RENAL OXIDATIVE STATUS IN DIABETIC WISTAR RATS EXPOSED TO METHANOL FRACTION OF ETHANOL EXTRACTS OF *DIALIUM GUINEENSE*

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SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE AWARD OF A BACHELOR OF SCIENCE DEGREE. B.Sc. (HONS)

IN

DEPARTMENT OF BIOCHEMISTRY,

FACULTY OF LIFE SCIENCES,

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BENIN CITY.

DECEMBER, 2022

CERTIFICATION

This is to certify that this project research was carried out by OZEDU MARTINS EHIZOJIE with Matriculation number, LSC1705252 under the supervision of Dr Osahon D. Abu and has been read and approved as meeting the requirements of the Department of Biochemistry, Faculty Of Life Sciences, University of Benin, Benin City, Edo state in partial fulfilment of the requirement for the award of a Bachelor of Science degree, BSc [Hons] in Biochemistry.

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(PROJECT COORDINATOR)

_____ _____

PROF MRS K.E IMAFIDON. Date

(HEAD OF DEPARTMENT)

DEDICATION

I dedicate this project to Almighty God who helped, strengthened and sustained from start to finish of this research work in good health.

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I want to extend a special thank you to the Almighty God, who has been my source of strength, understanding, and wisdom throughout this program. I have only been able to fly on his wings.

Through the challenges of school and life, my parents, Mr. and Mrs. Pius Ozedu, have been a constant source of support and encouragement, and their good examples have motivated me to work arduously toward the goals I have set for myself. I sincerely appreciate them.

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Last but not least, I want to express my gratitude to my brothers Polycarp, Kingsley, Blessed, and Jerry as well as my friends Nashville, Mims, Emmanuel, Joy, Lizzy, Sammy, Amblessed, Chizzy, and Antonia for their never-ending support and affection. Blessings from God to you

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CHAPTER TWO

ABSTRACT

In all of Africa, *Dialium guineesne* (Velvet tamarind, family: *Fabaceae*) is known for its culinary and nutritional benefits. Traditional systems of medicine are also well aware of the therapeutic benefits of *D. guineesne's* leaves and other plant components. Scientists and researchers have investigated the plant for its pharmacological properties and therapeutic uses, including antibacterial, anti-ulcer, anti-oxidant, analgesic, anti-hepatotoxic, antimicrobial, anti-plasmodia, anti-vibrio, anti-diarrheal properties. The equilibrium between pro- and anti-oxidants is maintained by antioxidant enzymes such superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), and glutathione peroxidase (GPx). These antioxidants aid in the cell's defense against oxidative stress. The current study is aimed at evaluating the renal protective properties of *Dialium guineesne* against streptozotocin-induced renal toxicity. A single dosage of 50 mg of freshly manufactured streptozotocin (STZ), diluted in 10 ml of normal saline was used to induce diabetes mellitus. After administering two groups with various dosages of the plant extract, the animals were sacrificed and blood samples were taken. The findings of this study demonstrated that treatment of diabetic Wistar rats with methanol fraction of ethanol stem bark extract of D. guineense significantly increased antioxidant enzyme activities as well as concentrations of GSH and NO levels ($p < 0.05$). The observed pharmacological activity may be the result of significant bioactive chemicals found in the extract fraction.

CHAPTER ONE

1.0 INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

The cells that make up the human body need energy and this energy is gotten from food. Glucose, a simple sugar obtained from the breakdown of carbohydrates, serves as the main energy source. It is crucial to control the synthesis and consumption of glucose, as well as the levels of glucose in the body. The metabolism of glucose is influenced by hormones like glucagon and insulin. The pancreas, which is situated in the rear of the abdomen behind the stomach, produces these hormones. Insulin acts as a doorway to drive glucose into cells, stimulating glucose utilization, whereas glucagon does the opposite, making glucose available for cells from glycogen, stimulating glucose production. When there is an excess of sugar in the blood, glucose is stored as glycogen. Following the consumption of carbs, insulin levels quickly increase while glucagon levels drop. (1981, Rizza et al.). Hyperglycemia, a sign of diabetes, it occurs when there is insufficient or aberrant insulin production. Dehydration results from the kidney's filtering and absorbing the extra glucose into the urine, which also carries fluids from the body (Jayakar and Suresh, 2003). By causing the beta cells to die, diabetogenic substances like streptozotocin and alloxan are used to cause diabetes in test compounds. Researchers have also noted that several plants have anti-diabetic characteristics. Dialium guineesne (Velvet tamarind, family: Fabaceae) is well-known for its culinary and dietary advantages throughout all of Africa. The medicinal advantages of the leaves and other plant parts of D. guineesne are also well known to traditional systems of medicine. Many medicinal plants have been advocated for the treatment of diabetes mellitus (Ghorbani, 2013).

