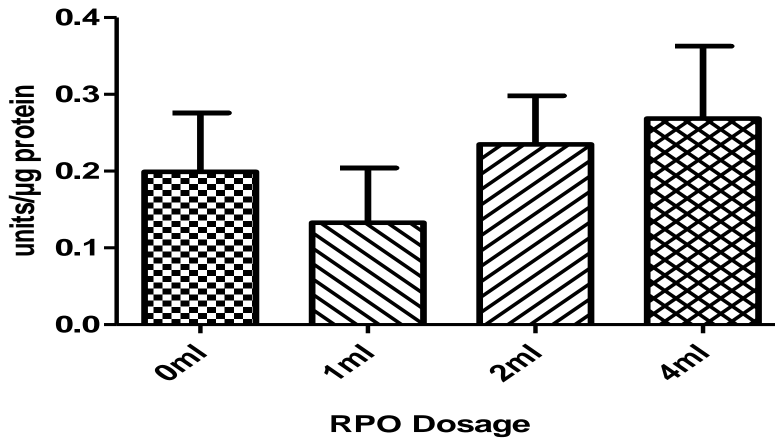


**Figure 6: Effect of dietary red palm oil on the activity of superoxide dismutase (SOD) in the red blood cells.**

(*) Indicates significant difference from control group at $p<0.05$

Table 1 indicates the effects of different doses of red palm oil on plasma and liver FRAP status, plasma polyphenol and GSHt in the RBCs are shown in Table 6. There were no significant (p>0.05) effects of red palm oil on the levels of FRAP, glutathione and plasma total polyphenols when compared with the control group.

**Table 1: Effect of different doses of red palm oil on FRAP status, total plasma polyphenol and GSHt levels in the rats.**

| RPO dosage | FRAP | | Total polyphenol | GSHt |
|---|---|---|---|---|
| | μmol/L | μmol/g tissue | mg/L | μmole/mg protein |
| | Plasma | Liver | Plasma | RBCs |
| 0 ml | 274.92 ± 95.62 | 1.16 ± 0.29 | 133.20 ± 19.33 | 0.060 ± 0.02 |
| 1 ml | 362.83 ± 21.77 | 0.90 ± 0.17 | 134.02 ± 11.53 | 0.044 ± 0.02 |
| 2 ml | 274.46 ± 97.62 | 1.22 ± 0.14 | 134.48 ± 14.58 | 0.059 ± 0.02 |
| 4 ml | 282.33 ± 53.79 | 1.36 ± 0.32 | 136.09 ± 32.37 | 0.058 ± 0.02 |

(*) Indicates significant difference from control group at p<0.05. FRAP, ferric reducing antioxidant power; GSHt, total glutathione.

Table 2 indicates the levels of total protein, albumin and globulin in rats fed with various doses of RPO were not significantly (p>0.05) different in all the groups when compared with the control group.

**Table 2: Effect of different doses of red palm oil on total protein, albumin and globulin in the rats.**

| RPO dosage | Total protein | Albumin | Globulin |
|---|---|---|---|
| | g/L | g/L | g/L |
| 0 ml | 58.47 ± 3.26 | 34.67 ± 0.82 | 23.79 ± 3.12 |
| 1 ml | 58.67 ± 1.98 | 35.24 ± 1.13 | 23.43 ± 1.69 |
| 2 ml | 55.17 ± 2.15 | 33.92 ± 1.29 | 21.24 ± 1.01 |
| 4 ml | 57.39 ± 2.89 | 34.08 ± 0.51 | 23.31 ± 2.94 |

Figures 7-10 indicate the histopathology of the liver of rats. The histopathology of the liver of control group and groups fed with 1ml, 2 ml and 4 ml RPO showed normal structures. No lipid accumulation was observed using H & E staining method in all the palm oil fed groups.

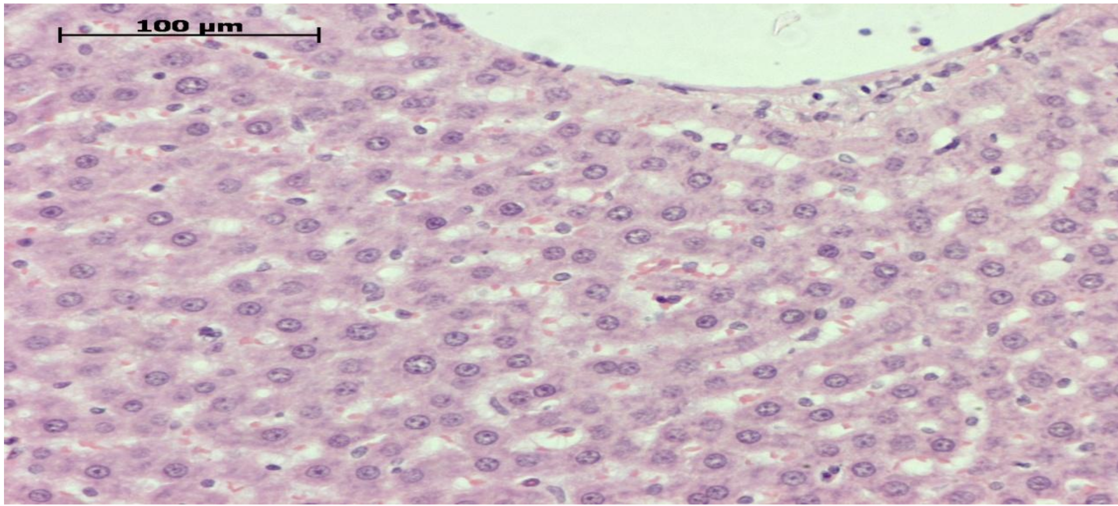


**Figure 7: The histopathology of the liver of the control group.**

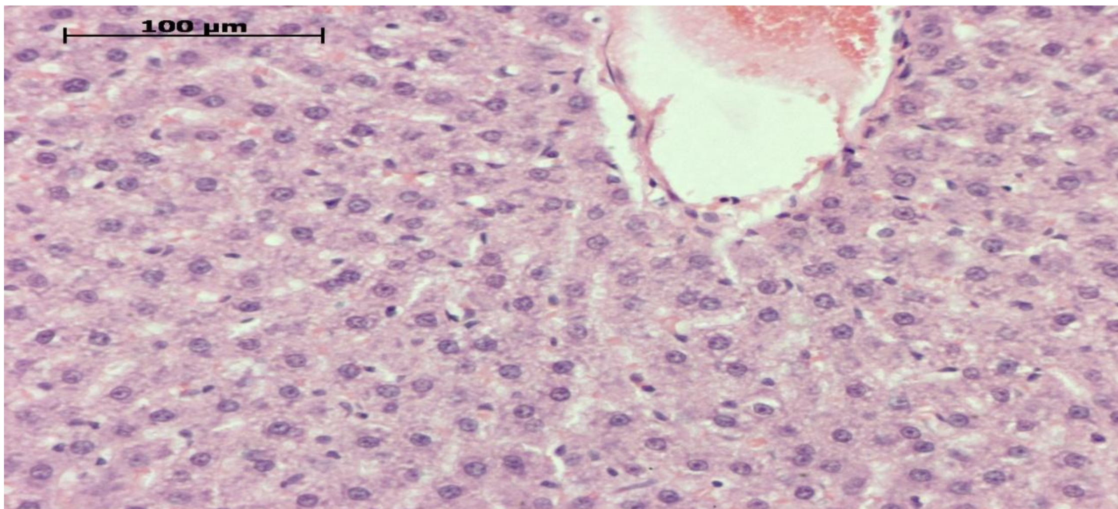


**Figure 8: The histopathology of the liver at 1ml RPO.**

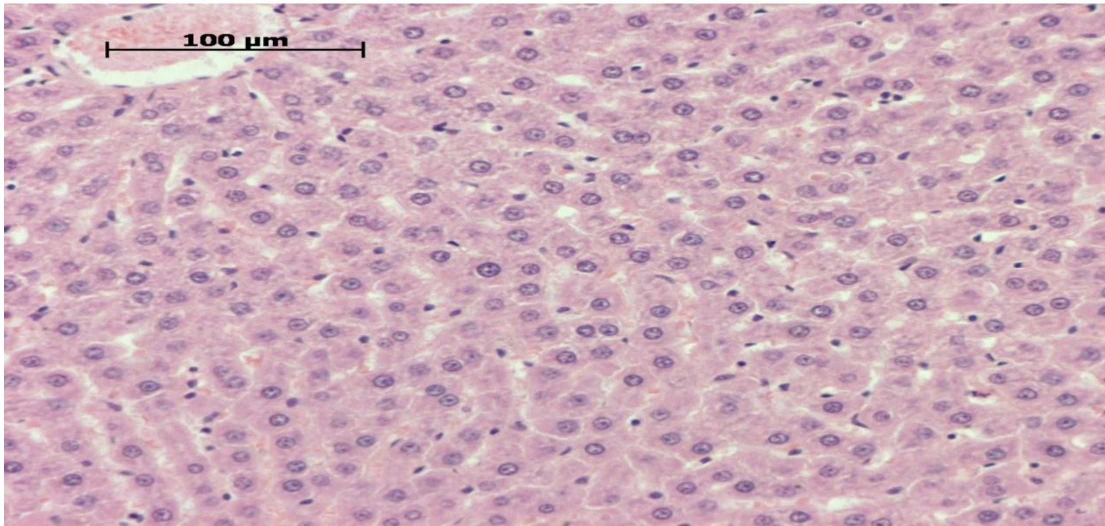


**Figure 9: The histopathology of the liver at 2ml RPO.**

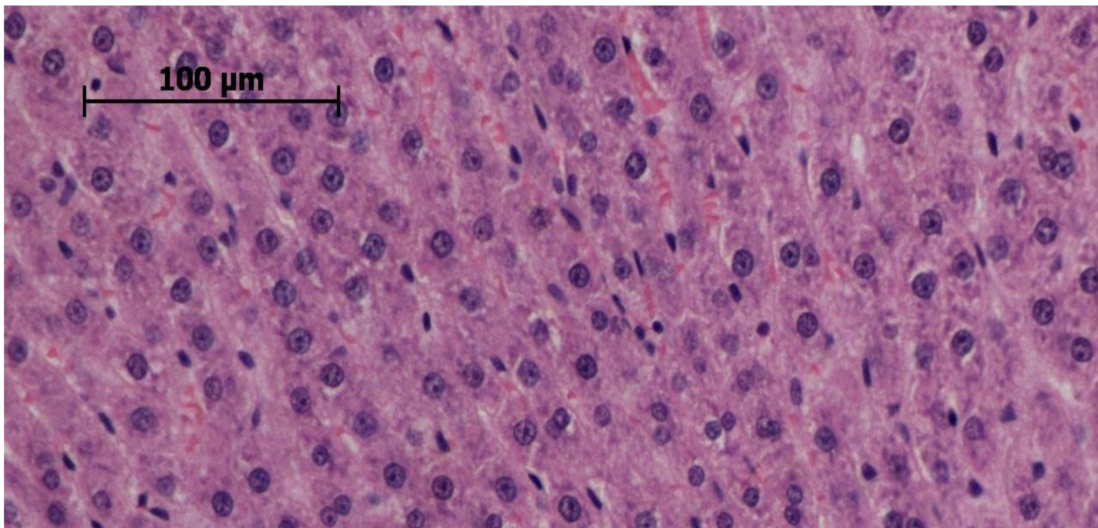


**Figure 10: The histopathology of the liver at 4ml RPO.**

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## DISCUSSION

Antioxidants are substances that, when present in foods at low concentrations compared with that of an oxidizable substrate, clearly delay or prevent the oxidation of the substrate (Sahidi, 2000). Antioxidant actions have been ascribed to different mechanisms such as prevention of chain initiation, decomposition of peroxides, reducing capacity and radical scavenging (Yildrim *et al.,* 2000; Hazra e*t al.,* 2008). They are believed to play an important role in the defence system to counteract ROS which are involved in the pathophysiology of the aging process in the body (Sakai *et al.,* 2010). The combined effect of carotenoids, tocopherols, tocotrienols and 50% unsaturation of the fatty acids gives palm oil a higher oxidative stability as compared to other vegetable oils (Arora *et al.,* 2006; Ayeleso *et al.,* 2012). Palm oil is a key source of vitamin E (having both tocopherols and tocotrienols) (Muharis *et al.,* 2010). Oguntibeju *et al.* (2010) reported that the connection between nutrition and health in oxidative stress has created more research interest in red palm oil. Tocotrienols have powerful neuroprotective, anti-cancer and cholesterol lowering properties that are frequently not shown by tocopherols (Sen *et al.,* 2006). Reduced risk of cancer, cardiovascular disease and age-related macular degeneration has been linked with dietary intake of carotenoids, even though, the overall evidence is inconsistent (Copper *et al.,* 1999; Copper, 2004). It has been suggested that a combination of carotenoids and vitamin E (tocopherols and tocotrienols) present in RPO plays a vital role in protecting against free radical damage (Dauqan *et al.,* 2011).

The FRAP assay is reproducible and linearly related to the molar concentration of the antioxidant (Ahmad and Khan, 2012). It measures the sample`s ability to reduce the intense blue ferric tripyri-dyltriazine complex to its ferrous form, thereby changing its absorbance (Benzie and Strain, 1996; Molan *et al.,* 2008). The reducing capacity of a compound could be used as an important indicator of its possible antioxidant activity (Hazra e*t al.,* 2008). In this study, the results showed that red palm oil did not alter the plasma FRAP status and total polyphenols levels in the experimental animals. The reason could be related to the fact that no disease condition was induced in the animals during the time of this study. Oxidative stress has been linked to cardiovascular diseases, cancer, and other chronic diseases that account for the majority of deaths (Wilcox *et al.,* 2004). SOD, CAT, and GPx are considered to be the most important endogenous enzymes in protecting oxidatively challenged tissues as they exhibit synergistic interactions by protecting each other from specific free radical attacks (Wijeratne *et al.,* 2005). Superoxide dismutase is a protective enzyme that can

selectively scavenge the superoxide anion radical by catalyzing its dismutation to hydrogen peroxide (Fridovich, 1983, Ceretta *et al.,* 2012).

Catalase breaks down hydrogen peroxide ($H_2O_2$) to water and molecular oxygen while GPx reduces $H_2O_2$ to water at the cost of oxidation of reduced glutathione (GSH) (Jena and Chainy, 2011). Hydrogen peroxide, a weak oxidizing agent inactivates a few enzymes directly by oxidation of essential thiol (-SH) groups (Hazra *et al.,* 2008). The present study shows that red palm was able to elevate the activities of some antioxidant enzymes in the experimental rats. The activities of CAT in the liver at 2 ml and 4 ml RPO and GPx in the RBCs at 4ml RPO were increased. SOD was significantly increased in the liver at 1ml and 2 ml RPO. The no significant difference in the liver SOD at 2 ml RPO could be due to the physiological conditions of the animals in the group. The results suggest that RPO could up-regulate the activity of these enzymes at the extracellular level and can potentially help the body to fight against oxidative stress mediated diseases. Narang *et al.* (2005) also showed a significant rise in myocardial SOD, CAT and GPx activities in rats fed with palm olein oil. However, the mechanism by which red palm oil could induce antioxidant enzymes is still unknown. Reduced glutathione is a major non-enzymatic and intracellular antioxidant that acts as reducing agent for the elimination of $H_2O_2$ and lipid hydroperoxide with the action of GPx and glutathione S-transferases (GST) (Jena and Chainy, 2011; Sadi *et al.,* 2012). The present study did not show any significant difference in the level of total glutathione in the RBCs of palm oil fed rats when compared with the control group.

Proteins have long been regarded as a principal target for oxidants due to their abundance in biological systems (Medina-Navarro *et al.,* 2010). This study showed no significant differences in the levels of total protein, albumin and globulin in comparison to the normal control rats. Albumin has numerous important physiological and pharmacological functions such as transportation of metals, fatty acids, cholesterol, bile pigments, and drugs (Roche *et al.,* 2008). It represents the key and predominant antioxidant in plasma, a body compartment known to be exposed to continuous oxidative stress (Roche *et al.,* 2008). Albumin concentrations could be found enhanced in sites of inflammation, for the protein to exert its multiple antioxidant properties (Halliwell, 1998; Roche *et al.,* 2008). Albumin, bound to bilirubin efficiently inhibited lipid oxidation and the antioxidant activity is likely due to an interaction of bilirubin with α-tocopherol incorporated within lipoproteins (Neuzil and Stocker, 1994). Histhopathological evaluations revealed a normal structure of the liver in all the red palm oil fed rats when compared with the control.

In conclusion, dietary intake of RPO may confer a wide variety of beneficial health effects and the different dosages of RPO used in this study did not cause any undesirable alterations in the biochemical parameters of RBCs and liver of the rats. It can be suggested that the increased activities of antioxidant enzymes is as a result of the inducing effect of RPO in the supplemented diet of the rats. Due to the fact that oxidative stress has been implicated in the development of various diseases such as diabetes, cardiovascular diseases, cancer, HIV/AIDS, further studies are required to explore the antioxidant potentials of palm oil as well as the mechanism by which red palm oil could induce the activation of antioxidant enzymes.

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# CHAPTER FIVE

## ASSESSEMENT OF LIPID PROFILES, ANTIOXIDANT STATUS AND LIVER HISTOPATHOLOGY IN MALE WISTAR RATS FOLLOWING CONSUMPTION OF ROOIBOS

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## ABSTRACT

### Background

Rooibos is a herbal tea that is known to contain a high and complex profile of antioxidants (polyphenols). The lipid profiles, antioxidant status and liver histopathology in rats fed with different concentrations of aqueous rooibos extract were studied.

### Methods

The rats were randomly divided into four groups (A-D). Group A served as the control group which consumed standard rat chow with tap water only, while groups B, C and D received standard rat chow with 2%, 4% and 6% rooibos extracts respectively orally as the only source of drinking. Liver and red blood cell antioxidant enzymes (catalase, glutathione peroxidase, superoxide dismutase), plasma ferric reducing antioxidant power, plasma total polyphenol levels and total glutathione in the red blood cells were investigated using established techniques. Total protein, albumin and globulin levels in the serum were carried out using an automated chemistry analyzer.

### Results

The results showed no significant differences in the plasma and liver ferric reducing antioxidant powers in all the rooibos fed groups. At 2% and 4% rooibos extracts, plasma total polyphenol did not show any significant difference ($p>0.05$) while it significantly increased at 6% rooibos extract when compared with the control group. There was a non-significant ($p>0.05$) decrease in total cholesterol, triglycerides, low density lipoprotein cholesterol and high density lipoprotein cholesterol levels in all the rooibos fed groups when compared with the control group. Liver catalase activity significantly ($p<0.05$) increased in all of the rooibos fed groups while there were no significant differences in the catalase and glutathione peroxidase activities in the red blood cells. Superoxide dismutase activities did not show any significant ($p>0.05$) difference in both the red blood cells and liver. No significant ($p>0.05$) difference was found in the total glutathione levels of the red blood cells in all the rooibos fed groups in comparison with the control group. However, there was a non-significant increase in glutathione levels at 2% rooibos extract while it significantly ($p<0.05$) increased at 4% and 6% rooibos extracts when compared with the control group. Total protein, albumin and globulin levels were not significantly ($p>0.05$) different in all the groups. Histopathological evaluations revealed no adverse effects in the structure of the liver in the rats.

**Conclusion**

It can be suggested from the overall results that rooibos can be helpful in diseased conditions due to its ability to enhance the body antioxidant system and therefore, further research studies are warranted.

**Key words- Lipid profiles, Antioxidant, Histopathology, Wistar rats, Rooibos**

## INTRODUCTION

Rooibos (*Aspalathus linearis)* is a herbal tea that can be found in the Cederberg mountain range area of the Western Cape, Republic of South Africa and it is known to contain a high and complex profile of antioxidants (polyphenols). It is an important source of flavonoids such as aspalathin and nothofagin. Phenolic and polyphenolic compounds in edible plants have been shown to exhibit potent antioxidant activities (Fang *et al.,* 2002). The favourable effects of tea polyphenolic compounds on scavenging free radicals and their role in the prevention and therapy of diseases such as coronary heart disease, hypertension, type 2 diabetes and cancer have been documented (Fang *et al.,* 2002). The expression of antioxidant enzymes and other detoxifying enzymes can be regulated by oxidative stress and by low concentrations of a broad variety of chemical agents which includes antioxidants (Matsumoto and Bastos, 2009). The induction of antioxidant enzymes by chemoprotective agents is an effective way of protection against multistage carcinogenesis in cellular models and experimental animals (Matsumoto and Bastos, 2009). During aerobic metabolism, defence against the reactive oxidants produced is a complex process which is provided by a system of antioxidant enzymes and antioxidant compounds (Szaleczky *et al.,* 1999). Superoxide radicals, the most abundant reactive oxygen species (ROS) generated in living systems is acted upon by superoxide dismutase (SOD) to produce hydrogen peroxide which in turn is inactivated by catalase and / or glutathione peroxidase (GPx) into water and oxygen (Narang *et al.,* 2004). Lipid profiles are risk indicators of coronary heart disease (Edem, 2002). Lipids are moved as lipid-protein complexes called lipoproteins, which are categorised according to their density and charges i.e. high density lipoprotein (HDL)-cholesterol carry lipids out of blood cells to the liver and low density lipoprotein (LDL)-cholesterol carry lipids from the liver to the cells and blood vessels (Owolabi *et al.,* 2010). Triacylglycerols have been found to be increased along with elevated total cholesterol (Owolabi *et al.,* 2010). Flavonoids preferentially enter the hydrophobic core of the membrane and exert a membrane-stabilizing effect by the modification of the lipid packing order and leads to a dramatic decrease in lipid fluidity in this region of the membrane (Arora *et al*.,

2000; Wojciech *et al.,* 2010). Tea and its components influence antioxidant capacity in biomembranes (Saija *et al.*, 1995; Wojciech *et al.,* 2010). The aim of this study was to investigate the biochemical effects of aqueous rooibos extract in male Wistar rats that were fed at different concentrations.

## MATERIALS AND METHODS

### Animal care

Male Wistar rats (192-240 g) were obtained from Stellenbosch University, Tygerberg, South Africa and used throughout the study. The study was conducted after obtaining Ethical Committee Clearance from Cape Peninsula University of Technology (CPUT/HAS-REC 2010/A002). The rats were individually housed in a well controlled environment set at 22$^{o}$C ± 2 with 50% ± 5% humidity and a 12-h hour light cycle. They were randomly placed in four groups. Group A (*n*=6) received only tap water and served as the control, while group B (*n*=6), C (*n*=6) and D (n=6) received 2%, 4% and 6% aqueous rooibos extracts (RTE) respectively substituting the drinking water. All the groups received standard rat chow. The fermented rooibos was supplied by Rooibos Ltd (Clanwilliam, South Africa). At the end of the seven weeks, all the animals were sacrificed after overnight fasting. Blood samples were collected from the abdominal aorta and then centrifuged to obtain the serum, plasma and red blood cells for biochemical analysis. The liver was removed, frozen in liquid nitrogen and stored at -80$^{o}$C until analysis.

### Preparation of rooibos extracts

Aqueous extracts of fermented rooibos was prepared by the addition of freshly boiled tap water to the leaves and stems (2 g/100 ml, 4 g/100 ml and 6 g/100 ml). The mixture was allowed to stand for 30 min at room temperature, cooled, filtered and dispensed into water bottles.

### Determination of total polyphenol, flavanol and flavonol content

The plasma was deproteinised using 0.5 M perchloric acid (PCA) (1:1 v/v). Folin–Ciocalteu method was used to determine the total polyphenol in the plasma and rooibos extracts according to the method of Singleton *et al.* (1999). The total polyphenols levels were expressed as mg gallic acid standard equivalents per litre. The flavanol content of the rooibos extracts was determined colorimetrically at 640 nm using p-dimethylaminocinnamaldehyde (DMACA) and expressed as mg catechin standard equivalents per litre extract (Delcour and de Varebeke, 1985; Treutter, 1989). The flavonol

content of the rooibos extracts was determined spectrophotometrically at 360 nm and expressed as mg quercetin standard equivalents per litre extract (Mazza *et al.,* 1999).