1.2 Aim and Objective

1.2.1 Aim

The aim of this study is to determine the renal oxidative status of diabetic male wistar rats treated with methanol fraction of ethanol extract of *Dialium guineense* stem bark in**.**

1.2.2 Objective

To determine the biochemical effect of methanol fraction of ethanol extract of *Dialium guineense* stem bark in diabetic male wistar rats**.**

1.3 Literature Review

1.3.1 Diabetes Mellitus

Diabetes mellitus is a chronic heterogeneous metabolic disorder characterized by hyperglycemia; elevated levels of blood sugar (glucose), which is as a result of either insulin deficiency or the impaired effectiveness of insulin's action or the combination both. For proper comprehension of diabetes mellitus, it is imperative to understand the basic physiological process that occurs during and after a meal. When food is ingested by humans, it passes through the digestive system where it is broken down to its simplest form and the nutrients released (including proteins, fat and carbohydrates) are absorbed into the bloodstream. The presence of sugar, a carbohydrate, signals to the endocrine pancreas to secrete the hormone insulin. Insulin facilitates the absorption and storage of sugar by almost all tissue types in the body, especially the liver, musculature and fat tissue [Roussel, 1998]. Therefore, this disorder is function of the production and(or) effectiveness of the hormone insulin, which leads over time to serious damages to the heart, eyes, blood vessels, kidney, nerves and may also lead to other complications. The cure for diabetes has not been discovered yet but control of blood sugar levels through healthy diet, exercises and

proper medications can lead to decrease of long-term diabetes complications. The long-term complications that can be experienced are:

- Cataract and retinopathy [gradual damaging of the eye] which may eventually lead to blindness.
- Kidney failures and kidney diseases.
- Neuropathy [which is the gradual damaging of newrves]
- Stiffness of the arteries which eventually leads to heart disease and stroke [Heart foundation,2003]
- Foot ulcers, infections, gangrene and many more.

1.3.2 Classification of diabetes mellitus

There is a modern categorization of diabetes as well as other types of glucose intolerance. This modern categorization is based on studies conducted by the National Diabetes Data Group in 1979. According to this research, diabetes mellitus is classified into two major categories: TYPE I diabetes mellitus, which is caused by an absolute or near absolute deficiency of insulin, and TYPE II diabetes mellitus, which is caused by an absolute or near absolute deficiency of insulin. Although there are few other cases that are difficult to classify, such as gestational diabetes mellitus (GDM); which is glucose intolerance with onset or first detection during pregnancy and a variety of uncommon and other forms of diabetes that are caused by infections, medications, pancreatic damage, and hereditary flaws. These distinct types of diabetes that are unrelated to one another are listed individually under "Other Specific Types."

1.3.3 Type I diabetes mellitus (T1DM)

Type I diabetes mellitus is sometimes known as juvenile diabetes or insulin-dependent diabetes mellitus (IDDM). In this circumstance, the pancreas either produces little or no insulin which is essential for the absorption of glucose (sugar) by cells where they may be stored as glycogen or use to produce energy. This subclass of diabetes (type 1 diabetes mellitus) is generally characterized by the abrupt onset of severe symptoms, dependence on exogenous insulin to sustain life and proneness to ketosis even in the basal state, all of which is caused by absolute insulin deficiency. The most common form of diabetes in children and young adults in developing nations is IDDM, often known as juvenile diabetes (Harris and Zimmet, 1997). About 10% of all instances of diabetes are type 1 cases. For the remainder of their lives, people with type 1 diabetes will require insulin injections. Additionally, they must maintain correct blood glucose levels by having routine blood tests and eating a specific diet (Piero *et al.,* 2014).

1.3.4 Type II diabetes mellitus (T2DM)

Type 2 diabetes is characterized by insulin resistance (cells not responding normally to insulin) and at least initially, a relative deficiency of insulin secretion. Approximately 90% to 95% of all diabetic cases are T2DM, with highest proportions in low- and middle income countries**.** T2DM most likely develops in people over age 45, but more and more children, teen and young adults are also developing it. Patients with T2DM are not dependent on exogenous insulin for prevention of ketonuria and are not prone to ketosis. However, if the correction of fasting hyperglycemia cannot be achieved with the use of diet or oral agent, they may require insulin for the correction and under special circumstances such as severe stress precipitated by infections or trauma (Harris and Zimmet, 1997) may lead to development of ketosis. Type 2 diabetes

pathogenesis, is that the body does not correctly utilize the insulin produce by the pancreas. This is caused primarily by peripheral insulin resistance where insulin receptors or other intermediates in the insulin signaling pathways within the body cells are insensitive to insulin and consequently glucose does not readily enter the tissues leading to hyperglycemia or elevated blood glucose concentration (Albright, 1997). Impaired insulin action leads to obesity, which is a common risk factor for type 2 diabetes and most patients with this type of diabetes are obese (Nolte and Karam, 2001) and they ultimately require multiple anti-diabetic agents to maintain adequate glycaemic control (Gerich, 2001).

1.3.5 Gestational diabetes

This type affects females during pregnancy. Some women have very high levels of glucose in their blood, and their bodies are unable to produce enough insulin to transport all of the glucose into their cells, resulting in progressively rising levels of glucose. Diagnosis of gestational diabetes is made during pregnancy. The majority of gestational diabetes patients can control their diabetes with exercise and diet. Between 10 to 20 percent of them will need to take some kind of blood-glucose-controlling medications. Undiagnosed or uncontrolled gestational diabetes can raise the risk of complications during childbirth (Piero *et al.,* 2014).

1.3.6 Biochemistry of diabetes mellitus

A regular energy source is a prerequisite for every cell to function in the human body. Glucose is the body's primary energy source, which circulates in the blood as a mobilizable fuel source for cells. Insulin is a pancreatic hormone responsible for blood glucose level regulation. The hormone binds to its receptor sites on peripheral side of the cell membranes. It allows glucose to

enter respiring cells and tissues through the necessary channels. Through glycolysis, insulin accelerates the breakdown of glucose into pyruvate. It also up-regulates glycogenesis from excessive cytosolic glucose and lipogenesis from excessive cytosolic acetyl-coA. When glucose level in the blood is above threshold, insulin facilitates the uptake of glucose by cells for storage. But in the case of diabetes where there's little or no insulin or cells not responding to insulin, glucose remain in the blood and the blood glucose level gradually builds up. The body attempts to arrest hyperglycemia, by drawing water out of the cells and into the bloodstream. The excess sugar is excreted in the urine. This explains why diabetics have polyuria, excessive water consumption, and persistent thirst as their cells work to eliminate the excess glucose. This subsequently leads to glucosuria (Piero *et al.,* 2014).

As hyperglycemia prolongs, the body cells are devoid of glucose due to the lack of insulin. This forces the cells to seek alternative energy sources. In this regard, the cells turn to fatty acids stored in adipose tissue. The fats are not fuel sources for the red blood cells, kidney cortex and the brain. The red blood cells lack mitochondria in which beta-oxidation pathway occurs. The fatty acids cannot pass the blood-brain barrier. To avail energy to such cells and tissues, the acetyl-CoA arising from catabolism of fatty acids is diverted to ketogenesis to generate ketone bodies, which can serve as alternative fuel sources for such cells and tissues. These ketone bodies are also passed in the urine, thereby leading to ketonuria, which characterizes diabetes mellitus. Build- up of ketone bodies in the blood produces ketosis. Ketone bodies are acidic in nature and therefore, their build up in blood lowers blood pH, leading to acidosis. A combination of ketosis and acidosis lead to a condition called ketoacidosis. If left untreated, ketoacidosis leads to coma and death (Belinda, 2004).