### Antioxidant enzymes assay

The activities of antioxidant enzymes in the liver and red blood cells were determined. Liver homogenates (10% w/v) were prepared in a phosphate buffer, centrifuged at 10,000g ($4^0$C) for 10 mins and the supernatant kept at -$80^0$C for enzyme analyses. Catalase (CAT) activity was determined spectrophotometrically at 240 nm by monitoring the decomposition of $H_2O_2$ and expressed as µmole $H_2O_2$/min/µg protein according to the method of Aebi (1984) while superoxide dismutase (SOD) activity was determined by the method of Crosti *et al.* (1987) modified for a microplate reader at 490 nm and expressed as the amount of protein (µg) required to produce a 50% inhibition of auto-oxidation of 6-hydroxydopamine. Glutathione peroxidase (GPx) activity was measured spectrophotometrically (340 nm) by the method of Ellerby and Bredesden (2000) and expressing activity as nmoles NADPH/min/µg protein.

### Ferric reducing antioxidant power (FRAP) assay

The ferric reducing antioxidant power was determined using the method described by Benzie and Strain (1996). Ten (10) µl of the plasma and liver homogenates was mixed with 300 µl FRAP reagent in a 96-well clear plate. The FRAP reagent was a mixture (10:1:1, v/v/v) of acetate buffer (300 mM, pH 3.6), tripyridyl triazine (TPTZ) (10 mM in 40 mM HCl) and $FeCl_3.6H_2O$ (20 mM). After incubation at room temperature for 30 min, the plate was read at a wavelength of 593 nm in a Multiskan Spectrum plate reader (Thermo Fisher Scientific). Ascorbic acid (AA) was used as the standard and the results expressed as $\mu$mol AAE/L for plasma and $\mu$mol AAE/g tissue for liver homogenates.

### Lipid profile determination

Triglycerides (TG), total cholesterol (TC) and high density lipoprotein (HDL)-cholesterol were evaluated with kits using a clinical chemistry analyzer (Easyra medical, USA) to manufacturer`s instructions. Very low density lipoprotein (VLDL)-cholesterol and LDL-cholesterol were calculated according to Friedewald's formula (Friedewald *et al.,* 1972). VLDL-cholesterol = TG/5 and LDL-cholesterol = TC – VLDL-cholesterol – HDL-cholesterol.

### Total glutathione, total protein, albumin and globulin analysis

The levels of total glutathione (GSHt) in the liver and red blood cells were determined according to the method of Asensi *et al.* (1999). Red blood cells were deproteinised using 5% metaphosphoric acid (MPA) solution. Liver samples were homogenized (1:10) in 15% TCA containing 1 mM EDTA. The homogenates were centrifuged at 15,000g for 10 min and

the supernatant collected. Total glutathione in the red blood cells and liver homogenates extracts was done by adding 50 µl of the samples into plate wells and 50 µl of 5, 5'-dithiobis-2-nitrobenzoic acid (DTNB) was added, followed by 50 µl of glutathione reductase. The reaction was initiated by the addition of 50 µl of nicotinamide adenine dinucleotide phosphate (NADPH) to a final volume of 200 µl. The change in absorbance was monitored at 412 nm for 5 min and levels of GSHt calculated using pure glutathione (GSH) as a standard and expressed as µmole/mg protein for red blood cells and µmole/g tissue for liver homogenates. Total protein and albumin levels in the serum were measured with kits using an automated chemistry analyzer (Easy RA Medical, USA) according to manufacturer`s instructions. Globulin level was determined by using the formula (Globulin = Total protein – Albumin).

**Histopathological evaluations**

At the end of treatment, the animals were sacrificed in order to collect the liver. The liver was blotted and freed from excess blood, fixed in 10% neutral formalin, trimmed, processed for paraffin embedment and 5 µm thick tissue sections were stained with haematoxylin. Histopathological structures of liver were examined using light microscopy at 20x magnification.

**Statistical analysis**

Data were expressed as the means ± standard deviations. Significant differences between mean values of different groups were determined by one-way analysis of variance (ANOVA) with MedCalc software. Data not normally distributed was log transformed and analyzed using the Kruskal–Wallis one-way ANOVA on ranks hypotheses. Differences were considered significant at $p<0.05$.

## RESULTS

Table 1 indicates the percentage body weight gain and liver weights of the rats. The results showed no significant ($p<0.05$) differences in the body weight of the rooibos fed rats in comparison with the control group. Similarly, there was no significant ($p<0.05$) differences in the percentage liver weight of the rooibos fed rats when compared with the control group.

**Table 1: Percentage body weight gain and liver weight in rats fed with the different concentrations of rooibos extracts**.

| Rooibos Extracts | Body weight gain (%) | Liver weight (%) |
|---|---|---|
| 0% | 55.77 ± 6.87 | 3.15 ± 0.14 |
| 2% | 65.00 ± 11.68 | 3.22 ± 0.13 |
| 4% | 61.37 ± 18.70 | 3.21 ± 0.15 |
| 6% | 51.15 ± 8.73 | 3.18 ± 0.19 |

Table 2 indicates the antioxidant profile of rooibos extracts of the different concentrations and daily intake of rooibos consumed by the rats. The higher the concentrations of the rooibos extracts, the more the antioxidants were consumed.

**Table 2: Daily intake of rooibos and antioxidant profile of rooibos extracts at different concentrations.**

| Rooibos Extracts | Rooibos intake/day (ml/day) | Polyphenol intake/day (mg/day) | Flavonol intake/day (mg/day) | Flavanol intake/day (mg/day) | FRAP status/day (µmol/day) |
|---|---|---|---|---|---|
| 0% | ND | ND | ND | ND | ND |
| 2% | 38.96 ± 3.78 | 21.05 ± 2.04 | 0.88 ± 0.09 | 0.43 ± 0.04 | 97.21 ± 9.43 |
| 4% | 35.39 ± 3.54 | 36.09 ± 3.61 | 1.48 ± 0.15 | 0.93 ± 0.09 | 180.24 ± 18.02 |
| 6% | 33.72 ± 3.44 | 47.64 ± 4.87 | 2.19 ± 0.22 | 1.37 ± 0.14 | 238.16 ± 24.32 |

ND - Not detected

Table 3 indicates the lipid profiles in the serum of rats fed with rooibos extracts at different concentrations. There were no significant (p>0.05) differences in total cholesterol (TC), triglycerides (TG), HDL-cholesterol, VLDL-cholesterol and LDL-cholesterol for all the groups when compared with the control group.

**Table 3: Effect of rooibos extracts on lipid profiles in rats at different concentrations.**

| Rooibos Extracts | TC<br>mmol/L | TG<br>mmol/L | HDL-cholesterol<br>mmol/L | VLDL-cholesterol<br>mmol/L | LDL-cholesterol<br>mmol/L |
|---|---|---|---|---|---|
| 0% | 1.72 ± 0.11 | 0.40 ± 0.11 | 0.52 ± 0.03 | 0.08 ± 0.02 | 1.12 ± 0.06 |
| 2% | 1.54 ± 0.10 | 0.33 ± 0.12 | 0.45 ± 0.04 | 0.07 ± 0.07 | 1.02 ± 0.04 |
| 4% | 1.64 ± 0.19 | 0.30 ± 0.03 | 0.47 ± 0.03 | 0.06 ± 0.01 | 1.11 ± 1.08 |
| 6% | 1.65 ± 0.13 | 0.3 2 ± 0.04 | 0.51 ± 0.04 | 0.06 ± 0.01 | 1.08 ± 0.08 |

TC, total cholesterol; TG, triglycerides; HDL, high density lipoprotein; LDL, low density lipoprotein; VLDL, very low density lipoprotein.

Table 4 indicates the total protein, albumin and globulin in the serum of rats fed with rooibos extracts at different concentrations. There were no significant (p>0.05) differences in total protein, albumin and globulin levels in all the rooibos fed groups when compared with the control group.

**Table 4: Effect of rooibos extracts on total protein, albumin and globulin in rats at different concentrations.**

| Rooibos Extracts | Total protein<br>g/L | Albumin<br>g/L | Globulin<br>g/L |
|---|---|---|---|
| 0% | 56.58 ± 1.88 | 33.98 ± 0.96 | 22.61 ± 0.92 |
| 2% | 54.17 ± 2.50 | 32.30 ± 1.03 | 21.87 ± 1.47 |
| 4% | 54.25 ± 3.17 | 32.63 ± 1.91 | 21.63 ± 1.27 |
| 6% | 54.33 ± 1.75 | 32.73 ± 0.88 | 21.60 ± 0.87 |

Table 5 indicates the effects of rooibos extracts on plasma and liver FRAP levels and plasma total polyphenol levels in the rats. No significant (p>0.05) differences in both liver and plasma FRAP status have been shown. There were no significant (p>0.05) increases in the plasma polyphenol levels in rats fed with 2% and 4% rooibos while a significant increase (p<0.05) at 6% rooibos extracts was shown when compared with the control group.

**Table 5: Effects of different concentrations of rooibos extracts on FRAP status and total polyphenols in the rats.**

| Rooibos Extracts | FRAP | | Total polyphenols |
|---|---|---|---|
| | μmol/L | μmol/g tissue | mg/L |
| | Plasma | Liver | Plasma |
| 0% | 313.80 ± 34.98 | 2.48 ± 0.27 | 108.45 ± 8.38 |
| 2% | 248.18 ± 23.32 | 2.39 ± 0.53 | 123.48 ± 32.01 |
| 4% | 254.39 ± 59.95 | 2.48 ± 0.17 | 110.06 ± 12.70 |
| 6% | 276.10 ± 40.09 | 2.43 ± 0.10 | 151.23 ± 21.84* |

(*) Indicates significant difference from control group at p<0.05. FRAP, ferric reducing antioxidant power.

Figure 1-8 indicates the effect of oral consumption of various concentrations of rooibos extracts on the activities of antioxidant enzymes at different concentrations. A significant ($p<0.05$) increase in the activity of liver CAT was shown while CAT activity in the RBCs was not significantly ($p>0.05$) different in all the rooibos fed groups when compared with the control groups. There were also no significant ($p>0.05$) differences in the activities of GPx and SOD in liver and RBCs in all the groups when compared with the control group. The total glutathione (GSHt) levels were significantly ($p<0.05$) increased in the liver at 4% and 6% rooibos extracts while an increase, though not significant, was observed for the 2% rooibos extract when compared with the control group. No significant ($p>0.05$) difference in the level of GSHt was shown in the red blood cells of treated groups when compared with the control group.

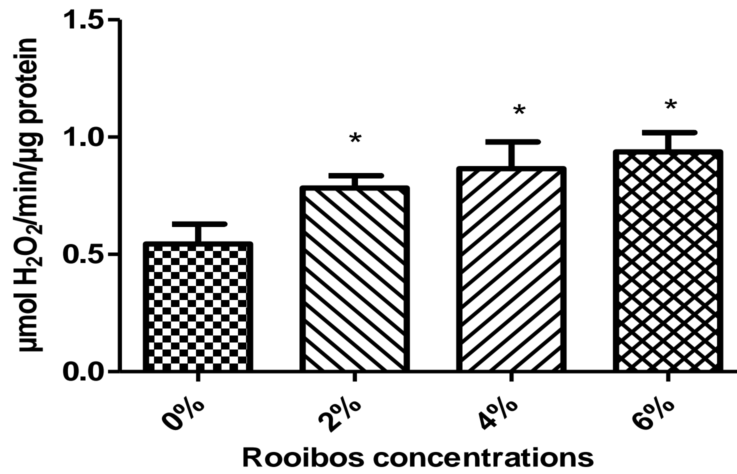


**Figure 1: Effect of rooibos extracts on the activity of catalase (CAT) in the liver.**

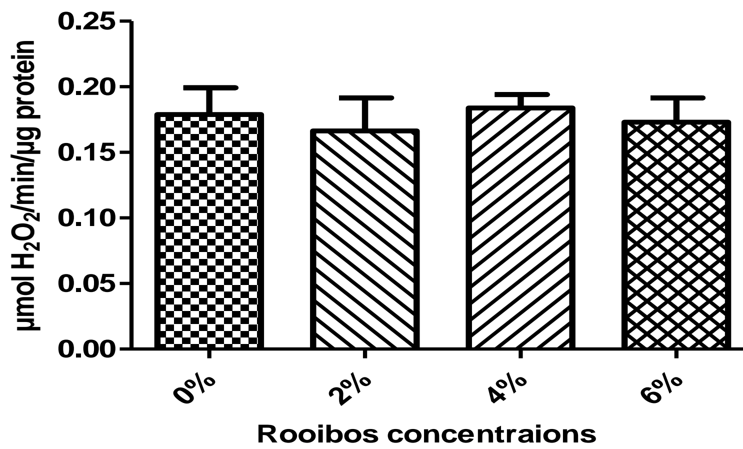


**Figure 2: Effect of rooibos extracts on the activity of catalase (CAT) in the red blood cells.**

(*) Indicates significant difference from control group at $p<0.05$

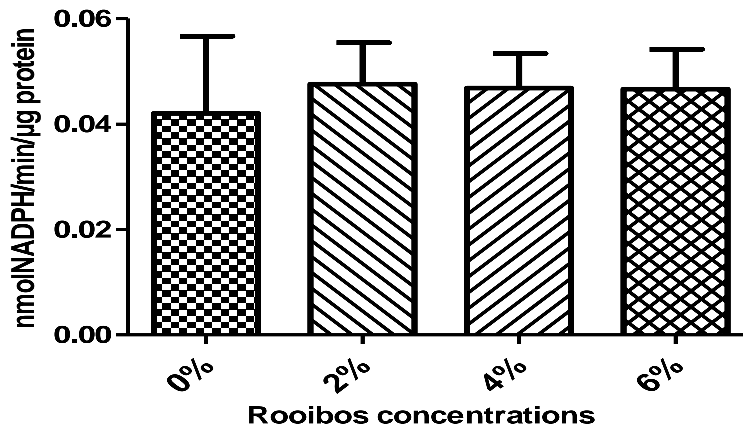


**Figure 3: Effect of dietary rooibos extracts on the activity of glutathione peroxidase (GPx) in the liver.**

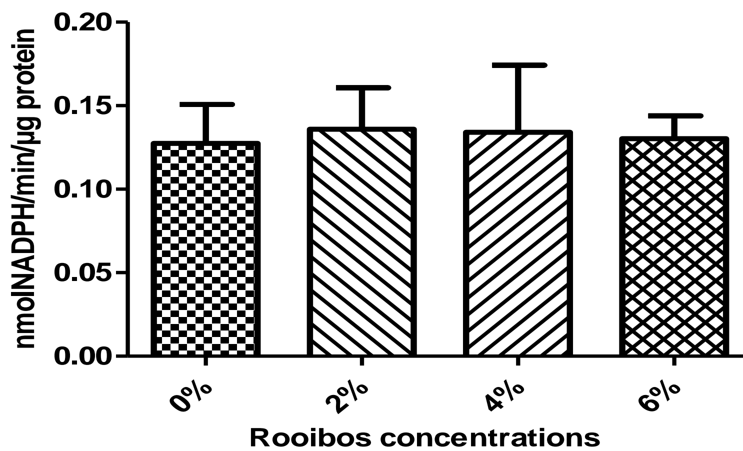


**Figure 4: Effect of dietary rooibos extracts on the activity of glutathione peroxidase (GPx) in the red blood cells.**

(*) Indicates significant difference from control group at $p<0.05$

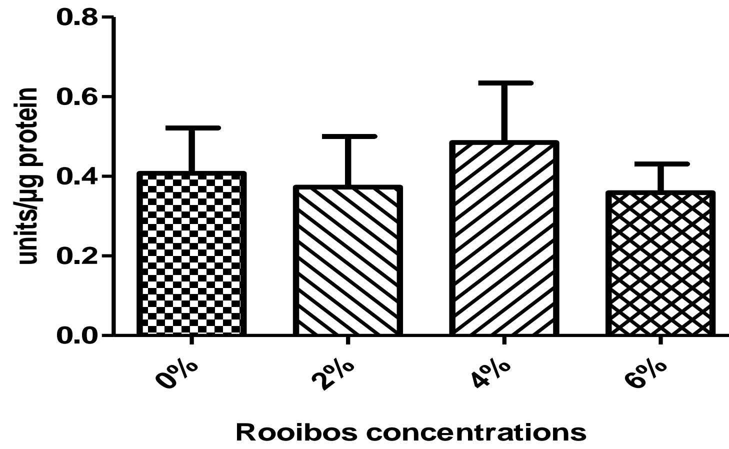


**Figure 5: Effect of rooibos extracts on the activity of superoxide dismutase (SOD) in the liver.**

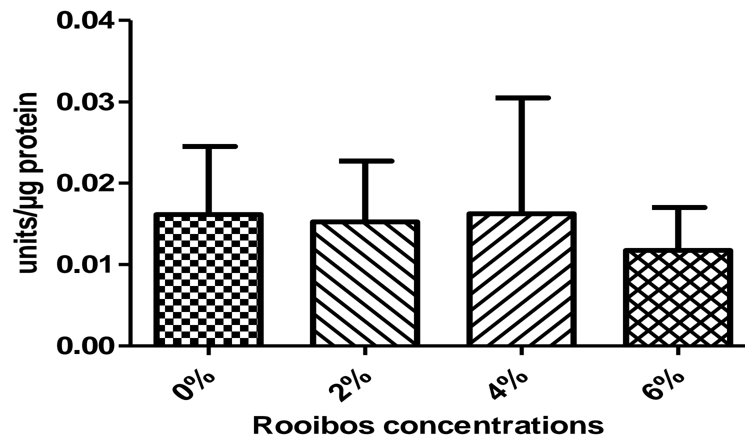


**Figure 6: Effect of rooibos extracts on the activity of superoxide dismutase (SOD) in the red blood cells.**

(*) Indicates significant difference from control group at p<0.05

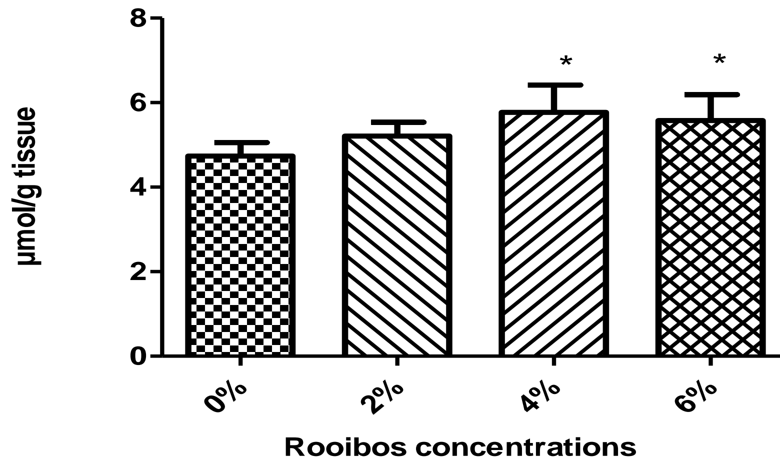


**Figure 7: Effect of rooibos extracts on the levels of liver total glutathione.**

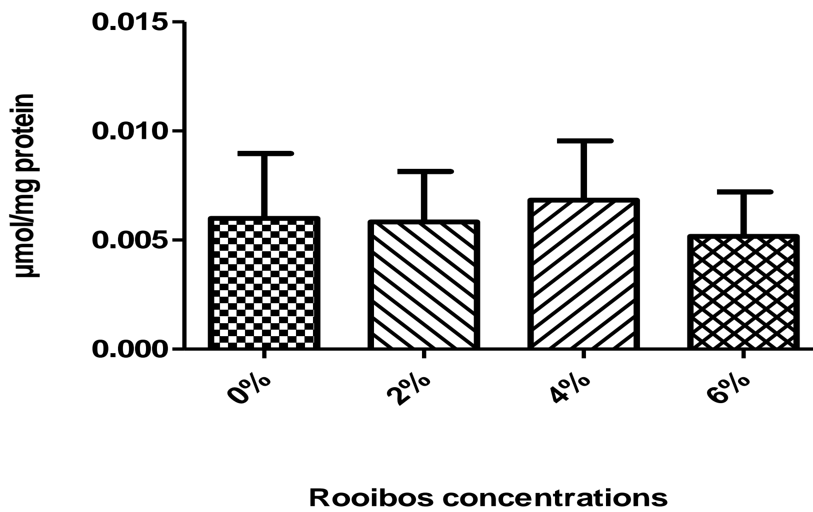


**Figure 8: Effect of rooibos extracts on the levels of total glutathione in the red blood cells.**

(*) Indicates significant difference from control group at $p<0.05$

Figures 9-12 indicate the histopathology of the liver of rats. The histopathology of the liver of control group and groups that received 2%, 4% and 6% rooibos extracts showed normal structures.