1.4. MEDICINAL PLANTS

Medicinal plants are plants which contain substances that could be used for therapeutic purposes or which are precursors for the synthesis of useful drugs (Abolaji *et al.,* 2007). A plant becomes medicinal only when its biological activity has been ethno botanically reported or scientifically established (Elujoba, 1997)

Since time immemorial, plants have been used virtually in all cultures as source of medicine. Its use is increasing worldwide, in view of tremendous expansion of medicine and a growing interest in the herbal treatment. In medicine, plants are used to maintain and augment health physically, spiritually and mentally as well as for treatment of specific conditions and ailments. Traditional systems of medicine continue to be widely practiced on many accounts. As a result of population rise, inadequate supply of drugs, prohibitive cost of treatments, side effects of several synthetic drugs and development of resistance to currently used drugs for infectious diseases. There have been increase emphases on the use of plant materials as a source of medicine for a wide variety of human activities. Mother nature gave us medicinal plants as divine gifts, who has kept those remedies in her plant kingdom for man to use to fight death from diseases and cure themselves from ailment. Natural products from plants is another potent source for the discovery of excellent activities such as blood booster, anti-oxidant, anti-ulcer, anti-cancer, anti-microbial e.t.c.

The importance of scientific research into herbal medicine has been emphasized by World Health Organization (WHO). Native medicinal plants have been looked upon by many developing countries as possible addition to the WHO's list of essential drugs once there is a clinical prove of their value. Our continued existence and survival depends on the efficiency with

which man, with all the available resources and technology, harnesses, develop, utilizes plants and its products (Ayoka *et al*.; 2008). One of these emerging interest is *Dialium guineense*.

1.4.1 *Dialium guineense* (Velvet tamarind)

1.4.2 Botanical description

Velvet tamarind also known as black velvet is a common name for dialiun guineense, a genus of a legume belonging to the family of Fabaceae and sub-family of Caesalpinioidae. The genus dialium comprises five species in West Africa but *D.guineense*, D.dinklagel, D.packyphylum are represented in Nigeria (Omotayo, 1999). It is a tree of average height of 30m with a densely leafy crown, but often shrubby. Bole without buttresses, bark smooth, grey, slash reddish yielding a little red gum (Hutchinson and Daniel, 1958). It has finely hairy leaves, with common stalk of 5 to 13cm long, with an odd terminal leaflet and usually two pairs of opposite or alternate leaflets, the lower pairs being smaller mostly 3.5 to 5cm, elliptic to broadly elliptic, sometimes slightly obovate, blunt at the apex or abruptly and shortly acuminate, symmetrical and rounded or slightly cuneate at base, leathery, glabrous above and with the midrib slightly sunken, sometimes finely hairy beneath. Its flowers are usually whitish in large terminal, or occasionally axillary, panicles up to 30cm long, branches spreading out widely and more or less horizontally (Szolnok, 1985). The whole inflorescence at first covered with very short, brownish hairs. Individual flowers with short stout stalks, the buds about 2cm. It has abundant fruits, which are more or less circular and flattened, but almost globose sometimes, up to 2.5cm in diameter, densely velvety, black, each fruit with a stalk about 6cm long with a little collar near the apex, with a brittle shell enclosing one seed or exceptionally two, embedded in a dry brownish, sweetly, acidic, edible pulp (Hong *et al*., 1996)

Fig 1.0a: The plant of Dialium guineense (Orwa et al., 2009)

Fig 1.0b: The plant of Dialium guineense (Orwa et al., 2009)

1.4.3 Origin of *Dialium guineense*

Origin of its generic name is not known. A noted 18th century English botanist J.E Smith, soughed it out but could not discover it, nor has other modern botanists. The specific name

means “of Guinea” suggesting it probably originated from Guinea, it was introduced as a plantation crop in tropical and subtropical regions subsequently.

1.4.4 Ecology

Velvet tamarind grows in dense savannah forests, shadowy canyons and gallery forests. It is found from Senegal to Sudan along the Southern border of the Sahel. It is naturally found on moist, sometimes brackish soils with mean annual rainfall of less than 2100-2600mm.