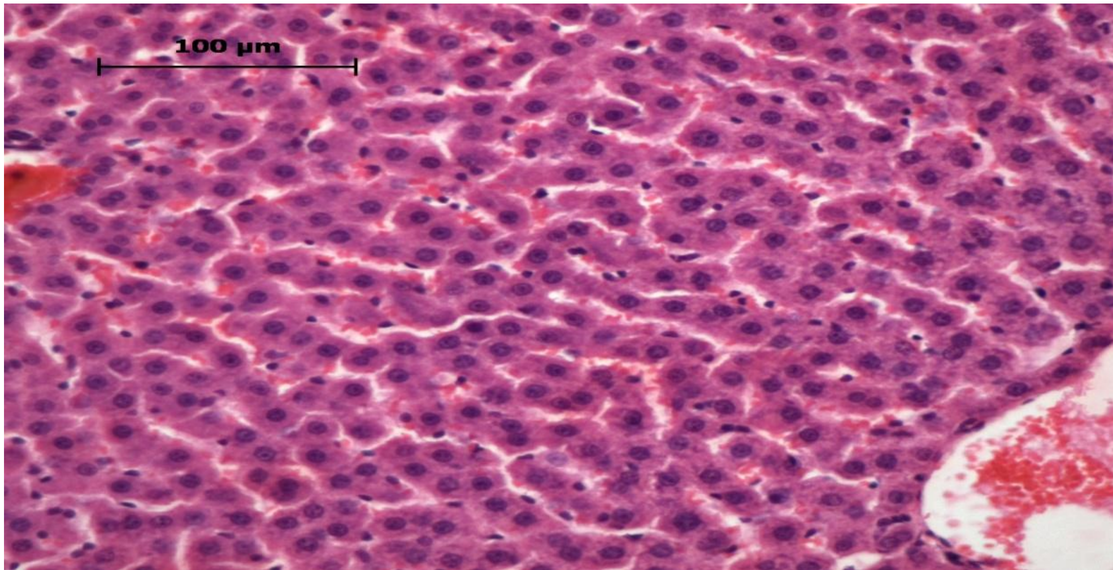


**Figure 9: The histopathology of the liver of the control group.**

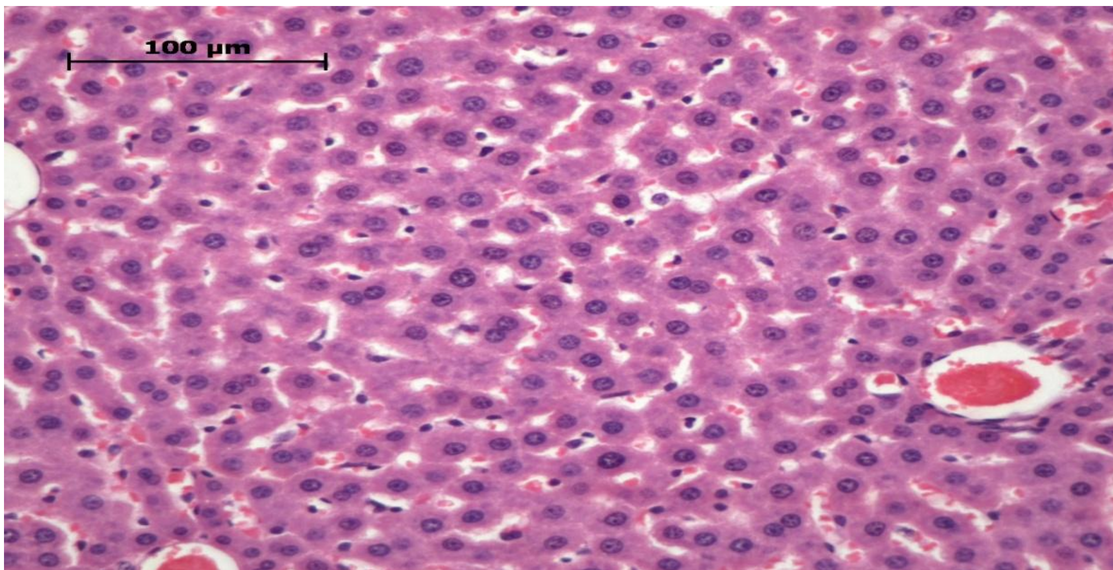


**Figure 10: The histopathology of the liver at 2% rooibos extract**

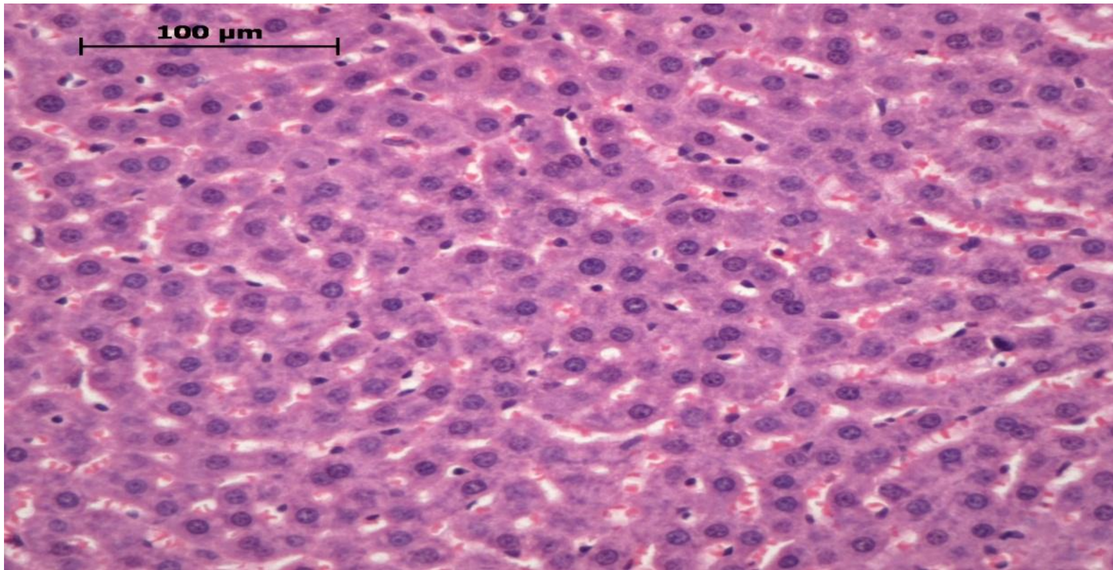


**Figure 11: The histopathology of the liver at 4% rooibos extract**

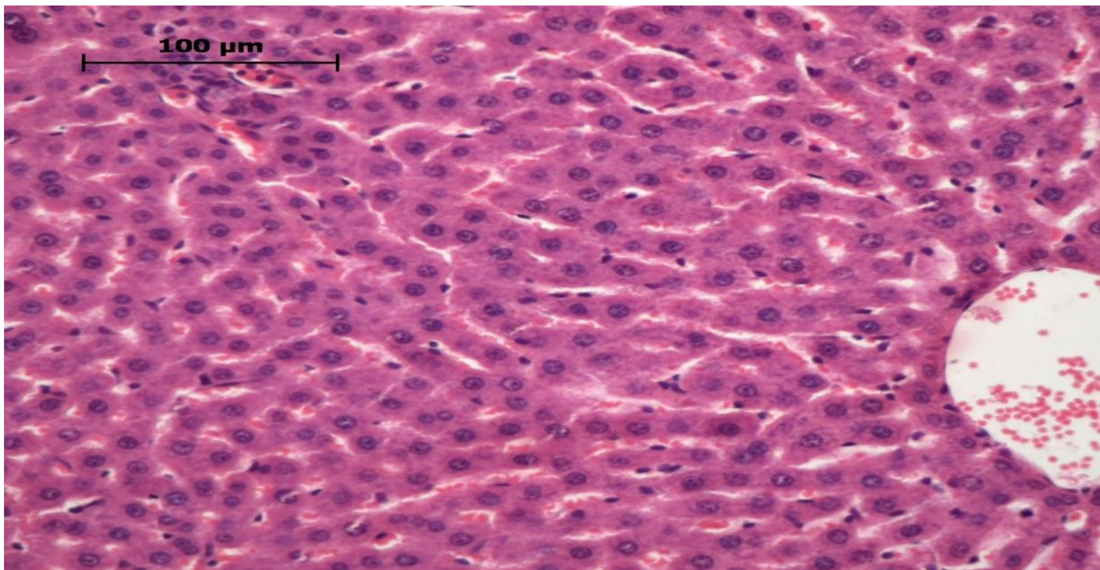


**Figure 12: The histopathology of the liver at 6% rooibos extract.**

## DISCUSSION

Rooibos is consumed for enjoyment and traditionally, it has been used to alleviate infantile colic, asthma, allergies and dermatological problems as well as certain malignancies and inflammatory disorders (Sinisalo *et al.,* 2010). There was no obvious toxicity found such as a significant decrease in body and organ weights in the rats that received rooibos extracts at different concentrations. Rooibos contains many polyphenol antioxidants that are potent free radical scavengers. The ability of rooibos to boost the liver antioxidant status and provide hepatoprotective effects on liver damage have been demonstrated (Ulicna *et al.,* 2003; Kucharska *et al.,* 2004). The involvement of active oxygen and free radicals is known in aging and diseases such as inflammation, cancer, and arterial sclerosis and hence, antioxidant enzymes such as catalase, glutathione peroxidase (GPx) and superoxide dismutase as well as some non-enzymatic enzymes such as vitamin C, vitamin E and flavonoid have anti-oxidative activity to prevent these oxidative reactions (Baba *et al.,* 2009).

In this study, there were no significant differences in the activities of GPx and SOD in both the RBCs and liver in the rats when compared with the control. The activity of CAT was significantly increased in rats receiving rooibos extracts. Similarly, there was a significant increase in glutathione levels in the liver at 4% and 6% rooibos extracts. The results suggest that rooibos contains phytochemicals that could induce the activation of CAT at the intracellular level as well as increasing liver glutathione levels. It further confirms that the administration of rooibos or its polyphenolic constituents could help to prevent or attenuate decreases in antioxidant enzyme activities in oxidative stress mediated diseases such as diabetes, cancer, cardiovascular diseases. The modulatory roles of rooibos consumption on antioxidant enzymes have been shown by several studies (Baba *et al.,* 2009; Marnewick *et al.,* 2009; Awoniyi *et al.,* 2011 and 2012). Similarly, its preventive roles on induced-oxidative stress using animal models have been reported (Marnewick *et al.,* 2003; Ulicna *et al.,* 2006; Awoniyi *et al.,* 2011 and 2012).

Fang *et al.* (2002) reported that the dietary supplementation of tea polyphenols decreased serum concentrations of total cholesterol and malondialdehyde (an indicator of lipid peroxidation) and increased serum concentrations of high density lipoprotein in humans. Though, not significantly different, the results from this study showed a decrease in the levels of cholesterol and triglycerides. Owolabi *et al.* (2010) reported that lipids and other substances are accumulated on the arterial wall and form plague, which occlude the vascular lumen and hinder the flow of blood to vital organs such as the heart, brain, liver, or

kidney. A good connection between increased plasma total cholesterol, low density lipoprotein cholesterol and increase in the occurrence of coronary heart disease has been documented (Edionwe and Kies, 2001; Kamisah *et al.,* 2005; Yakubu *et al.,* 2008). Elevated levels of all lipids except the high density lipoprotein (HDL) are associated with increased risk of atherosclerosis (Yakubu *et al,* 2009).

No significant differences in the levels of HDL-cholesterol and LDL-cholesterol were shown in all the rooibos fed rats when compared with the normal control group. In cholesterol homeostasis, HDL-cholesterol plays an important role (Wang and Peng, 2011). HDL protective effects are most widely attributed to its major role in mediating the reverse cholesterol transport from the peripheral tissues to the liver for reutilization (Eckarstein *et al.,* 2002). It is broadly known that low plasma HDL-cholesterol levels are inversely related to the risk of cardiovascular diseases (CVD) independent of other risk factors (Wang and Peng, 2011). It has also been reported that HDL-cholesterols are also carriers of enzymes that destroy the lipid hydroperoxides that oxidize LDL phospholipids (Navab *et al.,* 2002).

Albumin, the most abundant circulating protein in the plasma exerts important antioxidant activities and it acts through its multiple-binding sites and free radical-trapping properties (Roche *et al.,* 2008). A great proportion of total serum antioxidant properties can be attributed to albumin (Roche *et al.,* 2008). This study showed no significant differences in the levels of total protein, globulin and albumin in rooibos receiving groups in comparison to the control group. Albumin which consists of more than 60% of free serum proteins is synthesized and secreted by the liver and it has many vital functions such as maintaining plasma colloid osmotic pressure, anti-oxidation and substances transfer (Shi *et al.,* 2010). Serum concentrations of proteins, bilirubin and albumin can help to show the condition of the liver and also ascertain the different types of liver damage (Yakubu *et al.,* 2003). The exposure of liver to xenobiotic-induced damage is due to its central role in xenobiotic metabolism and its portal location within the circulatory system (Jones, 1996; Avwioro *et al.,* 2010). In this study, the results indicate no adverse effects on histopathology of the liver of rats subjected to different concentrations of rooibos extracts in comparison to control group.

In conclusion, the results from the present study indicated that rooibos consumption improved the antioxidant defence system while other biochemical indices measured did not show significant changes. Hence, it could be useful in the prevention and management of various diseases.

**AUTHORS' CONTRIBUTIONS**

OO and NL designed the work, supervised the work and edited the manuscript. AO performed the experiment, collated, analysed data and wrote the manuscript.

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# CHAPTER SIX

## AMELIORATIVE EFFECTS OF RED PALM OIL AND ROOIBOS ON HYPERGLYCAEMIA, LIPID PARAMETERS AND LIVER FUNCTION IN STREPTOZOTOCIN-INDUCED DIABETIC MALE WISTAR RATS

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## ABSTRACT

**Objective:** Diabetes mellitus is an endocrine disorder characterised by hyperglycaemia and results from defects in insulin secretion, insulin action, or both. The present study was designed to investigate the effects of the administration of red palm oil, aqueous rooibos extract and combined treatment of red palm oil and rooibos extract on the levels of glucose, glycogen, insulin, glycosylated haemoglobin, fructosamine, lipid profiles and liver function in streptozotocin-induced diabetic male Wistar rats.

**Materials and Methods:** Diabetes was induced by a single administration of streptozotocin (50 mg/kg) and the rats were treated for 7 weeks. The effects of these plant products on glucose, glycogen, insulin, glycosylated haemoglobin, fructosamine, lipid profiles and liver function were performed using established techniques. Pancreas histopathological evaluation was carried out using a hematoxylin and eosin stain.

**Results:** The levels of glucose, glycogen, glycosylated haemoglobin and fructosamine increased significantly while the level of insulin was significantly decreased in the diabetic control group in comparison with the normal control group. Administration of red palm oil and rooibos extract alone to diabetic rats did not reduce glucose and glycosylated haemoglobin levels while the combined treatment of red palm oil and rooibos extract significantly ($P<0.05$) decreased the levels of glucose, glycosylated haemoglobin, fructosamine and increased insulin levels in the diabetic rats. Liver function enzymes such as aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, and gamma glutamyl transpeptidase markedly increased in the diabetic rats. However, the combination of red palm oil and rooibos extract significantly ($P<0.05$) reduced alanine aminotransferase when compared with diabetic control group. Treatment of diabetic rats with red palm oil alone and the combined treatment of red palm oil and rooibos extract prevented the leakage of gamma glutamyl transpeptidase from the liver cells into the serum of the diabetic rats. Triglyceride and very high density lipoprotein-cholesterol levels were significantly ($P<0.05$) increased in the diabetic control group when compared with the normal control group. Diabetic rats treated with red palm oil showed a significant ($P<0.05$) increase in triglycerides in comparison to the normal control group while there were no significant ($P>0.05$) differences in the total cholesterol levels in both treated non-diabetic and diabetic groups. Red palm oil and rooibos extract significantly ($P<0.05$) elevated high density lipoprotein-cholesterol in the treated diabetic rats in comparison to the normal control group. The activity of pyruvate kinase was significantly ($P<0.05$) reduced in all diabetic groups when compared to normal

control group. However, combined treatment with red palm and rooibos significantly ($P<0.05$) increased the activity of pyruvate kinase when compared with the diabetic control group. There was no significant ($P>0.05$) effect on the activity of glucokinase in both the untreated and treated diabetic rats.

**Conclusion:** From these findings, it can be concluded that red palm oil and rooibos extract could help in the improvement of lipid metabolism while the combined treatment with red palm oil and rooibos extract produced pronounced beneficial effects on blood glucose control and liver functions.

**Key words: Red palm oil, Rooibos, Hyperglycaemia, Lipid parameters, Liver function, Streptozotocin**

## INTRODUCTION

Diabetes mellitus is a complex disorder arising from various causes which include dysregulated glucose sensing or insulin secretion, autoimmune-mediated β-cell destruction in type 1 diabetes or insufficient compensation for peripheral insulin resistance in type 2 diabetes (White, 2003). Hyperglycaemia which is the result of an uncontrolled glucose regulation is a link between diabetes and diabetic complications (Rolo and Palmeira, 2006). Hyperglycaemia and dyslipidaemia are the two devastating concomitants of diabetes that play a major role in creating the secondary disorders such as macro and micro vascular complications (Karvey *et al.,* 2006). Non-enzymatic glycation is referred to as the ability of reducing sugars to react with amines as well as with basic amino groups of proteins and nucleic acids without enzyme mediation (Turk *et al.,* 2001) and the compounds formed are called advanced glycation end products (AGEs) (Singh *et al.,* 2001). Hyperglycaemia plays a vital role in increased protein glycosylation (Brownlee, 2005; Ayeleso *et al.,* 2012). Oxidation of glucose produces free radicals that oxidize low density lipoproteins or favour lipoperoxidation of membrane lipids causing damage to cellular membranes (Alvarado-Vazquez *et al.,* 2003).

A majority of diabetic patients suffer from dyslipidaemia that is related to insulin resistance (Shahi *et al.,* 2011). In diabetes, one of the major pathogenesis of lipid metabolism disturbances is the increased mobilization of fatty acids from adipose tissue and secondary elevation of free fatty acid level in the blood (Singh *et al.,* 1987; Ravi *et al.,* 2005). An increase in blood cholesterol following a streptozotocin (STZ) injection could be as a result

of an increase in the concentration of acetyl CoA, a key substrate in the biosynthesis of cholesterol which arises most likely from enhanced β- oxidation stem of fatty acids (Yakubu *et al.,* 2009). In diabetes, lipid accumulation is mediated through a variety of derangements in the metabolic and regulatory processes, particularly insulin deficiency which makes the diabetic patient prone to hypercholesterolemia and hypertriglyceridaemia (Jaiprakash *et al.,* 1993; Ravi *et al.,* 2005).

High density lipoprotein (HDL) is considered to have anti-atherogenic properties and it has been reported that an increase in HDL levels correlates inversely with coronary heart disease while a decrease portends cardiovascular risk (Mayes, 1996; Yakubu *et al.* 2009; Proph *et al.,* 2012). High glucose or free fatty acids flux or both impair metabolic flexibility which may improve the supply of mitochondrial substrate and generation of reactive oxygen species (ROS) (Brownlee, 2001; Bouderba *et al.,* 2012). Hyperglycaemia seems to cause elevated levels of atherogenic cholesterol-enriched apolipoprotein B-containing remnant particles by reducing expression of the heparan sulphate proteoglycan perlecan in hepatocytes (Ebara, 2000; Brownlee, 2001). Liver disease and elevated liver enzymes are widespread in diabetic patients and the increasing level of enzymes indicates the severity of the hepatic injury (Sarkar *et al.,* 2011).

The fruit of the palm oil tree (*Elaeis guineensis*) is the source of palm oil (Mukherjee and Mitra, 2009) and it is broadly used as cooking oil in West and Central Africa and plays an essential role in energy and essential fatty acid needs supply in many regions of the world (Oguntibeju *et al.,* 2012). Red palm oil contains high level of antioxidants and the most abundant antioxidants are carotenoids and vitamin E (Bester *et al.,* 2012). It contains at least 500 ppm carotenoids of which the majority is in the form of α- and β- carotene and approximately 500 ppm vitamin E of which the majority is in the form of tocotrienols (Bester *et al.,* 2012).

Rooibos, a South African herbal tea, is made from the leaves and stems of the fynbos plant, *Aspalathus linearis* and its popularity as a health beverage are not only growing locally but also internationally (Marnewick *et al.,* 2011). The herbal tea is prepared from both the unfermented "green" and fermented "oxidised" plant material, though the usage of the traditional fermented product has a long history and its intake is more common (Beelders *et al.,* 2012). Secondary metabolites present in fermented rooibos plant include single ring phenolic acids and monomeric flavonoids such as dihydrochalcones, flavanones, flavones, and flavonols (Joubert *et al.,* 2008; Beelders *et al.,* 2012). Red palm oil and rooibos are both natural plant products known to have various health promoting benefits which can largely be

attributed to their antioxidant properties. The present study was designed to investigate the potential biochemical effects of red palm oil and rooibos and their combined treatment on glycaemic and lipidaemic parameters as well as biomarkers of liver functions on STZ-induced diabetic rats.

## MATERIALS AND METHODS

### Preparation of rooibos extract

Aqueous extracts of fermented rooibos was prepared by the addition of freshly boiled tap water to the leaves and stems (2 g/100 ml)  The mixture was allowed to stand for 30 min at room temperature, cooled, filtered and dispensed into water bottles.

### Animal care

Male Wistar rats (176-255 g) were bred and used at the Medical Research Council (MRC), Primate Unit, Tygerberg, South Africa. The study was conducted after obtaining Ethical Committee Clearance from Cape Peninsula University of Technology (CPUT/HAS-REC 2010/A002). The rats were individually housed and maintained in a temperature controlled room of 22-25 °C, humidity of 45-55%, 15-20 air changes per hour and on a 12 hour light/dark cycle and rats have free access to standard rat chow. The rats were treated by supplementing their diets with 2 ml red palm oil and/ or 2% rooibos for 7 weeks. The fermented rooibos was supplied by Rooibos Ltd (Clanwilliam, South Africa) and the red palm oil used was Carotino palm fruit oil from Malaysia.

### Induction of diabetes mellitus

Diabetes was induced by a single intramuscular injection of STZ (Sigma-Aldrich, South Africa) at the dose of 50 mg/kg of body weight into overnight fasted rats. Streptozotocin was dissolved in 0.1 M citrate buffer (pH 4.5). Diabetes was confirmed three (3) days after STZ injection by determining the blood glucose level using an Accu chek glucometer. Diabetic rats with blood glucose levels above 14 mmol/L were used for the experiment.

### Experimental design

The rats were divided into eight groups consisting of seven rats each for the non-diabetic groups and eight rats each for the diabetic groups.

**Group 1 (Normal control):** Rats received a single intramuscular injection of citrate buffer and given tap water orally for 7 weeks.

**Group 2 (Diabetic control):** Diabetes was induced by a single intramuscular injection of STZ at a dose of 50 mg/kg body weight and given tap water for 7 weeks.

**Group 3**: Rats received a single intramuscular injection of citrate buffer and fed with RPO (2 ml/day) and tap water for 7 weeks.

**Group 4:** Diabetes was induced by a single intramuscular injection of STZ at a dose of 50 mg / kg body weight and treated with RPO (2 ml/day) for 7 weeks.

**Group 5:** Rats received a single intramuscular injection of citrate buffer and fed with RTE (2%) for 7 weeks.

**Group 6:** Diabetes was induced by a single intramuscular injection of STZ at a dose of 50 mg/kg body weight and fed with RTE (2%) for 7 weeks.

**Group7:** Rats received a single intramuscular injection of citrate buffer and fed with both RPO (2 ml/day) and RTE (2%) for 7 weeks.

**Group 8:** Diabetes was induced by a single intramuscular injection of STZ at a dose of 50 mg / kg body weight and fed with both RPO (2 ml/day) and RTE (2%) for 7 weeks.

At the end of experimental period, the overnight fasted rats were sacrificed. Blood was collected from the dorsal aorta using a syringe into an EDTA tube for whole blood, potassium oxalate tube for plasma (glucose determination) and serum separator tube for serum collection. The serum and plasma were separated after centrifugation at 3,000 rpm for 15 min and then transferred into properly labelled vials and stored at -80° C until the analysis was carried out. The liver tissues were excised, rinsed in saline solution, blotted on filter paper, weighed and stored at -80°C. The percentage weight of the pancreas was calculated using the formula below:

$$\text{The percentage weight of organ} = \frac{\text{Absolute weight of organ}}{\text{Final body weight of rat}} \times 100$$

**Glucose and lipid profile determination**

The levels of glucose, triglycerides (TG), total cholesterol (TC) and high density lipoprotein (HDL)-cholesterol were evaluated with kits using a clinical chemistry analyzer (Easyra medical, USA) according manufacturer`s instructions. Very low density lipoprotein (VLDL)

and low density lipoprotein (LDL)-cholesterol were calculated according to Friedewald's formula (Friedewald *et al.,* 1972). VLDL-cholesterol = TG/5 and LDL-cholesterol = TC – VLDL-cholesterol – HDL-cholesterol.

**Insulin, glycosylated haemoglobin and fructosamine determination**

Serum insulin level was determined with a rat insulin radioimmunoassay kit (Millipore, USA). The glycosylated haemoglobin (HbA1c) in whole blood and serum fructosamine levels were determined with kits (Diazyme Laboratories, USA) using a chemistry analyser (Vitalab Selectra E) according to manufacturer`s instructions.

**Glycogen content determination**

Glycogen content in the liver was determined according to the method described by Ong and Khoo (2000) with modifications. Weighed amount of the liver tissues in 2 volumes of an ice-cold 30% (w/v) KOH solution were boiled in a boiling water-bath ($100^0$ C) for 30 min and precipitated with 0.625 ml of 95% ethanol. The solution was mixed and centrifuge at 10,000g for 30 min. The supernatant was discarded and the pellet solubilised with 500 ul distilled water and reacted with anthrone (1 g of anthrone dissolved in 500 ml of $H_2SO_4$), read at 620nm using a micro plate reader. The amount of glycogen present was determined from the glycogen standard and expressed as mg/g tissue.

**Liver function and glycolytic enzymes**

Liver function enzymes: Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), Alkaline phosphatase (ALP), Lactate dehydrogenase (LDH), Gamma glutamyl transpeptidase (GGT) were evaluated with kits using a clinical chemistry analyzer (EasyRA Medical, USA) according manufacturer`s instructions. Pyruvate kinase (PK) activity was determined with colorimetric assay kits (Bio Vision, USA) and glucokinase activity (GK) was assessed with enzyme immunoassay technique kits (Cusabio, China).

**Histopathological evaluations**

At the end of the treatment, animals were sacrificed to collect the pancreas. The pancreas was blotted to remove excess blood, fixed in 10% buffered formalin, trimmed and processed for paraffin embedment. Tissue sections of 5 μm thick were stained with haematoxylin and eosin. Histopathological evaluations of pancreatic sections were examined using light microscopy at 20x magnification.

### Statistical analysis

Data were expressed as the means ± standard deviations. Significant differences between mean values of different groups were determined by one-way analysis of variance (ANOVA) using MedCalc software. Data not normally distributed was log transformed and analyzed using the Kruskal–Wallis one-way ANOVA on ranks hypotheses. Differences were considered significant at $p<0.05$.

## RESULTS

Table 1 shows the effects of RPO and / or RTE treatments on the percentage weight of the pancreas and body weight gain. Significantly less weight was gained on a daily basis in the STZ control group and all the treated diabetic groups when compared with the normal control group. Diabetic rats treated with RPO+RTE showed a significant ($p<0.05$) increase in the body weight when compared with the STZ control group. A significant ($p<0.05$) increase in the pancreas weight in the STZ control group as well as the diabetic rats treated with RPO, RTE and RPO + RTE was shown. Similarly, non-diabetic rat fed with RTE and RPO + RTE showed an increase in pancreas weight when compared with the normal control group.

**Table 1: Effect of RPO, RTE and RPO + RTE treatments on the pancreas weight and body weight gain.**

| Treatment groups | Body weight gain/day (g) | Pancreas weight (%) |
|---|---|---|
| NORMAL CONTROL | 2.93 ± 0.48 | 0.36 ± 0.06 |
| STZ CONTROL | 0.05 ± 0.60 [a] | 0.51 ± 0.05 [a] |
| RPO | 2.68 ± 0.70 | 0.42 ± 0.04 |
| STZ + RPO | 0.73 ± 0.65[b] | 0.55 ± 0.08[b] |
| RTE | 2.86 ± 0.29 | 0.46 ± 0.07* |
| STZ + RTE | 0.22 ± 0.81[b] | 0.60 ± 0.09[bc] |
| RPO + RTE | 2.37 ± 0.16 | 0.49 ± 0.05* |
| STZ + RPO+ RTE | 1.11 ± 0.48[bc] | 0.50 ± 0.07[b] |

All significant differences are at $p<0.05$. STZ, streptozotocin-induced diabetes; RPO, red palm oil; RTE, aqueous rooibos extract.

(*) represents significant difference between non-STZ fed groups and normal control group.
([a]) represents significant difference between STZ control group and normal control group.
([b]) represents significant difference between treated STZ groups and normal control group.
([c]) represents significant difference between treated STZ groups and STZ control group.

Table 2 shows the effects of RPO and / or RTE treatments on glycaemic parameters. There was no significant ($p>0.05$) difference in glucose levels in the non-diabetic rats fed with RPO, RTE and RPO + RTE when compared with the normal control group. The levels of glucose in the diabetic rats treated with RPO and RTE alone did not show any significant ($p>0.05$) difference. However, the combined treatments (RPO + RTE) significantly ($p<0.05$) reduced the glucose level in comparison to the STZ control group. There was a significant ($p<0.05$) decrease in the insulin levels in all the diabetic rats when compared with the normal control group. However, a significant increase in insulin level was noted for STZ + RPO + RTE when compared with the STZ control group. The levels of glycogen significantly ($p<0.05$) increased in all the diabetic rats when compared with the control group. Hepatic glycogen levels in diabetic rats treated with RPO and RPO + RTE were not significantly ($p>0.05$) different in comparison to the STZ control group. However, diabetic rats treated with RTE alone significantly ($p<0.05$) decreased glycogen level when compared with the STZ control group. There was a significant ($p<0.05$) increase in the level of glycosylated haemoglobin in all the diabetic rats when compared with the normal control group. Combined treatment (RPO + RTE) significantly ($p<0.05$) decreased the Hb1Ac level when compared with the STZ control group while diabetic rats fed with either RPO or RTE alone did not show any significant ($p>0.05$) difference in the levels of Hb1Ac when compared with the STZ control group. Fructosamine was significantly ($p<0.05$) increased in all the diabetic groups when compared with the normal control group. However, treatment with RPO + RTE significantly ($p<0.05$) reduced fructosamine in comparison to the STZ control group. The fructosamine levels in rats fed with RTE and RPO + RTE were non- detectable.

**Table 2: Effect of RPO, RTE and RPO + RTE treatments on glycaemic parameters.**

| Treatment groups | FBG<br>mmol/L | Insulin<br>ng/mL | Glycogen<br>mg/g | Hb1Ac<br>% | Fructosamine<br>mmol/L |
|---|---|---|---|---|---|
| NORMAL CONTROL | 7.12 ± 1.02 | 1.82 ± 0.52 | 0.11 ± 0.01 | 9.35 ± 1.91 | 5.97 ± 3.65 |
| STZ CONTROL | 20.98 ± 6.46 [a] | 0.30 ± 0.09 [a] | 4.01 ± 1.77 [a] | 16.74 ± 2.73 [a] | 98.61 ± 23.35 [a] |
| RPO | 6.62 ± 1.19 | 1.15 ± 1.84 | 0.10 ± 0.01 | 9.61 ±0.65 | 4.88 2.50 |
| STZ + RPO | 22.41 ± 7.14[b] | 0.46 ± 0.16[b] | 4.13 ± 2.89[b] | 14.39 ± 3.44[b] | 95.14 ± 22.92[b] |
| RTE | 6.30 ± 0.80 | 1.63 ± 0.64 | 0.11 ± 0.01 | 9.74 ± 1.20 | - |
| STZ + RTE | 21.03 ± 5.98[b] | 0.31 ± 0.17[b] | 2.29 ± 0.35[bc] | 13.79 ± 3.59[b] | 110.84 ± 28.66[b] |
| RPO + RTE | 7.49 ± 1.13 | 1.87 ± 0.70 | 0.12 ± 0.01 | 8.63 ± 2.38 | - |
| STZ + RPO + RTE | 15.60 ± 5.94[bc] | 0.72 ± 0.21[bc] | 4.00 ± 1.14[b] | 12.41 ± 2.25[c] | 62.52 ± 28.41[bc] |

All significant differences are at $p<0.05$. STZ, streptozotocin-induced diabetes; FBG, fasting blood glucose; RPO, red palm oil; RTE, aqueous rooibos extract; Hb1Ac, glycosylated haemoglobin.

(*) represents significant difference between non-STZ fed groups and normal control group.

([a]) represents significant difference between STZ control group and normal control group.

([b]) represents significant difference between treated STZ groups and normal control group.

([c]) represents significant difference between treated STZ groups and STZ control group.

Table 3 shows the effects of RPO and / or RTE on serum lipid parameters. There was no significant ($p>0.05$) difference in the levels of TC in all the groups. The levels of TG were significantly ($p<0.05$) increased in the STZ control group and diabetic rats treated with RPO when compared with the normal control group. There was no significant ($p>0.05$) effect on the levels of TG in diabetic rats treated with RTE and RPO + RTE in comparison with the STZ control group. A significant ($p<0.05$) reduction in the level of triglyceride in non-diabetic rats fed with RTE was observed. There was a significant ($p<0.05$) increase in the levels of HDL-cholesterol in the diabetic rats fed with RPO and RTE alone while RPO + RTE did not have any significant ($p>0.05$) effect on HDL-cholesterol in the diabetic rats when compared with the normal control group. There was no significant ($p>0.05$) difference in the level of LDL-cholesterol in all the groups. The levels of VLDL-cholesterol significantly ($p<0.05$) increased STZ control group and diabetic rats treated with RPO. There was no ($p>0.05$) significant difference in the levels of VLDL-cholesterol in diabetic rats treated with RTE and RPO + RTE.

**Table 3: Effect of RPO, RTE and RPO + RTE treatments on serum lipid parameters.**

| Treatment groups | TC<br><br>mmol/L | TG<br><br>mmol/L | HDL-cholesterol<br><br>mmol/L | LDL-cholesterol<br><br>mmol/L | VLDL-cholesterol<br><br>mmol/L |
|---|---|---|---|---|---|
| NORMAL CONTROL | 1.21 ± 0.24 | 0.25 ± 0.06 | 0.36 ± 0.06 | 0.80 ± 0.19 | 0.05 ± 0.01 |
| STZ CONTROL | 1.25 ± 0.19 | 0.53 ± 0.31 [a] | 0.47 ± 0.06 | 0.67 ± 0.15 | 0.11 ± 0.06 [a] |
| RPO | 1.23 ± 0.13 | 0.44 ± 0.17 | 0.39 ± 0.04 | 0.77 ± 0.12 | 0.09 ± 0.03 |
| STZ + RPO | 1.34 ± 0.17 | 0.85 ± 0.48 [b] | 0.53 ± 0.10[b] | 0.65 ± 0.17 | 0.17 ± 0.10[b] |
| RTE | 1.27 ± 0.13 | 0.18 ± 0.07* | 0.39 ± 0.03 | 0.85 ± 0.10 | 0.04 ± 0.01 |
| STZ + RTE | 1.26 ± 0.25 | 0.50 ± 0.27 | 0.51 ± 0.11[b] | 0.64 ± 0.11 | 0.10 ± 0.05 |
| RPO + RTE | 1.27 ± 0.13 | 0.31 ± 0.08 | 0.38 ± 0.05 | 0.83 ± 0.08 | 0.06 ± 0.02 |
| STZ + RPO + RTE | 1.21 ± 0.28 | 0.45 ± 0.22 | 0.45 ± 0.13 | 0.64 ± 0.17 | 0.09 ± 0.04 |

All significant differences are at $p<0.05$. STZ, streptozotocin-induced diabetes; RPO, red palm oil; RTE, aqueous rooibos extract; TC, total cholesterol; TG, triglycerides; HDL, high density lipoprotein; LDL, low density lipoprotein; VLDL, very low density lipoprotein.

(*) represents significant difference between non-STZ fed groups and normal control group.

([a]) represents significant difference between STZ control group and normal control group.

([b]) represents significant difference between treated STZ groups and normal control group.

([c]) represents significant difference between treated STZ groups and STZ control group.

Table 4 shows the effects of RPO and / or RTE on liver function and glycolytic enzymes. Serum ALT, AST and ALP were significantly ($p<0.05$) increased in all the diabetic groups in comparison to the normal control group. There was a significant ($p<0.05$) decrease in ALT in diabetic rats treated with RPO + RTE when compared with the STZ control group. There was no significant ($p>0.05$) effect on ALP in diabetic rats treated with RPO, RTE and RPO + RTE when compared with the STZ control group. GGT was not detectable in the serum of all normal control and normal treated groups. Similarly, diabetic rats treated with RPO and RPO + RTE did not show the presence of GGT while it was found in the serum of STZ control group as well as the diabetic rats treated with RTE only. A non-significant increase ($p>0.05$) of LDH in STZ control group as well as treated diabetic rats fed with RPO in comparison to the normal control group was observed. The activity of pyruvate kinase (PK) was significantly ($p<0.05$) reduced in the STZ control group as well as treated diabetic groups in comparison to the normal control group. Diabetic rats treated with RPO and RTE alone did not show significant ($p>0.05$) increase in PK activity while RPO + RTE significantly increased the activity of PK when compared with the STZ control group. Non-diabetic rats fed with RPO and RPO + RTE showed a significant ($p<0.05$) decrease in activity of PK when compared with the normal control group. There was no significant ($p>0.05$) difference in the activities of glucokinase (GK) in all the groups.

**Table 4: Effect of RPO, RTE and RPO + RTE treatments on liver function and glycolytic enzymes.**

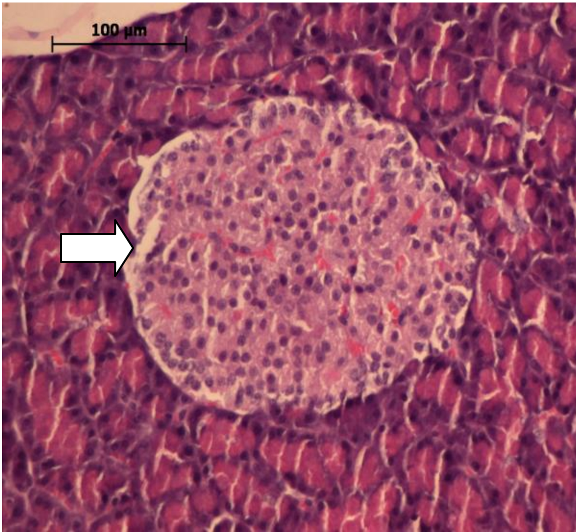
| Treatment groups | AST U/L | ALT U/L | ALP U/L | LDH U/L | GGT U/L | PK mU/mg tissue | GK ng/mg tissue |
|---|---|---|---|---|---|---|---|
| NORMAL CONTROL | 64.96 ± 9.38 | 45.60 ± 8.41 | 72.00 ± 15.52 | 105.00 ± 45.29 | - | 32.18 ± 2.29 | 3.71 ± 0.09 |
| STZ CONTROL | 166.96 ± 129.75 [a] | 110.58 ± 62.90[a] | 205.18 ± 112.09[a] | 245.50 ± 84.27 | 6.40 ± 1.67 | 16.24 ± 4.07 [a] | 3.61 ± 0.11 |
| RPO | 72.16 ± 15.20 | 49.82 ± 16.04 | 78.93 ± 18.55 | 204.14 ± 48.10 | - | 25.53 ± 3.77 | 3.75 ± 0.10 |
| STZ + RPO | 121.17 ± 60.59[b] | 102.38 ± 72.28[b] | 224.31 ± 70.65[b] | 252.80 ± 65.31 | - | 17.44 ± 1.87[b] | 3.66 ± 0.14 |
| RTE | 77.10 ± 18.09 | 43.75 ± 10.77 | 70.50 ± 11.63 | 163.25 ± 41.79 | - | 33.19 ± 3.47 | 3.55 ± 0.10 |
| STZ + RTE | 131.84 ± 49.45[b] | 86.81 ± 23.01[b] | 246.75 ± 132.05[b] | 195.50 ± 66.05 | 6.80 ± 0.97 | 17.03 ± 5.22[b] | 3.57 ± 0.10 |
| RPO + RTE | 64.29 ± 14.38 | 62.59 ± 7.46* | 60.07 ± 9.68 | 184.58 ± 68.25 | - | 25.99 ± 4.70* | 3.74 ± 0.11 |
| STZ + RPO + RTE | 84.93 ± 16.82[b] | 64.88 ± 22.84[c] | 216.88 ± 142.53[b] | 173.93 ± 107.72 | - | 26.48 ± 5.87[bc] | 3.62 ± 0.06 |

All significant differences are at $p<0.05$. STZ, streptozotocin-induced diabetes; RPO, red palm oil; RTE, aqueous rooibos extract; AST,aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; LDH, lactate dehydrogenase; GGT, gamma glutamyl transpeptidase; PK, pyruvate kinase; GK, glucokinase.

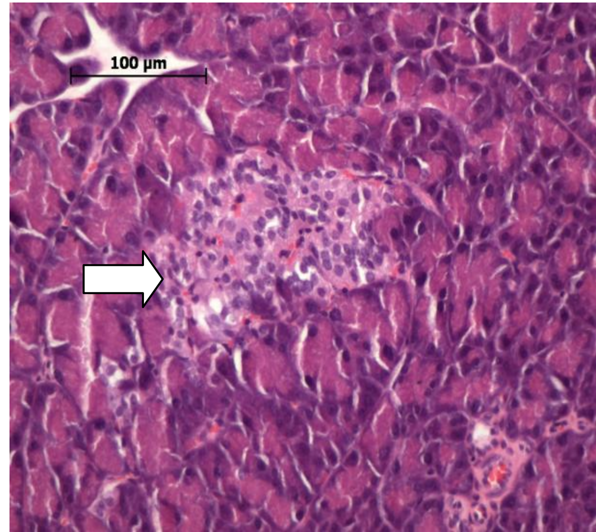
(*) represents significant difference between non-STZ fed groups and normal control group.
([a]) represents significant difference between STZ control group and normal control group.
([b]) represents significant difference between treated STZ groups and normal control group.
([c]) represents significant difference between treated STZ groups and STZ control group.

Figure 1a and 1b show the histopathological evaluations of the pancreas showing islets of Langerhans. The results from the histopathological evaluations of the pancreas as indicated by the arrows revealed that normal control and treated normal rats showed a greater presence of the islets compared to the non-treated and treated STZ diabetic rats as a result of destruction of β-cells by streptozotocin.

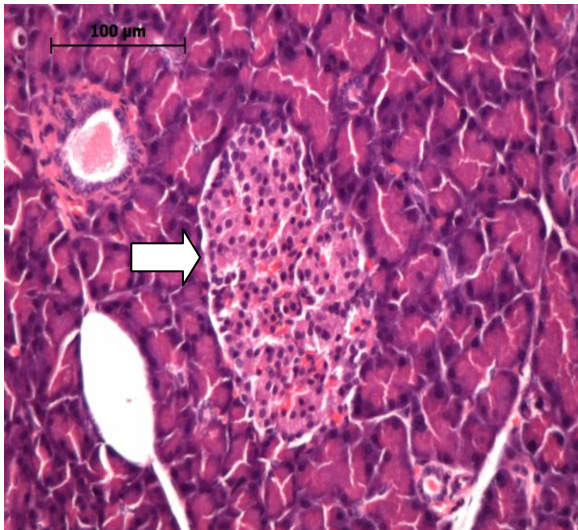
A




B




C

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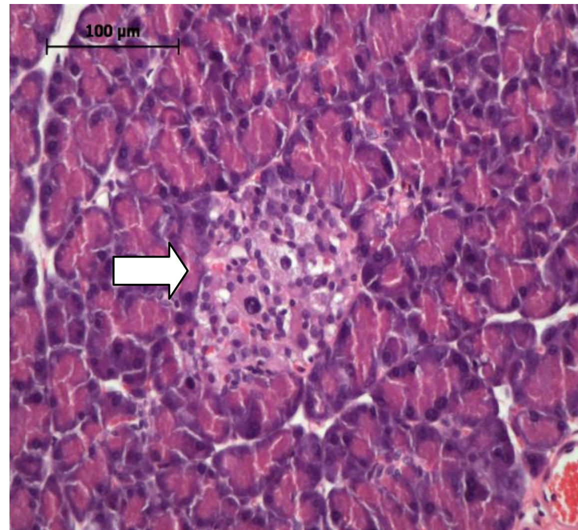
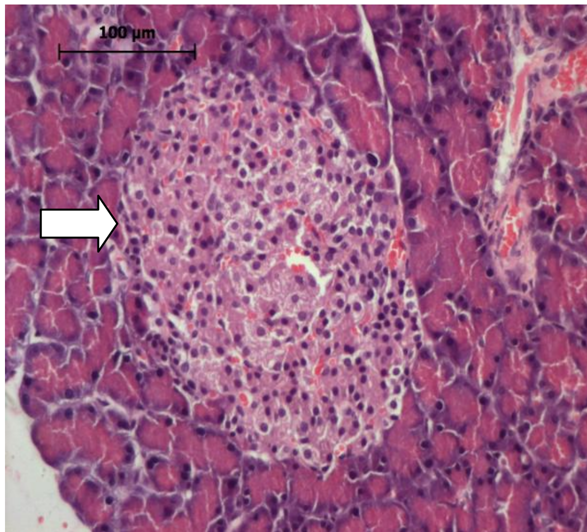
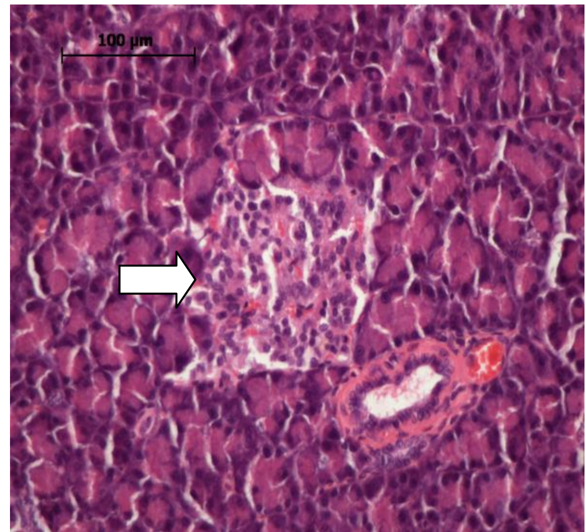



**Figure 1a: Histopathological evaluations of the pancreas showing islets of Langerhans in (A) Normal control group (B) Diabetic control group (C) RPO only group (D) Diabetes + RPO group.**

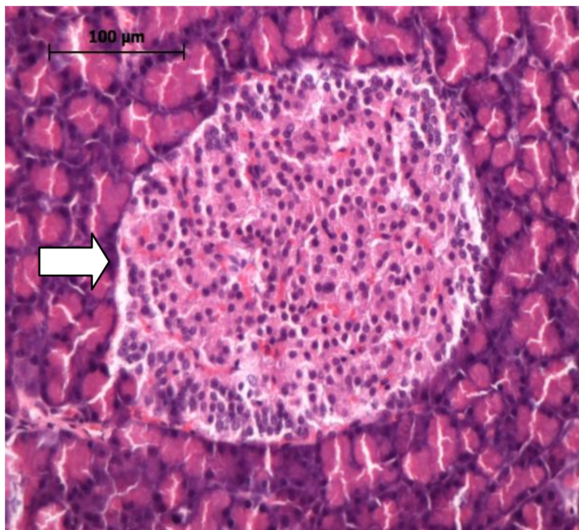
E




F




G




H

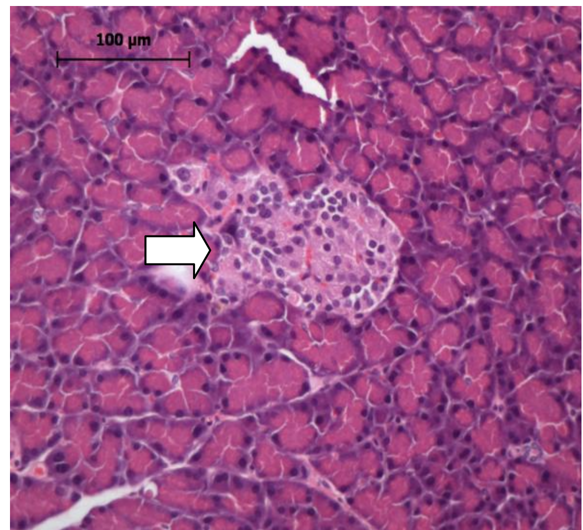



**Figure 1b: Histopathological evaluations of the pancreas showing islets of Langerhans in (E) RTE only group (F) Diabetes + RTE group (G) RPO + RTE group (H) Diabetes + RPO + RTE group.**

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## DISCUSSION

It has been suggested that experimental animal models are one of the best ways to understand the pathophysiology of any disease (Rees *et al.,* 2005; Chatzigeorgiou *et al.,* 2009; Ali *et al.,* 2011). In this study, the intra-muscular administration of streptozotocin effectively induced diabetes mellitus in rats which was confirmed by elevated levels of fasting plasma glucose. The biological effects of STZ may be ascribed to its hydrophilicity, glucose similarity and alkylation (Ali *et al.,* 2011). Administration of RPO and RTE alone to the animals did not prevent loss of body weight in STZ-diabetic rats, however, the combined treatment with RPO + RTE was able to increase the body weight when compared with the STZ control group. The glucose levels of the normal rats administered with RPO, RTE and RPO + RTE were not altered indicating their normoglycaemic effects. In this study, the increased levels of plasma glucose in the diabetic rats treated with RPO and RTE alone were not significantly different from the diabetic control group. However, there was a significant reduction in glucose level in the diabetic rats with combined treatment (RPO + RTE). Similarly, RPO + RTE significantly increased insulin level in the diabetic rats in comparison to the diabetic group. The results reveal the anti-hyperglycaemic effects of RPO + RTE which could be as a result of an increased responsiveness of tissues to insulin or increased release of insulin and possibly due to regeneration of islets of langerhans in the pancreas.