1.4.5 Distribution

It is also found in Central and West African Republic, Chad, Benin, Burkina Fasso, Ivory Coast, Ghana, Guinea, Liberia, Mali, Senegal, Sierra Leone and Togo. In Nigeria, it is the most common and widespread Dialium. Among the Igbo which is the eastern part of Nigeria, it is known as “icheku”, as “awin” among the Yoruba the western part of Nigeria, in Hausa the northern part, it is known as “tsamiyar Kurm” (Nwosu,2000; Akinpelu *et al.*, 2011). In Ghana it is known as “yoyi” “Sierra Leone tamarind” in Sierra Leone and in French as “tamarinier noir”. The tree flowers from September to October and fruits from October to January in Nigeria (Keay, 1998). For rural dwellers in Nigeria during dry season when fruits are scarce use its wild fruits as dietary supplement (George,2011). From January till May, the ripe fruits are available between March and April are the peak period for harvest according to Okafor, (1975). Dispersal of the fruits is carried out by animals which like to eat the pulp in which the seeds are embedded, however, the fruit can also be transported by water since it floats, transport by sea currents may lead to long distance dispersal.

1.4.6 Local uses of *Dialium guineens*

S/N	MORPHOLOGICAL PART	NON-MEDICINAL USES
1	Wood	It is used as fuel, firewood and charcoal in Africa, it is also used as construction materials for making vehicles, tools, weapons, furniture, packing cases, houses and flooring. Also used in making fufu pestle
2	Bark	Used in artwork in carving figures like amulet, statuettes and various ornamental objects. It is also used in making chewing sticks commonly used in eastern Nigeria.

3	Gum	Is obtained from seed and is added to many kinds of food to Improve their viscosity in Japan.
4	Seed	Its seed powder is used as coffee substitute by village people In India. Dehulled seed are soaked overnight in water and eaten in addition with sugar or salt in India.
5	Flowers	They are used for decoration
6	Leaves	Its young leaves are chewed for its tangy taste. It is used as tempering in India or in various cuisines as paste or whole.
7	Fruits	Edible can be soaked in water and drunk as beverage. It is also used to provide jam and jellies. In Nigeria, it is used as flavor in snacks and when peeled, pulp can be eaten raw

1.4.7 Non-medicinal uses of *Dialium gineense*

Commonly, it is used for food, its pulp is red, with sweet-sour astringent flavor similar to baobab but sweeter. When dry it can be eaten raw by man and animal [Matsuda, 2006]. In south-east Nigeria, the pulp is eaten raw peeled because of its refreshing properties and pleasant scorching taste [Ubbaonu et al, 2003]. Its thirst quenching, refreshing pulp can also be soaked in water and

drunk as a beverage and also provides jam and jellies (FAO, 2004). In snacks and alcoholic beverages, it could be used as flavor (Adame, 2002; Efiong *et al*., 2009)

The young leaves are sometimes chewed for its tangy taste. They are also used as paste and whole in various cuisines or as tampering in India. The bitter leaves are used to cook a Ghanians dish called "domoda" that taste both sweet and bitter. The seed are roasted, seed coats are removed mechanically, rural people of certain ethnic groups such as Kuvumba, Irulas, Malayali and Dravidan tribes or in India they soak the dehulled tamarind seed overnight in water and is eaten with the addition of salt or sugar (Siddhuraja *et al.,* 1995). Village people use hot water extract of dry heated tamarind seed powder as tamarind substitute.

In Japan, the flowers and leaves are eaten as vegetables and the gum obtained from the seed is added to many kinds of food to improve their viscosity (Siddhuraju, 2007), the low cost tamarindus kernel powder could be used as a good substitute for costly pectin for making jelly (Bhattacharya *et al.,* 1994).

The tree is used for fuel, it is used to make firewood and charcoal. It is also used for timber, sapwood is white with distinct ripple marks, the heartwood is red brown. Axes and saws get blunt because of the high silicate content of the timber. The wood has fine texture and is treated internationally because it's hard, durable, heavy and light brown. It is used for vehicles, for construction materials, for making houses and flooring, tools, weapons, furniture, packing cases and fufu pestle. Among Nigerian populace, the bark is used as chewing stick (indigenous tooth brush) (Akinpelu *et al.,* 2011). It is used in artwork for carving figures like amulet, statuettes and various ornamental objects.