Elevated serum glucose levels have been shown to stimulate the synthesis of advanced glycation end products (AGEs) and this leads to the continuous induction of oxidative stress as well as an increasing production of reactive oxygen species (ROS) (Diaz-Flores *et al.*, 2004; Alvarado-Vásquez *et al.,* 2006). Excess blood glucose during the course of diabetes reacts with haemoglobin to form glycosylated haemoglobin (Subramanian *et al.,* 2012). Administration of RPO and RTE individually showed a non-significant reduction in the glycosylated haemoglobin level in the rats. However, the combined treatment with RPO and RTE significantly reduced the glycosylated haemoglobin level indicating an improvement in glycaemic control following their administration. This reduction shows an anti-hyperglycaemic activity, since the concentration of HbA1c is more parallel to the observed blood glucose concentrations. We also observed a significant increase in the levels of fructosamine in all the diabetic groups. However, RPO+RTE significantly decreased fructosamine level while there was no significant reduction in the levels of fructosamine in the diabetic rats fed with RPO and RTE alone.

An increase in gluconeogenesis is the main mechanism responsible for increased glucose output while glycogenolysis has not been shown to be increased in patients with type 2 diabetes (Consoli *et al.,* 1989; Sarkar *et al.,* 2011). In this study, the results showed a significant increase in glycogen levels in all the diabetic groups. Ferrannini *et al.* (1990) reported a similar increase in liver glycogen in chronic diabetes and it was reported that the reason could be due to metabolic changes which improved gluconeogenesis and participated in the repletion of liver glycogen stores. The build up of excess glycogen is seen in 80% of diabetic patients (Stone and van Thiel, 1985; Levinthal and Tavill, 1999). Aljabri *et al.* (2011) reported that inactivation of glycogen phosphorylase owing to hyperglycaemia results in the inhibition of glycogenolysis and activation of glycogen synthase hence, leading to glycogen synthesis. Glycogen synthase (UDP-glucose-glycogen glucosyltransferase) is an enzyme involved in converting glucose to glycogen while glycogen phosphorylase is an enzyme that breaks up glycogen into glucose subunits. An increase in glycogen synthase (a) has been reported in long term diabetes while a decrease was found in short term diabetes in rats (Ferrannini *et al.,* 1990). It has been postulated that long standing insulin deficiency may assist synthase activity (Levinthal and Tavill, 1999). This study showed that RTE alone significantly decreased glycogen level in the diabetic rats. Diabetic patients showing too much glycogen deposition may show hepatomegaly and liver enzyme abnormalities as well as abdominal pain, nausea, vomiting and all these aberrations may be improved with glucose control (Chatila and West, 1996; Levinthal and Tavill, 1999). Aljabri *et al.* (2011) showed that accumulation of glycogen in glycogen hepatopathy (a rare cause of serum transaminase elevations in type 1 diabetes mellitus) has been found to be causing hepatomegaly and elevated liver enzymes, especially transaminase.

The liver plays a vital role in carbohydrate metabolism regulation and liver function tests are frequently used in clinical practice to screen for liver disease, monitor the progression of known disease, and monitor the effects of potentially hepatotoxic drugs (Sarkar *et al.,* 2011). Serum aminotransferases such as ALT indicates the concentration of intracellular hepatic enzymes that have seeped out into circulation which serves as a marker of hepatocyte injury (Aljabri *et al.,* 2011). Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities are used as the indicators of hepatocytes damage (Pratt and Kaplan 2009; Farokhi *et al.,* 2012). Primarily, ALT is mainly found in the liver but AST can be found in the liver and some other organs, so it is a less-specific marker for liver toxicity (Pratt and Kaplan, 2009; Farokhi *et al.,* 2012). This study showed elevated levels of AST, ALT, ALP, and GGT in the serum of the diabetic rats. The combined treatment (RPO + RTE) was able to reduce the level of ALT significantly in the diabetic rats. This reduction in ALT reveals the potential benefits of combined RPO + RTE treatment in the prevention of liver injury and this reduction could be as

a result of the antioxidant properties of the combined RPO and RTE. The gamma-glutamyl transpeptidase (GGT), another liver enzyme acts as a marker of biliary function and cholestasis (Aljabri *et al.,* 2011). In this study, GGT was found in the serum of the diabetic rats and the fact that it could not be detected in the diabetic rats treated with RPO suggests that RPO was able to prevent liver damage by the non-leakage of GGT into the serum.

Type 2 diabetes patients often have dyslipidaemia which causes them to be at risk of cardiovascular diseases and this dyslipidaemia is characterized by an increase in the level of TG and a reduced level of HDL-cholesterol while levels of TC and LDL-cholesterol may be either normal or elevated (Buse *et al.,* 2004). This study revealed a non-significant difference in the level of cholesterol in all the diabetic rats. The rate at which plasma fatty acids become triglycerides is greater than normal in hyperlipidaemia (a major complication in diabetes), leading to an increase in the plasma triglyceride concentration (Kim *et al.,* 2008; Kim *et al.,* 2010). Muthulingam *et al.* (2010) reported an abnormally increased concentration of serum lipids in diabetes is mainly owing to the increase in free fatty acids mobilization from the peripheral depots, since insulin inhibits the hormone sensitive lipase. In this study, the results indicate a significant increase in the level of TG in the diabetic control group as well as the RPO treated diabetic group. A similar increase in TG levels in RPO fed diabetic rats has been reported (Kochikuzhyil *et al.* 2010). It is suggested that the increase could be as a result of the high amount of palmitic acid that is present in red palm oil. However, RTE showed a significant decrease in the serum TG level of the non-diabetic rats when compared with the normal control group.

Marnewick *et al.* (2011) showed that rooibos significantly reduced serum levels of LDL-cholesterol and triacylglycerol levels and non-significantly reduced total cholesterol levels while HDL-cholesterol levels were significantly higher in adults at risk of cardiovascular diseases. The changes in the levels of major lipids such as cholesterol, high density lipoprotein, low density lipoprotein and triglycerides levels may well provide helpful information about lipid metabolism as well as predisposition of the heart to atherosclerosis and its associated coronary heart diseases (Yakubu *et al.,* 2008: Proph *et al.,* 2012). In contrary to several reports, the results from this study showed a non-significant increase in HDL-cholesterol levels in all the diabetic groups. Significant elevated HDL-cholesterol is due to the increase in the level of total cholesterol in the diabetic animals (O`Meara *et al.,* 1990; Kim *et al.,* 2010). Increased cholesterol levels in diabetic rats may be attributed to their lack of ability to metabolize carbohydrates as an energy source, and the subsequent use of free fatty acids for energy and cholesterol synthesis (Yao *et al.,* 2008; Kim *et al.,* 2010). Diabetic rats fed with

RPO and RTE alone showed a significantly increase in HDL cholesterol when compared with the normal control rats.

Pyruvate kinase is the last enzyme involved in glycolysis that catalyses the transfer of a phosphate group from phosphoenol pyruvate (PEP) to adenosine diphosphate (ADP), yielding one molecule of pyruvate and one molecule of adenosine triophosphate (ATP). In this study, the results showed a significant decrease in the activity of pyruvate kinase in the STZ diabetic rats in comparison with the normal control rats. A similar decrease in the activity of pyruvate kinase in diabetes has been reported following induction of diabetes (Aly and Mantawy, 2012). The inhibition of pyruvate kinase will prevent the conversion of phosphoenol pyruvate to pyruvate and hence, the metabolite is converted back to glucose in a series of gluconeogenesis reactions. Diabetic rats treated with either RPO or RTE did not show any significant difference while rats treated with the combination of RPO + RTE showed a significant increase in the activity of pyruvate kinase when compared with the STZ control group.

Glucokinase is an enzyme catalyzing the phosphorylation of glucose and other hexoses by means of phosphoryl donors (ATP, ADP, and inorganic polyphosphate) and are related homologously and by evolution to at least three other hexokinases (Kawai *et al.,* 2005). Glucokinase in the liver is an essential regulator of glucose storage and disposal (Saravanan and Pugalendi, 2005; Lee, 2006) and its activity was decreased in the liver of diabetic rats which may be due to a deficiency of insulin (Lee, 2006). In this study, glucokinase was not altered in the STZ induced diabetic rats. Glucokinase, which plays a role in the generation of a metabolic signal for glucose induced secretion of insulin, is not actively involved in mediating STZ toxicity [53]. This suggests that glucokinase may not be part of the observed hypoglycaemic property of RPO + RTE. Pancreas histopathological evaluations in the diabetic control group and treated diabetic groups showed a striking reduction in mass of pancreatic islets as a result of damage to the islets in comparison to the normal rats. Marked reduction in mass of islets has also been shown in non-treated STZ induced diabetic rats [54]. No significant changes in the histopathology of the pancreas were observed in the treated non-diabetic rats.

In conclusion, the results indicate that the continuous administration of antioxidant compounds of plant origin play a complementary role in the management of metabolic diseases such as diabetes mellitus. The combined treatments of red palm oil and rooibos was able to show more significant beneficial effects in the management of diabetes and this could be due to the

combination of different antioxidants (both fat soluble and water soluble) that are present in these two plant products.

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# CHAPTER SEVEN

## MODULATORY EFFECTS OF RED PALM OIL AND ROOIBOS ON ANTIOXIDANT STATUS IN STREPTOZOTOCIN-INDUCED HYPERGLYCAEMIC MALE WISTAR RATS

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## ABSTRACT

Oxidative stress, mediated chiefly by hyperglycaemia-induced generation of free radicals has been reported to contribute to the progression of diabetes and its complications. The present study was aimed to investigate the modulatory role of red palm oil, aqueous rooibos extract and their combined treatment on antioxidant status in the red blood cells and liver of normal and streptozotocin-induced diabetic rats. Antioxidant enzymes (catalase, glutathione peroxidase, superoxide dismutase), antioxidant capacity such as ferric reducing antioxidant power, oxygen radical absorbance capacity and trolox equivalent antioxidant capacity as well as total protein, albumin, globulin, total glutathione, conjugated diene and thiobarbituric acid reactive substances were investigated. Treatment of diabetic rats with red palm oil and the combination of red palm oil and rooibos extract improved the activities of glutathione peroxidase in the red blood cells and liver. There was a significant ($p<0.05$) decrease in the activity of liver superoxide dismutase in the diabetic control group when compared with the normal control group. Treatment with red palm oil, rooibos extract and the combination of red palm oil and rooibos extract significantly ($p>0.05$) increased the activity of superoxide dismutase in the diabetic rats. Similarly, red palm oil, rooibos extract and the combination of red palm oil and rooibos extract significantly ($p<0.05$) increased ferric reducing antioxidant power and oxygen radical absorbance capacity values in the plasma of diabetic rats. There was a significant ($p<0.05$) decrease in the levels of albumin in the diabetic control group and diabetic treated groups with red palm oil and rooibos extract alone when compared with the normal control group. There was no significant ($p>0.05$) decrease in the liver total glutathione in the diabetic rats in comparison to the normal control group. Plasma total glutathione was non-significantly increased in treated diabetic rats. A significant ($p<0.05$) increase in the plasma thiobarbituric acid reactive substances in the diabetic control group was observed when compared with the normal control group. Treatment of diabetic rats with rooibos extract and the combination of red palm oil and rooibos extract reduced plasma thiobarbituric acid reactive substances to a level not significantly different at $p<0.05$ from the normal control group. Liver thiobarbituric acid reactive substances did not show any significant ($p>0.05$) difference in all the groups. This study revealed the anti-oxidative potentials of red palm oil, rooibos extract and the combination of red palm oil and rooibos extract in diabetic conditions.

**Key words: Rooibos, Red Palm Oil, Antioxidant, Streptozotocin, Hyperglycaemia**

## INTRODUCTION

Streptozotocin (STZ) is known for its selective cytotoxicity on pancreatic islet β-cells and has been broadly used to induce diabetes mellitus in experimental rat models (Latha and Daisy, 2011). Its diabetogenic action has been explained to cause the alkylation of DNA, production of nitric oxide and free radicals which leads to decreased insulin biosynthesis (Shi and Pan, 2010). The failure of insulin action or insulin production resulting in hyperglycaemia leads to a number of complications (Renard *et al.*, 2006; Shi *et al.*, 2011). Diabetes does not only lead to hyperglycaemia but also causes hyperlipidaemia, hyperinsulinaemia, hypertension, and atherosclerosis (Shi and Pan, 2010). Oxidative stress can occur as a result of excess ROS production to the available antioxidant buffering capacity (Adly, 2010). Elevated levels of glucose can induce oxidative stress through various mechanisms which include glycation, PKC activation and sorbitol pathway (Wiernsperger, 2003).

An increase in oxidative glucose metabolism leads to increased mitochondrial generation of the superoxide anion which is converted to hydroxyl radicals and hydrogen peroxide (Nishikawa *et al.* 2000; King *et al.,* 2004). Increased production of reactive oxygen species such as superoxide anion and hydrogen peroxide has been linked with cellular injury due to an increase in lipid peroxidation, DNA damage and protein modification or altered gene expression (Ganafa *et al.,* 2002). Oxidative stress acts on signal transduction and affect gene expression through NF-kB, thereby reducing the expression of antioxidant enzymes (Wiernsperger, 2003). An increase in lipid peroxidation and the reduction in antioxidant enzyme activity have been linked with progression of albuminuria in diabetes (Mastan rao *et al.,* 2010). The reduction of oxidative stress in diabetic rats may, in itself, offset hyperglycaemia (Gao *et al.,* 2012a).

Red palm oil is obtained from the fleshy orange-red mesocarp of the fruit of a tropical plant known as oil palm (*Elaeis guineensis*) (Edem, 2009). It is reported to contain antioxidants vitamins such as vitamin A (carotenes) and vitamin E (tocopherols and tocotrienols) (Badmus *et al.,* 2008; Ayeleso *et al.,* 2012) and has been reported to prevent oxidative stress in both *in vitro* and *in vivo* systems (Serbinova *et al.,* 1992; Aboua *et al.,* 2009). Red palm oil contains unsaturated and saturated fatty acids in the ratio that is close to one (Badmus *et al.,* 2008; Ayeleso *et al.,* 2012). On the other hand, rooibos (*Aspalathus linearis*) is a rich source of polyphenols that is used in making a mild-tasting tea containing no caffeine and low in tannins compared to green or black teas (Iswaldi *et al.,* 2011). It contains different bioactive phenolic compounds which include dihydrochalcones, flavonols, flavanones, flavones, and flavanols

(Krafczyk *et al.,* 2009). Polyphenols are broadly distributed throughout the plant kingdom and represent an abundant antioxidant component of the human diet (Iswaldi *et al.,* 2011).

Antioxidants are substances that can directly or indirectly offer protection against adverse effects of xenobiotics, drugs, carcinogens, and toxic radical reactions (Halliwell *et al.,* 2005; Matsumoto and Bastos, 2009). Various antioxidants either scavenge superoxide and free radicals and/or stimulate the detoxification mechanisms within cells, resulting in the prevention of many pathophysiological processes (Matsumoto and Bastos, 2009). The antioxidant activities of vitamins, phenolic compounds and foods containing them have been shown in different *in vivo* systems (Cao *et al.,* 1998; Prior, 2003; Kapsokefalou *et al.,* 2006; Bucioli *et al.,* 2011; Bilbis *et al.,* 2012). Epidemiological evidence suggests that antioxidant properties of phenolic compounds may have health benefits (Kapsokefalou *et al.,* 2006). Total antioxidant capacity has been used for the assessment of antioxidant status which would provide useful information for health care (Kambayashi *et al.,* 2009). Prevention of oxidative damage is important for health care because oxidative stress is involved in various diseases (Halliwell and Gutteridge, 1999; Kambayashi *et al.,* 2009). The purpose of this study was to investigate the potential modulatory effects of red palm oil (RPO) and aqueous rooibos extract (RTE) as well as their combined effects on the antioxidant status in STZ-induced hyperglycaemic rats.

## MATERIALS AND METHODS

### Preparation of rooibos extract

Aqueous extracts of fermented rooibos was prepared by the addition of freshly boiled tap water to the leaves and stems (2 g/100 ml). The mixture was allowed to stand for 30 min at room temperature, cooled, filtered and dispensed into water bottles.

### Animal care

Male Wistar rats (176-255 g) were bred and used at the Medical Research Council, Primate Unit, Tygerberg, South Africa. The study was conducted after obtaining Ethical Committee Clearance from Cape Peninsula University of Technology (CPUT/HAS-REC 2010/A002). The rats were individually housed and maintained in a temperature controlled room of 22-25 °C, humidity of 45-55%, 15-20 air changes per hour and on a 12 hour light/dark cycle and rats have free access to standard rat chow. The rats were treated by supplementing their diets with 2 ml red palm oil and/ or 2% rooibos for 7 weeks. The fermented rooibos was supplied by Rooibos Ltd (Clanwilliam, South Africa) and the red palm oil used was Carotino palm fruit oil from Malaysia.

**Induction of diabetes mellitus**

Diabetes was induced by a single intramuscular injection of STZ (Sigma-Aldrich, South Africa) at the dose of 50 mg/kg of body weight into overnight fasted rats. Streptozotocin was dissolved in 0.1 M citrate buffer (pH 4.5). Diabetes was confirmed 72 hours after STZ injection by determining the blood glucose levels using an Accu chek glucometer. Only diabetic rats with blood glucose levels above 14 mmol/L were used for the experiment.

**Experimental design**

The rats were divided into eight groups consisting of seven rats each for the non-diabetic groups and eight rats each for the diabetic groups.

**Group 1 (Normal control):** Rats received a single intramuscular injection of citrate buffer and given tap water orally for 7 weeks.

**Group 2 (Diabetic control):** Diabetes was induced by a single intramuscular injection of STZ at a dose of 50 mg/kg body weight and given tap water for 7 weeks.

**Group 3**: Rats received a single intramuscular injection of citrate buffer and fed with RPO (2 ml/day) and tap water for 7 weeks.

**Group 4:** Diabetes was induced by a single intramuscular injection of STZ at a dose of 50 mg/kg body weight and treated with RPO (2 ml/day) for 7 weeks.

**Group 5:** Rats received a single intramuscular injection of citrate buffer and fed with RTE (2%) for 7 weeks.

**Group 6:** Diabetes was induced by a single intramuscular injection of STZ at a dose of 50 mg/kg body weight and fed with RTE (2%) for 7 weeks.

**Group7:** Rats received a single intramuscular injection of citrate buffer and fed with both RPO (2 ml/day) and RTE (2%) for 7 weeks.

**Group 8:** Diabetes was induced by a single intramuscular injection of STZ at a dose of 50 mg/kg body weight and fed with both RPO (2 ml/day) and RTE (2%) for 7 weeks.

At the end of the experimental period, the overnight fasted rats were sacrificed. Blood was collected from the dorsal aorta by using a 10ml syringe and transferred into EDTA tubes for

plasma and RBCs collection. The plasma was separated by centrifugation at 3,000 rpm for 15 min and then transferred into properly labelled sterile vials. The red blood cells were lysed with distilled water, centrifuged at 2,500 rpm for 15 min and washed three times with phosphate-buffered saline. Liver tissues were excised, rinsed in saline solution, blotted on filter paper and weighed. All the samples collected were stored at -80° C until analysis was performed. The percentage weight of the liver was calculated with the formula below:

$$\text{The percentage weight of organ} = \frac{\text{Absolute weight of organ}}{\text{Final body weight of rat}} \times 100$$

**Determination of total polyphenol, flavanol and flavonol content**

The total polyphenol levels in the plasma and rooibos extracts were determined using the Folin Ciocalteu's phenol reagent according to the method described by Singleton *et al.* (1999). The total polyphenols levels were determined spectrophotometrically using a micro plate reader and expressed as mg gallic acid standard equivalents per litre. The flavanol content of the rooibos extracts was determined colorimetrically at 640 nm using the aldehyde DMACA and expressed as mg catechin standard equivalents per millimetre extract (Delcour and de Varebeke, 1985; Treutter, 1989). The flavonol content of the extracts was determined spectrophotometrically at 360 nm and expressed as mg quercetin standard equivalents per millimetre extract (Mazza *et al.,* 1999).

**Antioxidant enzymes assay**

The activities of antioxidant enzymes in the red blood cells and liver were determined. Liver homogenates (10% w/v) were prepared in a phosphate buffer, centrifuged at 10,000g (4$^{o}$C) for 10 mins and supernatant kept at -80$^{o}$C for enzyme analyses. Catalase (CAT) activity was determined spectrophotometrically at 240 nm by monitoring the decomposition of $H_2O_2$ and expressed as µmole $H_2O_2$/min/µg protein according to the method of Aebi (1984) while superoxide dismutase (SOD) activity was determined by the method of Crosti *et al.* (1987) modified for a microplate reader at 490 nm and expressed as the amount of protein (µg) required to produce a 50% inhibition of auto-oxidation of 6-hydroxydopamine. Glutathione peroxidase (GPx) activity was measured spectrophotometrically (340 nm) by the method of Ellerby and Bredesden (2000) and the activity expressed as nmoles NADPH/min/µg protein.

**Ferric reducing antioxidant power (FRAP) assay**

The ferric reducing antioxidant power was determined using the method described by Benzie and Strain (1996). Ten (10) µl of the plasma and liver homogenates were mixed with 300 µl FRAP reagent in a 96-well clear plate. The FRAP reagent was a mixture (10:1:1, v/v/v) of

acetate buffer (300 mM, pH 3.6), tripyridyl triazine (TPTZ) (10 mM in 40 mM HCl) and $FeCl_3.6H_2O$ (20 mM). After incubation at room temperature for 30 min, the plate was read at a wavelength of 593 nm in a Multiskan Spectrum plate reader (Thermo Fisher Scientific, USA). Ascorbic acid (AA) was used as the standard and the results expressed as µmol AAE/L for plasma and *µ*mol AAE/g tissue for liver homogenates.

**Trolox equivalence antioxidant capacity (TEAC) assay**

Trolox equivalence antioxidant capacity was determined using the principle of 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical scavenging activity according to a method described by Re *et al.* (1999). $ABTS^+$ solution was prepared a day before use by mixing ABTS salt (8 mM) with potassium persulfate (3 mM) and then storing the solution in the dark until the assay could be performed. The $ABTS^+$ solution was further diluted with distilled water. Twenty five microlitres (25 µl) of the samples were mixed with 300 µl $ABTS^+$ solution in a 96-well clear microplate. The plate was read after 30 min incubation at room temperature in a Multiskan Spectrum plate reader (Thermo Fisher Scientific, USA) at 734nm. Trolox was used as the standard and results expressed as *m*ol TE/L for plasma and µmol TE/g tissue for liver homogenates.

**Oxygen radical absorbance capacity (ORAC) assay**

Liver samples were homogenized in 10 volumes of sodium phosphate buffer (75 mM, pH 7.0) in a Thomas homogenizer and centrifuged at 10,000g for 10 mins at $4^oC$. The liver homogenates and plasma samples were deproteinised using 0.5M perchloric acid (PCA), centrifuged at 15,000g for 10 min. The ORAC assay was conducted according to the method of Ou *et al.* (2001) on a 96-well microplate using a Fluorescence plate reader (Thermo Fisher Scientific, Waltham, Mass., USA). The reaction consisted of 12 µl of diluted sample and 138ul of fluorescein (14 µM), which was used as a target for free radical attack. The reaction was initiated by the addition of 50 µl AAPH (768 µM) and the fluorescence (emission 538 nm, excitation 485 nm) recorded every 1 min for 2 hours. Trolox was used as the standard and results expressed as µmol/L for plasma and µmol/g tissue for liver homogenates.

**Total glutathione, total protein, albumin and globulin analysis**

The levels of total glutathione (GSHt) in the whole blood and liver were determined according to the method of Asensi *et al.* (1999). The whole blood was deproteinised using 5% metaphosphoric acid (MPA) solution. Liver samples were homogenized (1:10) in 15% (w/v) trichloroacetic acid (TCA) containing 1 mM ethylenediaminetetraacetic acid (EDTA). The homogenates were centrifuged at 15,000g for 10 min and the supernatant collected. Total glutathione in the whole blood and liver homogenates was performed by placing 50µl of the

samples into plate wells and 50µl of 5, 5'-dithiobis-2-nitrobenzoic acid (DTNB) was added, followed by 50 µl of glutathione reductase. The reaction was initiated by the addition of 50µl of nicotinamide adenine dinucleotide phosphate (NADPH) to a final volume of 200 µl. The change in absorbance was monitored at 412 nm for 5 min and levels of GSHt calculated using pure glutathione (GSH) as a standard and expressed as µmole/L for whole blood and µmole/g tissue for liver samples. Total protein and albumin levels in the serum were measured with kits using an automated chemistry analyzer (Easy RA Medical, USA) according to manufacturer`s instructions. Globulin level was determined by using the formula (Globulin = Total protein – Albumin).

**Conjugated diene determination**

Conjugated dienes (CD) concentrations in serum samples were determined as described by Recknagel & Glende (1984). Thawed liver and plasma samples (100 µl) were mixed with 405 µl of a chloroform/ ethanol mixture (2:1) and kept on ice. Solutions were vortexed (1 min) and centrifuged (10,000 g; 10 min; 4°C). The bottom organic chloroform layers were dried under nitrogen ($N_{2)}$ gas. To each of the dried residues, cyclohexane (1 ml) was added and vortexed. Thereafter, 300 µl of the solution and cyclohexane as blank were transferred into 96-well microplates and the absorbance was determined at 234 nm spectrophotometrically. The CD calculations were done according to the equation given below and expressed as nmol CD/L for plasma and nmol CD/g tissue for liver homogenates.

$$\frac{A_{234}s - A_{234}b}{\xi} \times 10$$

Where $A_{234}s$: absorbance of sample at 234 nm

$A_{234}b$: absorbance of blank at 234 nm

$\xi$: coefficient of extinction= $2.95 \times 10^4$

Quoted $\xi$ is based on a 1 cm curvette; since 300 µl in a microplate well has a length of 0.9 cm, appropriate factoring was done in the calculations.

**Estimation of thiobarbituric acid reacting substances (TBARS)**

Malondialdehyde (MDA) which is a part of thiobarbituric acid reacting substances (TBARS) is commonly used as an indicator of lipid peroxidation (Seljeskog *et al.,* 2006). Thiobarbituric acid reacting substances was performed according to a modified method of Khoschsorur *et al.* (2000) using a micro plate reader. Fifty microlitres (50 µl) of plasma or liver homogenates was mixed with 375 µl of 0.44 M $H_3PO_4$ and 125 µl of 42 mM aqueous 2-Thiobarbituric acid (TBA) and 225 µl of distilled water were added. The mixture was heated in boiling-water in a water bath for 60mins. After cooling on ice, alkaline methanol (5ml + 45 ml 1M NaOH) was added to

the reaction mixture in ratio (1:1). The samples were centrifuged for 3 min and absorbance read at 535nm using a micro plate reader. Malondialdehyde was used as the standard and results expressed as nmol MDA/L for plasma and nmol MDA/g tissue for liver homogenates.

### Histopathological evaluations

At the end of the treatment, animals were sacrificed to collect the liver. The liver was blotted and freed from blood, fixed in 10% neutral formalin, trimmed, processed for paraffin embedment and 5 μm thickness of tissue sections were stained with haematoxylin and eosin. Histopathological evaluations of liver were examined using light microscopy at 20x magnification.

### Statistical analysis

Data were expressed as the means ± standard deviations. Significant differences between mean values of different groups were determined by one-way analysis of variance (ANOVA) with MedCalc software. Data not normally distributed was log transformed and analyzed using the Kruskal–Wallis one-way ANOVA on ranks hypotheses. Differences were considered significant at $p<0.05$.

## RESULTS

Table 1 shows the effects of RPO and / or RTE treatments on the percentage weight of the liver and body weight gain in diabetic and non-diabetic rats. A significant $p<0.05$ increase in the relative liver weights in all the diabetic groups was noted when compared with the normal control group. Diabetic rats treated with RPO + RTE significantly $p<0.05$ increased the body weight of the diabetic rats when compared with the STZ control group. The non-diabetic rats fed with RPO, RTE and RPO + RTE did not show any significant $p>0.05$ difference on weight gain in comparison to the normal control group.

**Table 1: Effect of RPO, RTE and RPO + RTE treatments on the liver weight and body weight gain.**

| Treatment groups | Liver weight (%) | Body weight gain (%) |
|---|---|---|
| NORMAL CONTROL | 3.10 ± 0.53 | 74.00 ± 11.19 |
| STZ CONTROL | 4.32 ± 0.41$^{a}$ | 1.64 ± 12.48$^{a}$ |
| RPO | 2.79 ± 0.12 | 66.54 ± 15.26 |
| STZ + RPO | 4.59 ± 0.62$^{b}$ | 17.37 ± 15.66$^{b}$ |
| RTE | 2.72 ± 0.18* | 70.67 ± 8.11 |
| STZ + RTE | 4.77 ± 0.41$^{bc}$ | 5.79 ± 19.45$^{b}$ |
| RPO + RTE | 2.93 ± 0.20 | 57.91 ± 5.63 |
| STZ + RPO + RTE | 4.00 ± 0.72$^{b}$ | 27.44 ± 13.51$^{bc}$ |

All significant differences are at $p<0.05$. STZ, streptozotocin-induced diabetes; RPO, red palm oil; RTE, aqueous rooibos extract.

(*) represents significant difference between non-STZ fed groups and normal control group.
($^{a}$) represents significant difference between STZ control group and normal control group.
($^{b}$) represents significant difference between treated STZ groups and normal control group.
($^{c}$) represents significant difference between treated STZ groups and STZ control group.

Table 2 shows the antioxidant profiles of the rooibos extracts consumed daily by the rats. It can be observed that the rate of consumption of rooibos and water was higher in all the diabetic rats than normal rats.

**Table 2: Daily intake and antioxidant profile of rooibos extract.**

| Treatment groups | RTE / water intake/day (ml/day) | Polyphenol intake/day (mg/day) | Flavonol intake/day (mg/day) | Flavanol intake/day (mg/day) | FRAP status/day (µmol/day) | TEAC status/day (µmol/day) | ORAC status/day (µmol/day) |
|---|---|---|---|---|---|---|---|
| NORMAL CONTROL | 40.57 ± 5.29 | ND | ND | ND | ND | ND | ND |
| STZ CONTROL | 121.70 ± 8.80 | ND | ND | ND | ND | ND | ND |
| RPO | 37.92 ± 4.98 | ND | ND | ND | ND | ND | ND |
| STZ + RPO | 101.60 ± 26.12 | ND | ND | ND | ND | ND | ND |
| RTE | 40.12 ± 3.83 | 33.38 ± 3.19 | 0.95 ± 0.09 | 0.58 ± 0.06 | 13.27 ± 1.27 | 19.07 ± 1.82 | 169.71 ± 16.22 |
| STZ + RTE | 122.85 ± 12.54 | 102.21 ± 10.43 | 2.90 ± 0.30 | 1.78 ± 0.18 | 40.64 ± 4.15 | 58.41 ± 5.96 | 519.72 ± 53.05 |
| RPO + RTE | 33.00 ± 1.67 | 27.46 ± 1.39 | 0.78 ± 0.04 | 0.48 ± 0.02 | 10.92 ± 0.55 | 15.69 ± 0.79 | 139.62 ± 7.05 |
| STZ + RPO + RTE | 86.51 ± 29.06 | 71.97 ± 24.18 | 2.04 ± 0.69 | 1.25 ± 0.42 | 28.62 ± 9.61 | 41.13± 13.82 | 365.95 ± 122.93 |

ND- Not detected

Table 3 shows the effect of RPO and / or RTE treatments on the antioxidant enzymes in the RBCs and liver of rats. A non-significant ($p>0.05$) increase in the activity of CAT in the RBCs of STZ control group was shown when compared with the normal control group. There was significant ($p<0.05$) increase in the activity of liver catalase in rats fed with only RPO when compared with the normal control group. Catalase activity in the liver was significantly reduced in RPO fed diabetic rats in comparison with RPO fed normal group. Diabetic rats fed with RTE and combined treatment (RPO + RTE) did not indicate any significant effects on liver CAT activity in comparison to the normal control and STZ control groups. No significant ($p>0.05$) increase in the activity of GPx in the RBCs of STZ control group was shown when compared with the normal control group. A significant increase in the activity of of GPx in the RBCs was noted in the diabetic rats fed with RPO and RPO + RTE when compared with the STZ control group. Liver GPX activity significantly ($p<0.05$) increased in RPO, RTE and RPO + RTE treated diabetic rats in comparison to the normal control group. There was a significant ($p<0.05$) increase in liver GPX activity in the diabetic rats treated with RTE and the combination of RPO + RTE when compared with the STZ control group. No significant increase in the activity of SOD in the RBCs of non-diabetic fed rats was shown. There was no significant difference in the activity of SOD in the RBCs of all the treated diabetic groups when compared with STZ control group. However, the activity of liver SOD reduced significantly in the STZ control group when compared with the normal control group. A significant ($p<0.05$) increase in the activity of liver SOD was observed in the diabetic rats treated with RPO, RTE and RPO + RTE. A significant ($p<0.05$) increase in liver SOD was also observed in rats fed with RTE and RPO + RTE when compared with the normal control group.

**Table 3: Effect of RPO, RTE and RPO + RTE treatments on the antioxidant enzymes in the red blood cells and liver.**

| Treatment groups | CAT µmol $H_2O_2$/min/µg protein | | GPx nmol NADPH/min/µg protein | | SOD units/µg protein | |
|---|---|---|---|---|---|---|
| | RBCs | Liver | RBCs | Liver | RBCs | Liver |
| NORMAL CONTROL | 0.168 ± 0.030 | 0.621 ± 0.127 | 0.006 ± 0.002 | 0.003 ± 0.000 | 0.017 ± 0.009 | 0.226 ± 0.062 |
| STZ CONTROL | 0.216 ± 0.079 | 0.670 ± 0.073 | 0.010 ± 0.002 | 0.003 ± 0.000 | 0.015 ± 0.007 | 0.160 ± 0.028[a] |
| RPO | 0.294 ± 0.128 | 0.819 ± 0.116* | 0.009 ± 0.002 | 0.004 ± 0.000 | 0.031 ± 0.018 | 0.266 ± 0.048 |
| STZ + RPO | 0.205 ± 0.029 | 0.503 ± 0.038[b] | 0.018 ± 0.003 [bc] | 0.004 ± 0.000[b] | 0.011 ± 0.004 | 0.246 ± 0.068[c] |
| RTE | 0.223 ± 0.061 | 0.694 ± 0.075 | 0.007 ± 0.002 | 0.003 ± 0.001 | 0.027 ± 0.025 | 0.351 ± 0.087* |
| STZ + RTE | 0.202 ± 0.047 | 0.655 ± 0.079 | 0.010 ± 0.003 | 0.004 ± 0.001[bc] | 0.014 ± 0.008 | 0.293 ± 0.043[c] |
| RPO + RTE | 0.266 ± 0.095 | 0.710 ± 0.089 | 0.011 ± 0.004* | 0.005 ± 0.000* | 0.036 ± 0.026 | 0.389 ± 0.093* |
| STZ + RPO + RTE | 0.240 ± 0.078 | 0.651 ± 0.107 | 0.017 ± 0.007 [bc] | 0.005 ± 0.001[bc] | 0.017 ± 0.01 | 0.339 ± 0.058[bc] |

All significant differences are at $p<0.05$. STZ, streptozotocin-induced diabetes; RPO, red palm oil; RTE, aqueous rooibos extract; CAT, catalase; GPx, glutathione peroxidase; SOD, superoxide dismutase.

(*) represents significant difference between non-STZ fed groups and normal control group.

([a]) represents significant difference between STZ control group and normal control group.

([b]) represents significant difference between treated STZ groups and normal control group.

([c]) represents significant difference between treated STZ groups and STZ control group.

Table 4 shows the effects of RPO and / or RTE treatments on the antioxidant capacity in the plasma and liver of the diabetic rats. A significant ($p<0.05$) increase in the FRAP status of RPO, RTE as well RPO + RTE fed diabetic rats when compared with both normal and STZ control groups was shown. Similarly, there was a significant increase in the FRAP status of normal rats fed only with RTE and RPO + RTE in comparison to the normal control group. However, the results showed no significant ($p>0.05$) difference in the liver FRAP status when compared with the normal and STZ control groups. The plasma TEAC status did not show any difference in all the groups in comparison to the normal and STZ control groups. Similarly, there was a significant increase in the liver TEAC status of diabetic rats that were fed with RTE. Non-diabetic rats fed with RPO and RTE showed a significant increase in liver TEAC status when compared with the normal control group. There was a significant ($p<0.05$) decrease in the plasma ORAC status of the STZ control group while it significantly ($p<0.05$) increased in the diabetic rats treated with RPO, RTE and RPO + RTE. Furthermore, the liver ORAC status was not significantly ($p>0.05$) altered in diabetic rats treated with RPO and RPO + RTE. A significant ($p<0.05$) decrease in the ORAC status was noted for the non-diabetic rats fed with RPO when compared with the normal control group. A decrease in liver ORAC was observed in non-diabetic rats fed with RTE and RPO + RTE in comparison to the normal control group. Liver ORAC was significantly ($p<0.05$) reduced for the group of diabetic rats fed with RTE when compared with the normal and STZ control groups. Plasma total polyphenols was significantly ($p<0.05$) higher in the STZ control group than the normal control group. Treatment of the diabetic rats with RPO, RTE and RPO + RTE showed a significant ($p<0.05$) reduction in plasma polyphenols when compared with the STZ control group.

**Table 4: Effect of RPO, RTE and RPO + RTE treatments on the antioxidant capacity and plasma total polyphenols.**

| Treatment groups | FRAP | | TEAC | | ORAC | | Total polyphenols |
|---|---|---|---|---|---|---|---|
| | µmol/L | µmol/g tissue | µmol/L | µmol/g tissue | µmol/L | µmol/g tissue | mg/L |
| | Plasma | Liver | Plasma | Liver | Plasma | Liver | Plasma |
| NORMAL CONTROL | 184.42 ± 27.20 | 2.301 ± 0.13 | 6858.83 ± 100.48 | 37.38 ± 1.85 | 613.91 ± 39.58 | 6.07 ± 0.34 | 145.04 ± 29.37 |
| STZ CONTROL | 221.55 ± 63.25 | 2.365 ± 0.24 | 6846.17 ± 293.21 | 39.79 ± 1.29 | 567.83 ± 55.60[a] | 6.03 ± 0.31 | 185.90 ± 33.52[a] |
| RPO | 193.43 ± 30.24 | 2.411 ± 0.24 | 6939.14 ± 461.35 | 41.09 ± 1.31* | 540.60 ± 41.37* | 5.53 ± 0.30* | 156.58 ± 43.20 |
| STZ + RPO | 317.41 ± 118.06[bc] | 2.264 ± 0.22 | 6799.22 ± 483.27 | 38.99 ±1.30 | 668.48 ± 50.78[c] | 5.90 ± 0.24 | 105.97 ± 14.43[c] |
| RTE | 231.05 ± 21.61* | 2.528 ± 0.10 | 7066.12 ± 149.18 | 42.93 ± 0.60* | 587.74 ± 20.20 | 5.29 ± 0.16* | 113.11 ± 13.33 |
| STZ + RTE | 340.10 ± 69.68[bc] | 2.217 ± 0.15 | 7306.43 ± 515.61 | 40.79 ± 1.91[b] | 664.48 ± 60.74[c] | 5.46 ± 0.35[bc] | 131.69 ± 15.99[c] |
| RPO + RTE | 242.81 ± 42.06* | 2.300 ± 0.15 | 6964.76 ± 370.12 | 39.65 ± 1.69 | 639.22 ± 58.56 | 5.09 ± 0.51* | 109.28 ± 21.90 |
| STZ + RPO + RTE | 242.81 ± 90.86[bc] | 2.505 ± 0.15 | 7081.01 ± 339.59 | 38.08 ± 1.74 | 641.57 ± 88.69[c] | 5.67 ± 0.21 | 134.14 ± 4.91[c] |

All significant differences are at $p<0.05$. STZ, streptozotocin-induced diabetes; RPO, red palm oil; RTE, aqueous rooibos extract; FRAP, ferric reducing antioxidant power; TEAC, trolox equivalence antioxidant capacity; ORAC, oxygen radical absorbance capacity.

(*) represents significant difference between non-STZ fed groups and normal control group.

([a]) represents significant difference between STZ control group and normal control group.

([b]) represents significant difference between treated STZ groups and normal control group.

([c]) represents significant difference between treated STZ groups and STZ control group.

Table 5 shows the effects of RPO and / or RTE treatments on the oxidative stress biomarkers in the diabetic rats. No significant ($p>0.05$) increase in the plasma GSHt level in all the non-diabetic and diabetic treated rats was shown in comparison to the normal and STZ control groups. The results also showed no significant ($p>0.05$) reduction in the liver GSHt level in all diabetic treated rats. There were no significant ($p>0.05$) differences in the plasma conjugated dienes in all the groups. Diabetic rats treated with RTE showed a significant ($p<0.05$) reduction in liver CDs in comparison to the normal and STZ control groups. Plasma TBARS significantly ($p<0.05$) increased in the STZ control group and RPO treated diabetic rats in comparison to the normal control group. Treatments of diabetic rats with RTE and RPO + RTE showed no significant ($p>0.05$) reductions in the level of plasma TBARS when compared with the normal control group. The level of liver TBARS was not significantly ($p>0.05$) different in all the diabetic and non-diabetic treated rats when compared with both normal and STZ control groups.

**Table 5: Effect of RPO, RTE and RPO + RTE treatments on the oxidative stress biomarkers.**

| Treatment groups | GSHt | | CD | | TBARS | |
|---|---|---|---|---|---|---|
| | µmole/L WB | µmole/g tissue Liver | nmolCD/L Plasma | nmolCD/g tissue Liver | nmol MDA Plasma | nmol MDA/g tissue Liver |
| NORMAL CONTROL | 1046.24 ± 45.42 | 3.56 ± 0.85 | 0.12 ± 0.01 | 1.21 ± 0.02 | 12.44 ± 2.51 | 0.25 ± 0.24 |
| STZ CONTROL | 1082.52 ± 115.82 | 2.36 ± 1.36 | 0.13 ± 0.01 | 1.20 ± 0.06 | 18.70 ± 4.23 [a] | 0.24 ± 0.02 |
| RPO | 1142.05 ± 63.36 | 3.07 ± 0.65 | 0.12 ± 0.01 | 1.20 ± 0.03 | 15.26 ± 2.60 | 0.28 ± 0.01 |
| STZ + RPO | 1325.31 ± 186.72 | 3.39 ± 1.53 | 0.13 ± 0.02 | 1.19 ± 0.03 | 17.88 ± 3.41[b] | 0.25 ± 0.02 |
| RTE | 1123.54 ± 111.59 | 3.56 ± 0.55 | 0.12 ± 0.01 | 1.17 ± 0.03 | 12.57 ± 0.07 | 0.25 ± 0.02 |
| STZ + RTE | 1167.53 ± 211.63 | 2.48 ± 1.45 | 0.13 ± 0.01 | 1.14 ± 0.04[bc] | 16.34 ± 3.55 | 0.22 ± 0.03 |
| RPO + RTE | 1145.50 ± 58.50 | 3.61 ± 0.47 | 0.13 ± 0.01 | 1.18 ± 0.02 | 14.01 ± 1.37 | 0.27 ± 0.07 |
| STZ + RPO + RTE | 1168.03 ± 195.48 | 2.87 ± 0.57 | 0.14 ± 0.01 | 1.19 ± 0.03 | 15.92 ± 2.78 | 0.26 ± 0.02 |

All significant differences are at $p<0.05$. STZ, streptozotocin-induced diabetes; RPO, red palm oil; RTE, aqueous rooibos extract; CD, conjugated dienes; TBARS, thiobarbituric acid reactive substances.

(*) represents significant difference between non-STZ fed groups and normal control group.

([a]) represents significant difference between STZ control group and normal control group.

([b]) represents significant difference between treated STZ groups and normal control group.

([c]) represents significant difference between treated STZ groups and STZ control group.

Table 6 shows the effects of RPO and / or RTE on serum total protein, albumin and globulin. No significant ($p>0.05$) decrease in the level of total protein was observed in the STZ control group when compared with the normal control group. There was also a significant ($p<0.05$) increase in the total protein of the diabetic rats treated with RPO alone when compared with the STZ control group. There was significant ($p<0.05$) reduction in the levels of albumin in the STZ control group and diabetic treated groups with RPO and RTE alone when compared with the normal control group. Similarly, albumin levels in the RPO + RTE fed non-diabetic rats was decreased when compared to the normal control group. The effect of RTE on the level of albumin in treated diabetic rats was significantly ($p<0.05$) lower when compared with the normal and STZ control groups. The results also showed a significant ($p<0.05$) increase in serum globulin in diabetic rats fed with RPO. However, diabetic rats fed with RTE and RPO + RTE did not have any significant ($p>0.05$) effects on globulin when compared with the STZ control group. The globulin level was significantly higher ($p<0.05$) in the RTE non-diabetic group when compared with the normal control group.

**Table 6: Effect of RPO, RTE and RPO+RTE on serum total protein, albumin and globulin.**

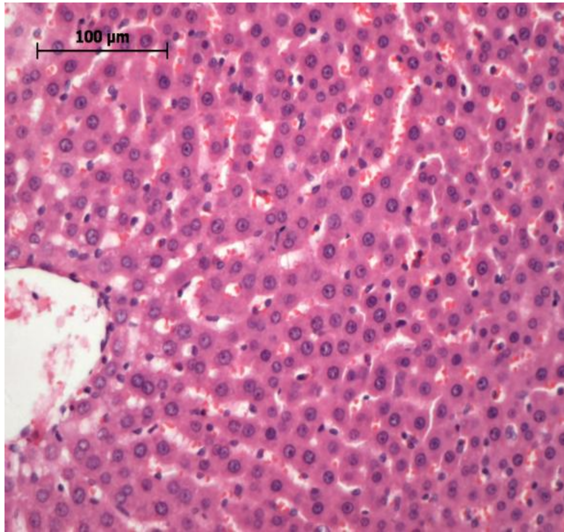
| Treatment groups | Total protein g/L | Albumin g/L | Globulin g/L |
|---|---|---|---|
| NORMAL CONTROL | 51.21 ± 1.70 | 30.66 ± 0.83 | 20.55 ± 0.97 |
| STZ CONTROL | 49.25 ± 2.27 | 28.88 ± 1.14 [a] | 20.38 ± 1.33 |
| RPO | 50.29 ± 2.48 | 29.69 ± 0.88 | 19.84 ± 1.50 |
| STZ + RPO | 53.21 ± 3.81 [c] | 29.21 ± 0.99 [b] | 23.43 ± 3.41 [bc] |
| RTE | 55.14 ± 1.21* | 30.59 ± 0.47 | 24.56 ± 1.16* |
| STZ + RTE | 48.06 ± 3.37 | 27.49 ± 0.98 [bc] | 20.57 ± 3.14 |
| RPO + RTE | 51.50 ± 1.22 | 29.06 ± 0.51* | 20.89 ± 0.97 |
| STZ + RPO + RTE | 51.13 ± 0.01 | 29.49 ± 0.89 | 21.63 ± 2.47 |

All significant differences are at $p<0.05$. STZ, streptozotocin-induced diabetes; RPO, red palm oil; RTE, aqueous rooibos extract.

(*) represents significant difference between non-STZ fed groups and normal control group.
([a]) represents significant difference between STZ control group and normal control group.
([b]) represents significant difference between treated STZ groups and normal control group.
([c]) represents significant difference between treated STZ groups and STZ control group.

Figure 1a and 1b show the histopathological evaluations of the liver. The results from the histopathological evaluations of the liver in all the groups indicated no spectacular lesions in the liver sections except for a very mild inflammatory activity around the portal area characterised by periportal cellular infiltration by mononuclear cells in the diabetic rats as indicated by the arrows.

A

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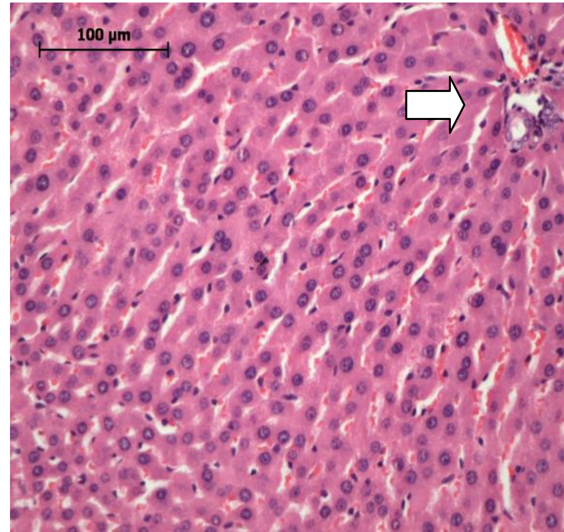


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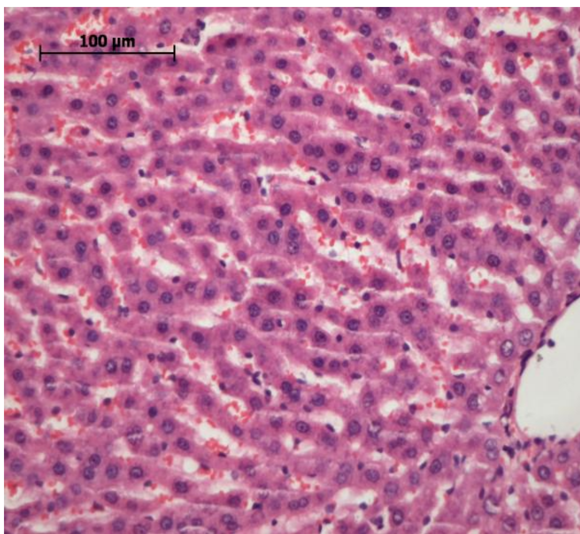



D

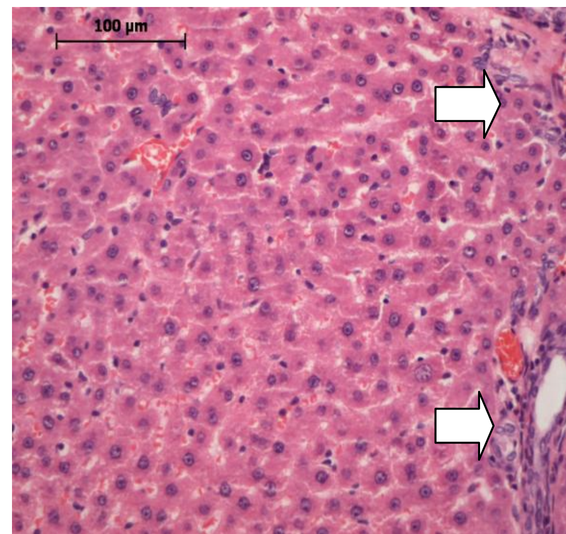
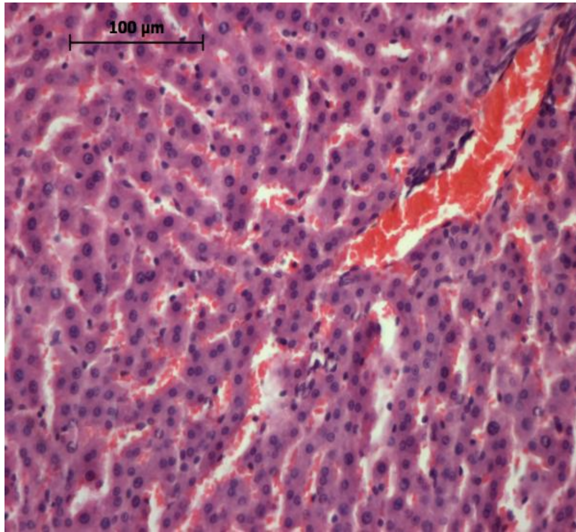
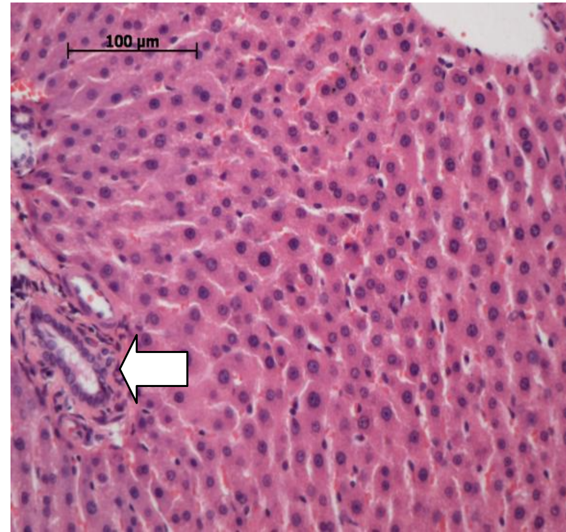



**Figure 1a: Histopathological evaluations of the liver in (A) Normal control group (B) Diabetes control group (C) RPO only group (D) Diabetes + RPO group.**

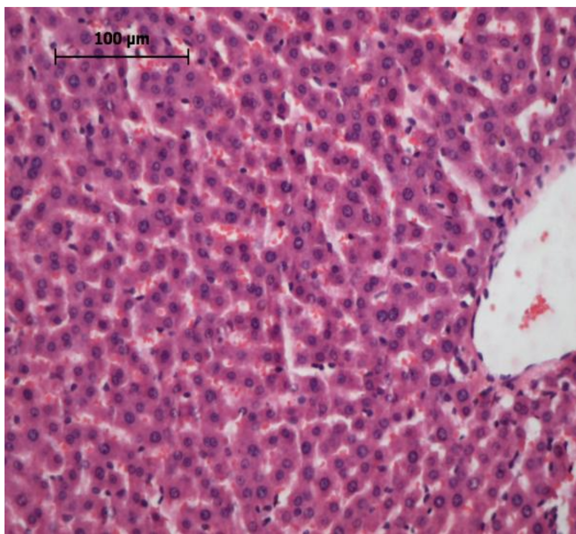
E




F




G




H

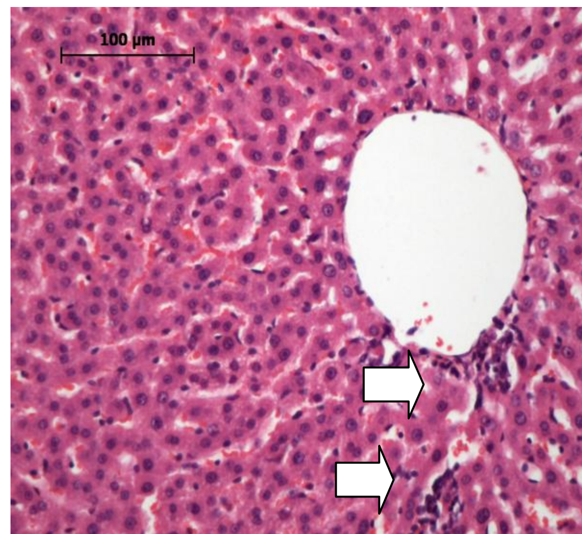



**Figure 1b: Histopathological evaluations of the liver in (E) RTE only group (F) Diabetes + RTE group (G) RPO + RTE group (H) Diabetes + RPO + RTE group.**

## DISCUSSION

In this study, the results indicated a decrease in body weights of the diabetic rats in comparison to the normal control rats. The decrease in body weight is as a result of loss of tissue proteins and muscle mass in diabetes (Mishra *et al.,* 2012). It is known that glycosuria causes a significant loss of calories for every gram of glucose excreted and most likely, this loss results in severe weight loss in spite of increased appetite, particularly when it is coupled with loss of muscle and adipose tissue due to excessive breakdown of protein (Akpaso *et al.,* 2011). Diabetic rats fed with RPO and RTE gained more body weight than those of the STZ group in this study. There was significant increase in the liver weight in the diabetic groups when compared with normal control group. Increased fluid intake by the diabetic rats was also observed and this is probably due to polyurea and dehydration. Streptozotocin generates oxygen radicals *in vivo* and cause oxidative damage to pancreas, liver, kidney, and haemopoietic systems (Halliwell and Gutteridge 1985; Gao *et al.,* 2012a). The key organ of oxidative and detoxifying processes, as well as free radical reactions is the liver and thus, oxidative stress biomarkers are elevated in the liver at the early stages of many diseases (Stadler *et al.,* 2003).

The mechanism of antioxidant defence against oxidative stress can be classified into: antioxidant, preventative, repair mechanisms and physical defences (Khansari *et al.,* 2009). Vitamin A, for example, acts directly by an intrinsic free radical scavenging mechanism and also inhibits nitric oxide production through inhibition of iNOS gene transcription in different tissues (Vertuani *et al.,* 2004). Priyadarsini, (2005) reported that vitamin E is a chain-breaking antioxidant which acts by scavenging chain propagating free radicals such as peroxyl radicals and convert the reactive free radicals to inactive products. It has been documented that α-tocopherol has the ability to terminate chain reactions of polyunsaturated fatty acid free radicals generated by lipid oxidation (Havaux *et al.,* 2005). Vitamin E has also been reported to act by up-regulating antioxidant enzymes (Vertuani *et al.,* 2004). Possible mechanisms of flavonoids against oxidative stress is by the direct scavenging of free radicals, inhibition of xanthine oxidase, interfering with inducible nitric-oxide synthase, immobilization and firm adhesion of leukocytes to the endothelial wall and by interaction with various enzyme systems (Nijveldt *et al.,* 2001).

Increased antioxidant capacity confirms the idea of the presence of functional recovery, at least in part, in the antioxidant defence systems in rats during chronic diabetes (Houcher *et al.,* 2007). FRAP measures the ferric reducing ability of the antioxidant molecule and the

antioxidant properties of many compounds are directly linked to their reducing power (Matsinkou *et al.,* 2012). In this study, non-significant increase in the plasma FRAP of the diabetic control group when compared with normal control group was shown. However, significant trends have been reported by Sasvari and Nyakas (2003) and Houcher *et al.* (2007). Sasvari and Nyakas (2003) showed elevated rates of plasma FRAP after induction of diabetes by streptozotocin in rats. Houcher *et al.* (2007) showed an increase in plasma FRAP values in alloxan-induced diabetic rats. The increase in plasma FRAP concentration in the course of diabetes is in accordance with the decline in ketosis (Sasvari and Nyakas, 2003; Houcher *et al.,* 2007). However, significant increases in the plasma FRAP status of the diabetic rats treated with both RPO and RTE as well the combined treatment (RPO + RTE) was observed. In plasma antioxidant, uric acid is estimated to participate in 60% of FRAP value while, ascorbic acid, proteins, α-tocopherol, bilirubin and others contribute about 15, 10, 5, 5 and 5% respectively (Benzie and Strain, 1996). There was no significant difference in the liver FRAP status in all the diabetic groups when compared with the normal control group. The trolox equivalent antioxidant capacity (TEAC) assay or 2, 2-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) assay is based on scavenging of the ABTS•+ radical cation by the antioxidants present in test sample (Zulueta *et al.,* 2009). It evaluates the relative ability of antioxidant to scavenge the ABTS+ generated in aqueous and organic solvent systems (Matsinkou *et al.,* 2012). There was no significant difference in plasma TEAC in treated diabetic rats. However, liver TEAC in treated diabetic rats with RTE as well as non-diabetic rats fed alone with RPO and RTE were significantly increased. The ORAC assay is found to give a good index of the total antioxidant capacity in patients with diabetes (Mancino *et al.,* 2011). A decrease in blood ORAC values are strongly linked with poor glycaemic control in diabetic patients (Therond *et al.,* 2000; Merzouk *et al.,* 2004; Mancino *et al.,* 2011). In this study, we observed a similar significant decrease in plasma ORAC status in the STZ group. However, plasma ORAC status of the diabetic rats treated with RPO, RTE and combined treatment (RPO + RTE) was significantly increased and therefore, suggest their ability to boost antioxidant levels in diabetic conditions.

CAT is regarded as a major determinant of hepatic antioxidant status and catalyzes the reduction of hydrogen peroxides and protects the tissue from highly reactive hydroxyl radicals (Sarkhail *et al.,* 2007). A decrease in the activity of CAT in diabetic conditions has been reported (Sarkhail *et al.,* 2007; Subramanian *et al.,* 2012). The activity of catalase in the RBCs of diabetic rats treated with RTE and RPO + RTE increased, though not significant. The no significant increase in the activity of catalase in the RBCs of diabetic control and treated diabetic rats may still be a response to an elevated production of $H_2O_2$. On the contrary, catalase activity in diabetic rats fed with RPO alone significantly decreased. This could be

attributed to the induction of the catalase which is involved in the decomposition of $H_2O_2$. The results from this study also showed a significant increase in liver catalase of non-diabetic rats fed with RPO. This supports the fact that RPO or its phytochemical constituents could have the ability to up-regulate the activity of CAT at the intracellular level. SOD catalyses the dismutation of superoxide radicals to hydrogen peroxide and molecular oxygen (Droge, 2002). The location of SOD in the mitochondria and its position in the antioxidant chain make the enzyme to be particularly important as a slight decrease in SOD is sufficient to provoke cell damage (Wiernsperger, 2003). From this study, a significant decrease in the activity of liver SOD in the diabetic rats was observed compared with the normal control group and this could be due to an excessive formation of superoxide anions in the diabetic rats. The decrease in liver SOD could as well as be related to inactivation by $H_2O_2$ or by glycation of enzymes. Kumawat *et al.* (2005) reported that auto-oxidation of glucose results in the formation of $H_2O_2$ that inactivates SOD. Non- diabetic rats fed with RTE and RPO + RTE showed an increase in the activity of SOD in comparison to the normal control group. The results from this study also showed that RPO, RTE and RPO + RTE were able to significantly increase the activity of SOD in the diabetic rats. Elevated SOD activity may protect CAT and GPx against inactivation by superoxide radicals as these radicals have been shown to inactivate CAT and GPx (Selvam and Anuradha, 1990; Sarkhail *et al.,* 2007). Glutathione peroxidase (GPx) is a selenoprotein, first described as an enzyme that protects haemoglobin from oxidative degradation in red blood cells (Subramanian *et al.,* 2012). The activity of liver GPX significantly increased in all diabetic treated rats when compared to the control group while only RPO and RPO + RTE significantly increased GPx activity in the RBCs of treated diabetic rats.

The reaction of hydroxyl radicals and singlet oxygen with the methylene groups of polyunsaturated fatty acids (PUFA) produces conjugated dienes, lipid peroxy radicals and hydroperoxides (Smirnoff, 1995; Blokhina *et al.,* 2003). In this study, there was no significant effect on conjugated dienes in the diabetic control and diabetic treated rats. The cytotoxic effects of oxygen free radicals is exerted on membrane phospholipids and lead to the formation of MDA, a product of lipid peroxidation (Gao *et al.,* 2012b) and the levels of MDA reveal the degree of oxidation in the body. Lipid peroxidation could cause protein damage and the inactivation of membrane bound enzymes either through direct attack by free radicals or through chemical modification by its end products, malondialdehyde and 4-hydroxynonenal (Bohr *et al.,* 2004). In this study, TBARS was used as a measure of the estimation of MDA. There was a significant increase in the plasma TBARS in STZ diabetic rats while liver TBARS did not show any difference in the diabetic control group in comparison to the normal control group. A similar result of non-accumulation of TBARS in liver tissue of diabetic rats has been shown (Sudnikovich *et al.,* 2007). A possible reason for this might be as a result of reduction in

lipid content (lipolysis) in cell membranes during the long-term diabetes in the rats (Lapshina *et al.,* 2006; Sudnikovich *et al.,* 2007). Lapshina *et al.* (2006) further argued that TBARS accumulation, which shows the degree of oxidative stress and antioxidative defense, may be tissue-specific and also depends upon duration of the diabetes conditions. Administration of RTE and RPO + RTE to the diabetic rats reduced the plasma TBARS to a level that is not significantly different from the normal control group.

Indirectly, hyperglycaemia is the cause of GSH depletion and these results in oxidative stress (Hamdy, 2012). A decrease in GSH levels could signify an increased utilization due to oxidative stress and elevated activity of GSH protection of cellular proteins against oxidation through the glutathione redox cycle, that could also directly detoxify reactive oxygen species the generated from exposure to STZ (Pari and Latha, 2004). Several studies have reported a decrease in GSH level as an indicator of oxidative stress in diabetic conditions (Sugiura *et al.,* 2006; Tirgar *et al*., 2010; Hamdy, 2012). However, this study showed no significant reduction in the GSHt levels in the diabetic control and diabetic treated rats compared to normal control group. Singh *et al.* (2001) reported no significant change in GSH levels either in blood or liver of diabetic animals and in treated diabetic animals, GSH levels were marginally high in both blood as well as the liver. The results indicate no significant increase in the plasma GSH levels in diabetic treated animals. In another study, Sudnikovich *et al.* (2007) did not observe any appreciable change in GSH levels in diabetic red blood cells or liver tissue when compared to normal rats.

Diabetes mellitus is grossly reflected by intense changes in the protein metabolism and by a negative nitrogen balance and loss of nitrogen from most organs (Prakasam *et al.,* 2004; Pasupathi *et al.,* 2009). Reduction in serum albumin, alpha and beta globulin, plasma albumin/globulin ratio and a concomitant elevation in gamma globulin have been shown in diabetic rats (El-Shenawy and Abdel- Nabi, 2006). A reduction in protein content in the serum of diabetic patients has been reported and this is indicated by an increase in the lipid peroxidation and a decreased antioxidant defense system (Chandramohan *et al.,* 2009). In diabetes, increased blood nitrogenous substances may be accounted for by the enhanced breakdown of both liver and plasma proteins (Prakasam *et al.,* 2004). The results indicate a significant decrease in the level of albumin while the total protein and globulin levels were non-significantly reduced in the diabetic control group in comparison to the normal control group. The reason for the reduction in the level of albumin could be as a result of the regulation of albumin catabolism by neonatal Fc receptor (FcRn) which binds albumin in acidic environments and increases albumin catabolism and hence, leads to a decreased level of serum albumin (Chaudhury *et al.,* 2003). The present study revealed that RPO+RTE could not

significantly increase the level of albumin when compared with the diabetic control group. Similarly, RPO significantly increased the level of globulin in comparison to both the normal and diabetic control groups. Indirectly, antioxidant activity of albumin comes from its ability to transport bilirubin which binds with high affinity to the molecule at lysine at position 240 (Lys 240) and such albumin-bound bilirubin has been shown to be helpful in the prevention of oxidative damage to lipids and proteins (Jacobsen, 1978; Neuzil and Stocker, 1994: Roche *et al.,* 2008). Albumin could also exert its antioxidant activity due to its capacity to bind homocysteine, a sulphur-containing amino acid, which results from the catabolism of methionine residues (Roche *et al.,* 2008). The histopathological evaluations of the liver in both the non-diabetic groups and diabetic groups revealed no spectacular visible pathology in the liver sections. The diabetic groups showed very mild inflammatory activity around the portal areas of the liver in the diabetic rats which are characterised by periportal cellular infiltration by mononuclear cells.

**Conclusion**

This study confirms the involvement of oxidative stress in the progression of diabetes. The activities of antioxidant enzymes critically influence the susceptibility of various tissues to oxidative stress in diabetes. These results confirms the ability of the RPO, RTE or the combined treatment (RPO + RTE) to up-regulate the activities of some antioxidant enzymes intracellular activities in non-diabetic and diabetic conditions. It also suggests that these plant products could offer protective roles against oxidative stress. The antioxidant beneficial effects of red palm and rooibos could be as a result of inhibition of specific pathways that are activated as a consequence of increased oxidative stress in the progression of diabetes. Therefore, antioxidant therapy in diabetes may therefore be helpful in relieving many symptoms and complications observed in diabetes patients.

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# CHAPTER EIGHT

# GENERAL DISCUSSION AND CONCLUSION

## 8.1 Biochemical effects of consumption of RPO and RTE at different doses in normal rats (a preliminary investigation)

Normal rats were subjected to oral consumption of three different doses of red palm oil (RPO) and three different concentrations of aqueous rooibos extracts (RTE) for a period of 7 weeks. There was a significant increase in body weights of the rats that were fed with 2 ml and 4 ml RPO. No significant changes in body weights were observed in the RTE fed animals. The various parameters investigated in these studies were used as indices for evaluating the biochemical effects of RPO and RTE in non-diseased rats at different doses. No adverse effects of these two plant products were observed. However, the plant products tended to help in the improvement of the antioxidant defence system in the rats. Chandratre *et al.* (2012) reported that a connection between biological activity and use in traditional medicine has been established in numerous cases of diseases. Several phytochemicals are reported to increase antioxidant enzymes through the induction of gene expression of the enzymes (Bellamkonda *et al.,* 2011). Antioxidant enzymes are known to be a primary defence that prevents biological macromolecules from oxidative damage (Oloyede *et al.,* 2012). Superoxide dismutase (SOD) can convert superoxide to hydrogen peroxide ($H_2O_2$) which is then converted into water (Shukla *et al.,* 2012). $H_2O_2$ can lead to the generation of hydroxyl radicals in the cells and hence, its removal is essential for antioxidant defence in cells and food systems (Shukla *et al.,* 2012). GPx and CAT participate in the removal of $H_2O_2$. Oloyede *et al.* (2012) reported that GPx is the main controller of $H_2O_2$ metabolism.

The increased activities of both RBCs and liver catalase in the rats fed with RPO at different doses used in this study could suggest that some constituents of RPO might be activating or inducing the synthesis of this enzyme at the intracellular level. An increase in the activity of GPx in the RBCs at both 2ml and 4 ml RPO was observed while there was no difference in the activities of the liver GPx in RPO fed rats. These effects could possibly be an important mechanism of protection by RPO since by having an increased ability to remove peroxides, the cells may be less susceptible to oxidative damage. In another vein, rooibos extracts at all the concentrations used significantly increased the activity of liver catalase while that of the RBCs was not significantly different from the control group. Its effects on the activities of RBCs and liver GPx did not show any significant difference in this study. These plant products, being

rich in antioxidants, were able to provide some beneficial health effects by boosting the antioxidant system in the rats in these studies.

Enhanced lipid risk factors are a direct risk factor for atherosclerosis (Chandratre *et al.,* 2012). Choi and Hwang (2005) reported that the therapeutic approach which is aimed at increasing the efflux of cholesterol from the arterial wall, may be an added advantage for patients with atherosclerosis. A state of continual hypercholesterolemia leads to enhanced oxidative stress causing atherosclerosis and coronary artery disease (Maruthappan and Shakthishree, 2010; Ramya *et al.,* 2012). Red palm oil at the different doses used in this study did not significantly alter the levels of total cholesterol, triglycerides and LDL-cholesterol in the rats. The rats fed with 1 ml RPO had a significant increase in HDL-cholesterol which could be as a result of the non-significant increase in the total cholesterol in this group of animals. There were no significant differences in the levels of triglycerides and total cholesterol in the rooibos fed rats at the different concentrations. HDL-cholesterol was not significantly different in the rooibos fed rats.

Previously, It has been reported that the determination of total protein, albumin and globulin levels is aimed at evaluating the toxicological nature of various chemicals (Abbas *et al.,* 2012). Albumin is an essential component of plasma antioxidant activity that binds free fatty acids, divalent cations and hydrogen oxychloride (HOCl) (Llesuy and Tomaro, 1994; Akinbinu *et al.,* 2008). From the results, there were no significant effects on the serum levels of total protein, albumin and globulin in the RPO and RTE fed rats in both phases of the study. A decrease in serum protein could be attributed to an increased binding of plant components to serum albumin and hence, probably depicts hepatocellular damage (Sodipo *et al.,* 2011). Essien *et al.* (2012) suggested that the non-significant effect in the levels of total protein and albumin in rats after herbal treatment revealed that the secretory ability and normal functioning of the liver in relation to these substances was not affected and this confirms the non-toxicity effects of RPO and RTE in the rats in this study.

Fat accumulation is the net result of absorption, de novo synthesis, and oxidation of fatty acids and the liver plays a dominant role in deposition and oxidation of fatty acids (Smink *et al.,* 2010). Body fat accumulation may also be referred to as the net result of the balance among dietary absorbed fat, endogenous fat synthesis (lipogenesis) and fat catabolism through β-oxidation (lipolysis) (Sanz *et al.,* 2000). Accumulation of liver lipids in hepatocytes is the hallmark of non-alcoholic fatty liver disease (NAFLD), an essential factor that can induce insulin resistance, lipid peroxidation, changes in energy metabolism, hepatic cell damage and inflammation (Wang *et al.,* 2011). It has been reported that 20% of dietary fatty acids are

secreted as VLDL triglycerides within 6 hours after a meal and this suggests that a significant fraction of fatty acids are taken up by the liver during the postprandial period (Heath *et al.,* 2003; Westerbacka *et al.,* 2005). Vegetable oil rich in saturated fatty acids in comparison with a vegetable oil rich in linoleic acid was found to increase fat deposition in broiler chickens and affected synthesis or oxidation, or both, of individual fatty acids (Smink *et al.,* 2010).

Red palm oil is known to contain an equal proportion of both saturated and unsaturated fatty acids (Oguntibeju *et al.,* 2010). It is rich in the saturated fatty acid-palmitic acid (C16:0) with the content of about 45% of the total fatty acids (Smink *et al.* 2008) and unsaturated fatty acids such as oleic acid (39%) and linoleic acid (10%) (Cottrell, 1991). In this study, the results indicated no excessive accumulation in total fatty acids in the liver of rats fed with palm oil at the different doses. The saturated fatty acid, palmitic acid, which is predominantly present in red palm oil, was found not to be significantly different in the liver of rats in all the palm oil fed groups when compared with the normal control group. Smink *et al.* (2008 and 2010) reported that a high fraction of palmitic acid in palm oil is bound at the *sn*-1 or *sn*-3 position of the glycerol molecule which makes it absorption less than those bound on the *sn*-2 position. This may suggest a reason why palmitic acid, absorbed in the liver, due to intake of red palm oil is efficiently metabolised in the body. The monounsaturated fatty acid, oleic acid, was not excessively accumulated at 1% and 2% RPO while the polyunsaturated fatty acid, linoleic acid, was signicantly reduced at 2% and 4% RPO in the liver of rats. Sanz *et al.* (2000) showed that dietary polyunsaturated fatty acid increased β-oxidation and inhibited *de novo* fatty acid synthesis despite higher dietary fat absorption which resulted into lower fat deposition in the abdominal fat pad of broiler chickens (Sanz *et al.,* 2000). This indicates that RPO which contains linoleic acid (polyunsaturated fat) could help to maintain an efficient fat metabolism in the body.

### 8.2 Effects of RPO, RTE and RPO + RTE on various biochemical parameters on STZ-induced hyperglycaemia in male Wistar rats.

The primary fundamental mechanism in diabetes mellitus is the lack of biologically active insulin which results in the impairment of uptake and storage of glucose and reduced usage of glucose for energy purposes (Saravanan and Ponmurugan, 2012). The most frequent and key symptoms of diabetes mellitus are hyperphagia, polyuria, polydipsia and reduced body weight (Unwin *et al.,* 2009; Islam, 2011). In this study, the volume of fluid intake in the diabetic control group and the treated diabetic rats was very high. The higher consumption of water and RTE extract in the diabetic was due to prolong and stable diabetic condition. The body weights of the normal control group as well as the normal rats fed with RPO, RTE and RPO + RTE were

found to be stable throughout the period of study while there was a significant reduction in the body weights of non-treated and treated diabetic rats. Treatment with the RPO and RTE singly did not have any significant effect on the body weights of the diabetic rats. It has been previously reported that the decreased body weight in diabetic rats is as a result of dehydration and breakdown of fats and proteins (Hakim *et al.,* 1997; Punithavathi *et al.,* 2011). The reduction in the body weight might also be due to increased catabolic reactions resulting into muscle wasting (Rajkumar *et al.,* 1991, Punithavathi *et al.,* 2011). Combined treatment with red palm oil and rooibos significantly increased the body weight gain of the diabetic rats and this shows the protection of animals from the diabetic conditions. It could also be suggested that, the restoration in the body weight could be a result of reduced hyperglycaemia.

The liver is an insulin dependent organ that has a crucial role to play in glucose and lipid homeostasis and it is seriously affected during diabetes (Seifter and England, 1982; Pari and Latha, 2002). It is a major organ that maintains systemic glucose homeostasis in mammals (Rolo and Palmeira, 2006). There were no alterations in the levels of blood glucose and insulin in normal rats fed with RPO, RTE and RPO + RTE when compared with the normal control rats in this study. The induction of diabetes in rats with streptozotocin showed an increase in the blood glucose levels and a decrease in the serum insulin levels. Diabetic rats fed only with either RPO or RTE did not have any significant effect on the blood glucose and insulin levels. However, the combined treatment (RPO + RTE) was able to significantly decrease the glucose level and increase the level of insulin. A higher insulin level could be as a result of the stimulatory effect of RPO + RTE and thus, potentiating the existing β cells of the islets of Langerhan's in the treated diabetic rats.

The antihyperglycaemic potency of the RPO and RTE in streptozotocin-induced diabetic rats with improved fasting blood glucose and insulin levels could be as a result of the effects of the profile of antioxidants present in the two plant products. The possible mechanism by which the combined effects of both RPO and RTE could bring about its antihyperglycaemic action is by increased pancreatic secretion of insulin from β-cell of islets or due to enhanced transport of blood glucose to peripheral tissue. Islam (2011) concluded from several reports that tea polyphenols ameliorate diabetic conditions via decreasing insulin resistance and / or by increasing insulin sensitivity rather than by increasing insulin secretion. Rooibos, which is also very rich in polyphenols, may be exhibiting the same mechanism of action in diabetic conditions. Deficiency of insulin leads to derangement in carbohydrate metabolism and reduces the activities of a number of key enzymes, including glucokinase,

phosphofructokinase, and pyruvate kinase (Hikino *et al.,* 1989; Kalaivanan and Pugalendi, 2011).

There was a significant increase in the glycosylated haemoglobin and fructosamine levels in the diabetic control and diabetic treated groups. However, diabetic rats treated with RPO + RTE showed a significant reduction in glycosylated haemoglobin and fructosamine. The reduced levels of blood glucose led to lowered levels of glycosylated haemoglobin and fructosamine and these indicate the antiglycosylative potentials of the synergistic effect of RPO and RTE in diabetes. Possibly, the decrease in glycosylated haemoglobin and fructosamine might also have been due to improved glycaemic control produced by the synergistic effects of the two plant products. Glycogen is the major intracellular storable form of glucose and its level in various tissues is a direct reflection of insulin activity (Gandhi *et al.,* 2011). However, in this study, there was a significant increase in all the diabetic groups. This is consistent with an earlier report that about 80% of diabetic patients have a built up glycogen in the liver (Lavanthi and Tavill, 1999). The marked increased liver glycogen levels were not in proportion to insulin deficiency and all the treatments used in this study could not bring the glycogen levels to normal. However, diabetic rats treated with RTE alone showed a significant decrease in the glycogen level.

Glycolysis and gluconeogenesis are the two main complementary events balancing the glucose load in our body (Punithavathi *et al.,* 2011). The levels of key carbohydrate metabolic enzymes are altered during diabetes which disturbs carbohydrate metabolism (Shukla *et al.,* 2007). Hepatic glucokinase is the most sensitive indicator of the glycolytic pathway in diabetes and increased levels could increase the utilization of blood glucose for glycogen storage in the liver (Iynedjian *et al*., 1988). On the contrary, the results from this study showed non-significant effects on the activity of glucokinase in the diabetic control and diabetic treated groups. Lenzen *et al.* (1987) reported that the mechanism of induction of diabetes with streptozotocin does not have immediate and inhibitory effect upon glucose phosphorylation through glucokinase (Lenzen *et al.,* 1987). Glucokinase, the low affinity glucose phosophorylating enzyme and glucose sensor of the beta cell is not actively involved in mediating the toxic action of streptozotocin (Elsner *et al.,* 2000). It can be inferred that the anti-hyperglycaemic potentials of the effects of RPO and RTE observed in this study was not due to the role of glucokinase activity. There was a significant reduction in the activity of pyruvate kinase in the STZ induced diabetic rats. A similar decrease in the activity of pyruvate kinase in diabetes has been reported (Aly and Mantawy, 2012). Pyruvate kinase is regulated at the mRNA level in insulin dependent diabetes (Sellamuthu *et al.,* 2009). Treatment of the diabetic rats with RPO and RTE singly showed a non-significant increase in the activities of the pyruvate kinase.

However, diabetic rats treated with RPO + RTE displayed a significant increase in the activity of pyruvate kinase.

The antioxidant defence system is made up of endogenous antioxidants which include antioxidant enzymes, glutathione, vitamins, small molecules and micronutrients (Sies, 1991; Halliwell and Gutteridge, 2007; Erejuwa, 2012). The balance between the generation of free radicals and the antioxidant defences in the body has crucial health implications (Astaneie *et al.,* 2005). It has been reported that the antioxidants status of tissues is an essential factor in the development of complications in diabetes (Wohaeib and Godin, 1987; Mary Jelastin Kala *et al.,* 2012). Antioxidant enzymes such as CAT, SOD, GPx and GR are primary enzymes that are involved in the direct elimination of free radicals (Oguntibeju *et al.,* 2010). In this study, there was no decrease in the activity of CAT in the RBCs of the diabetic control group. The activity of liver SOD was significantly reduced in the diabetic control group. The decrease in the activity of SOD and CAT can lead to the accumulation of superoxide ions and hydrogen peroxide which results in the generation of hydroxyl radicals that leads to initiation and propagation of lipid peroxidation (Rao *et al.,* 2012).

Circulating red blood cells act as a sink for free radicals since both superoxide radicals ($O_2^-$) and hydrogen peroxide ($H_2O_2$) have the ability to penetrate membranes of the cells (Andallu and Varadacharyulu, 2003). They are also subject to a continuous flux of $O_2$ and $H_2O_2$ which results from auto-oxidation of haemoglobin (Arai *et al.,* 1989; Andallu and Varadacharyulu, 2003). The decreased activity of liver SOD observed in the diabetic control rats was significantly unregulated by RPO, RTE and RPO + RTE treatments, indicating their modulatory effects on SOD. The increase may be attributed to the inhibition of the generated of active oxygen species from auto-oxidation of glucose, generated as a result of the hyperglycaemic state. It is therefore likely that RPO and RTE exert their beneficial effects as a result of their antioxidant components which act as strong free radicals quenchers. There was no significant difference in the activity of SOD in the RBCs of the diabetic control and diabetic treated groups in this study. The reason for this could possibly be due to the efficient system of the enzymes or decreased production of free radicals in the cells. There was also a significant increase in the activity of GPx in the RBCs of diabetic rats treated with RPO and RPO + RTE. Diabetic rats treated with RTE and RPO + RTE also showed an increase in liver GPx. This clearly reveals the potential effects of red palm oil and rooibos in the up-regulation of GPx in the diabetic rats.

The plasma TEAC status in both the diabetic control rats as well as the treated diabetic rats did not show any significant differences. The TEAC assay is based on the suppression of the

absorbance of radical cations of 2, 2 -azinobis (3-ethylbenzothiazoline 6-sulfonate) (ABTS) by antioxidants in the sample when ABTS incubates with a peroxidase (metmyoglobin) and $H_2O_2$ (Rice-Evans and Miller, 1994; Wang *et al.,* 2004). This could imply that the antioxidant enhancing ability of RPO and RTE in the blood of diabetic rats does follow the principle of assessing for TEAC status. However, the liver TEAC was significantly increased in the diabetic rats treated with RTE alone. The principle of FRAP is based on the reduction of ferrous ions by the effect of the reducing power of plasma and this is made possible by low molecular weight antioxidants of hydrophilic and/or hydrophobic nature (Shukla *et al.,* 2012). There was no significant increase in the plasma FRAP status of diabetic control rats. Furthermore, RPO, RTE and RPO + RTE significantly increased plasma FRAP status in treated diabetic rats. This suggests that RPO and RTE could help to boost the antioxidant capacity in diabetic conditions. The increasing effect of medicinal plants on FRAP status has been reported (Shukla *et al.,* 2012). Similarly, there was an increase in the plasma ORAC in both the non-diabetic and diabetic rats treated with RPO, RTE and RPO + RTE. The increased antioxidant capacity in the plasma of treated diabetic rats may indicate an enhanced antioxidant activity.

Oxidative stress causes a biomolecular damage as a result of the attack of reactive species on components of living organisms and is known as oxidative damage (Halliwell and Gutteridge, 2007; Erejuwa, 2012). This is caused by increased production and / or reduction in the removal of reactive species by the antioxidant defences (Erejuwa, 2012). Hyperglycaemia leads to generation of free radicals due to auto-oxidation of glucose and glycosylation of proteins (Al-Faris *et al.,* 2010) and induces oxidative stress which becomes the chief factor that leads to diabetic complications (Kumawat *et al.,* 2009). Abnormal elevated levels of free radicals and the simultaneous reduction of antioxidant defence can result in damage of cellular organelles and enzymes, increased lipid peroxidation and development of insulin resistance (Tirgar *et al.,* 2010).

The elevated level of lipid peroxidation causes oxidative damage by increasing peroxy radicals and hydroxyl radicals (Shukla *et al.,* 2012) and is usually measured through the catabolite, malonaldehyde (MDA), in terms of TBARS as a maker of oxidative stress (Kumar *et al.,* 2012). In this study, a significant increase in the plasma TBARS in the diabetic control group was observed. This increase in plasma TBARS indicated enhanced lipid peroxidation which could cause injury to the cells. Increased levels of lipid peroxides in the plasma is usually considered to be the consequence of high production and liberation of tissue lipid peroxides into circulation due to pathological changes (Selvam and Anuradha, 1990; Ravi *et al.,* 2004). The diabetic rats treated with RTE and RPO + RTE show no significant decrease in TBARS. No

significant differences in the level of liver TBARS in STZ-induced diabetic control and treated diabetic rats were observed.

Glutathione is a strong cell antioxidant present in many metabolic pathways and reduces different oxidants after donating its hydrogen atom (Klepac *et al.,* 2006). It is the non-protein compound-containing thiol group that acts as a substrate for glutathione transferase and glutathione peroxidase and this plays an essential role in prevention of the damaging effect by oxygen radicals (Zhang and Tan, 2000; Al-Faris *et al.,* 2010). In this study, the results showed a non-significant decrease in the total glutathione levels in the liver of non-treated and treated diabetic rats. This could mean that hyperglycaemia has the tendency to reduce the glutathione levels in the liver in the diabetic state possibly due to increased generation of free radicals. A non-significant increase in the plasma glutathione levels in all the treated groups was observed. Albumin, the most abundant plasma protein is secreted into the portal circulation when it is produced and accounts for about 55-60% of the measured serum proteins in humans (Nayyar *et al.,* 2012). Serum albumin is a key antioxidant agent and its structural modification as a result of induction by glucose or free radicals can impair its antioxidant potentials (Faure *et al.,* 2008). In this study, there was a non-significant decrease in the levels of total protein while albumin levels were reduced significantly in the diabetic control group. Treatment with RPO + RTE appreciably increased the serum albumin in the diabetic rats. The ability of the RPO + RTE to normalize the levels of albumin in the hyperglycaemic state may be attributed to their free radical scavenging properties.

Alterations in lipid metabolism and increased mobilization of free fatty acids from muscle and fat deposition occur in tissues such as liver and heart in diabetes mellitus (Bloomgarden, 2003; Shukla *et al.,* 2012). Hyperlipidaemia, a risk factor in diabetes mellitus is frequently seen among diabetic patients (Mengesha, 2006). Serum lipid levels are commonly increased in diabetes mellitus and such an elevation represents a risk factor for coronary heart disease (Al-Shamaony *et al.,* 1994, Muthulingam, 2010). Insulin increases receptor-mediator removal of LDL-cholesterol in normal conditions while decreased activity of insulin, during diabetes results to hypercholesterolaemia (Mary Jelastin Kala *et al.,* 2012). In this study, the non-significant reduction in the levels of triglycerides in diabetic rats treated with RTE and RPO + RTE show their possible protective effects against complications that may arise as a result of this metabolic disorder.

An increase in the levels of triacylglycerols, cholesterol and lipoprotein (LDL and VLDL-cholesterol) in the serum of the diabetic rats has been documented (Fernandes *et al.,* 2010). Marked increases in the level of triglycerides and VLDL were observed in the diabetic control

rats. Diabetic rats treated with RPO alone showed an increase in TG and VLDL-cholesterol levels. Kochikuzhyil *et al.* (2010) also showed a similar increase in the levels of triglyceride in RPO fed diabetic rats and suggested that it could be attributed to the presence of saturated fatty acid-palmitic acid. Treatments with RPO + RTE brought the levels of TG and VLDL-cholesterol to levels that were not significantly different from normal rats fed with RPO + RTE. The level of cholesterol was non-significantly different in all non-diabetic and diabetic rats. Furthermore, diabetic rats treated with RPO and RTE singly were able to increase the levels of HDL-cholesterol.

Increased gluconeogenesis and ketogenesis might be due to an elevated activity of transaminase (Ghosh and Suryawansi, 2001; Gandhi *et al.,* 2011). Abolfathi *et al.* (2012) reported that the elevation in markers of liver injury such as ALT, AST, ALP and bilirubin indicate hepatocyte damage in experimental diabetes. ALP and AST are biomarkers of damage to the plasma membrane and endoplasmic reticulum and are often used to assess the integrity of the plasma membrane and tissues after being exposed to certain pharmacological agents (Esien *et al.,* 2012). In a similar vein, a significant increase in the levels of ALT, AST, ALB and GGT in the serum of diabetic rats was observed in this study. The ability of the combined treatment (RPO + RTE) to significantly decrease the AST serum level suggests their hepato-cellular protective function and this can be attributed to their synergistic effects. We also observed an increase in GGT in the diabetic control rats and RTE treated diabetic rats. However, GGT was below the detection limit in the diabetic rats treated with RPO and RPO + RTE. This clearly reveals that RPO could protect the liver from hepato biliary injury as a result of the non-leakage of GGT into the serum from the liver. A study conducted by Abolfathi *et al.* (2012) showed the improved effects of green tea extracts on serum biomarkers of liver tissue injury and it was suggested that green tea extracts is prophylactic against diabetic complications and ameliorates diabetic hepatopathy through its antioxidant potential. The antioxidant potentials of red palm oil and rooibos can also be suggested to be responsible for the protection conferred on the liver as demonstrated in the current study.

The histopathological evaluations of the pancreas showed that normal control and treated rats showed a greater presence of the islets compared to the non-treated and treated STZ diabetic rats. This is the result of the destruction of β-cells by streptozotocin which causes selective destruction of pancreatic β-cells in the diabetic rats and hence, diminishes insulin secretion. The histopathological evaluations of the liver in all the groups revealed no visible pathology in the liver sections, apart from very mild inflammatory activity around the portal areas of the liver

in the diabetic rats which are characterised by periportal cellular infiltration by mononuclear cells.

## Conclusion

Chronic hyperglycaemia is the hallmark of diabetes mellitus, a serious metabolic disorder chiefly mediated by the actions of oxidative stress. Red palm oil and rooibos could help to improve lipid metabolism and the body antioxidant defence system with their characteristic physiological and biochemical properties. It can be suggested that the abnormally high levels of serum lipids observed in the diabetic rats is as a result of increased mobilization of fatty acids from fat tissue. The red palm oil and rooibos, most especially their combined treatment appear to contribute positively to blood glucose control and by enhancing lipid metabolism as well as the red blood cells and hepatic antioxidant defence system. In this study, results indicate that red palm oil and rooibos contain free radical scavenging activities which could exert beneficial effects against pathological alterations due to the impact of superoxide radicals and hydrogen peroxide radicals. The reason for these potential health effects could be connected to the modulatory actions of lipid-soluble and water-soluble antioxidants that are present in red palm oil and rooibos respectively. The possible antihyperglycaemic mechanism of actions of red palm oil and rooibos could be due to stimulation of synthesis and / or release of insulin from the pancreatic beta cells and hence, prompting the uptake of glucose by the cells and the protection of the remaining β-cells against further oxidative damage as a result of glucose toxicity. Further studies will be needed in future to determine which one or more of the active components of red palm oil and rooibos could be responsible for antihyperglycaemic and antioxidative effects. Furthermore, there is need for future research studies on the use of antioxidant therapy in the management of diabetes mellitus.

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## ADDENDUM 1: RESEARCH OUPUT

### PUBLISHED ARTICLES:

i) **Ayeleso, A.O.**, Oguntibeju, O.O. & Brooks, N. 2012. Flavonoids and their antidiabetic potentials. *In: Bioactive Phytochemicals: Perspectives for Modern Medicine*, Volume 1. Daya Publishing House, New Delhi, ISBN: 978-81-7035-779-7.

ii) **Ayeleso, A.O.**, Oguntibeju, O.O., Brooks, N.L. 2012. Effects of dietary intake of red palm oil on lipid profile and fatty acid composition in male Wistar rats. *African Journal of Biotechnology,* 11(33): 8275-8279 (Refer to the next page).

### PUBLISHED ABSTRACT:

i) **Ayeleso**, A.O., Oguntibeju, O.O., Brooks, N.L. 2012. Influence of dietary red palm oil on antioxidant status in male Wistar rats. *BMC Complementary and Alternative Medicine* 12 (Suppl 1): P50. Scientific Abstracts Presented at the International Research Congress on Integrative Medicine and Health, Portland, USA.

### CONFERENCES ATTENDED:

i) Cape Peninsula University of Technology Post Graduate Research Conference, Bellville, South Africa, 2012.
Theme: To enhance postgraduate students' competency, skill and knowledge in presentation and publication.
Paper presented: **Ayeleso, A.O.,** Oguntibeju, O.O., Brooks, N.L. 2012**.** Effects of red palm oil and rooibos on glycaemic and lipidaemic parameters in streptozotocin induced-hyperglycaemic male Wistar rats.

ii) International Research Congress on Integrative Medicine and Health, Portland, USA, 2012.
Theme: Strengthening Research in Integrative Heath Care around the World.
Paper presented: **Ayeleso, A.O.,** Oguntibeju, O.O., Brooks, N.L. 2012. Influence of dietary red palm oil on antioxidant status in male Wistar rats.