1.4.8 Ethno medicinal uses of *Dialium guineense*

For treatment of different diseases, different parts of the tree have been used on folkloric medicine. The bark for cancer, headache and pains treatment (Idu *et al.,* 2009) reported the usefulness of the bark for oral hygiene and stomach ache among the Esan tribe of Edo state. In fever, prenatal pains and edema the leaves are used as remedy, while in diarrhea, the fruits are used (Arbonnier *et al.,* 2004). There is a present of fairly low levels of ascorbic acid and tannin in the edible and sweet pulp of the fruit. It is a good source of protein and minerals (Arogba *et al.,* 2006). In order to improve lactation and to check genital infection, women of South-East Nigeria chew *the* fruit of the plant (Nwosu, 2000). In severe cough, bronchitis, stomach ache, malaria fever, wound, jaundice, anti-ulcer and hemorrhoids, the leaves and stem barks are used as folklore remedies (Bero *et al*., 2009). Wolof and Senegal squeeze and apply the leaves on wounds (Devendra, 1988). Its seed coat and extracts of leaves are rich in vitamin C (Maduaka, 1988). Presence of vitamin C and antioxidant makes it an ideal food addictive to boost the body's immunity. *D.guineense* is used as chewing stick in Nigeria (Akinpelu *et al*., 2011). From previous studies, it has been shown that the plant contains saponins presumed to add cleaning effect of teeth and also prevent plague (Okwu and Okeke, 2008). Among some tribes in southern part of Nigeria, *D.guineense* is used as antiulcer and as vitamins supplements, and this was reported from the findings of Lawal *et al* (2010). Its tannin component was reported by Lawrence *et al* (1997) to possess excellent cardio protective qualities in addition to the antioxidant action. It reduces the level of intake of cholesterol by precipitating lipoprotein that carries cholesterol. Among the Esan people of Edo state, the usefulness of tannins in management of hypertension was reported by Mensah *et al.,* (2009). There is 13% of dietary fiber in the fruit pulp which increases its bulk and augments bowel movement, thereby help in

prevention of constipation. Colon mucus membrane is protected from cancer causing chemicals when the fiber binds to toxins in food. It binds to bile salt and decrease their reabsorption in the colon thereby help in expulsion of bad cholesterol.

MEDICINAL USES OF DIFFERENT MORPHOLOGICAL PARTS OF DIALIUM GUINEENSE

S/N	MORPHOLOGICAL PARTS	MEDICINAL USES
1.	Fruits	It is used to improve lactation and check genital infection in South-east Nigeria. Also remedy for diarrhea
2.	Leaves	Vitamin supplement among some tribes in southern Nigeria. Remedy for fever, prenatal pains, edema and infections such a diarrhea, severe cough etc
3.	Barks	Used in the prevention of dental carrus and plague formation (mouth wash) in south east Nigeria. Chewed for oral hygiene stomach ache among Esan people. It is also used as remedy for cancer, pains and infections such as bronchitis, wound, malaria fever etc

1.4.9 Biological activities of *Dialium guineense*

Different parts of the plant possess remarkable therapeutic actions that can support its traditional usage in treatment of some ailments. Based on modern scientific investigations, there are several reports on the therapeutic properties and pharmacological actions of D.guinense.

1.4.9.1 Antioxidant activity

In vivo antioxidant of methanolic leaf extract of *D.guineense* was studied using 2, 2-diphenyl-l-picnylhyclrazyl (DPPH) free radical scavenging activity and reducing power assay. In addition, the total phenolic content was also analyzed Result of DPPH scavenging activity of the extract showed a concentration dependent antioxidant activity with maximum scavenging activity observed at 250µg/ml concentration comparable to those of ascorbic (95.75%) and gallic acids (93.67%). There was also a comparison between the reducing potential of the extract (0.069±0.003nm) to that of gallic acid (0.078±0.022nm), while the total phenolic content was 69.45±0.02mg/g gallic acid equivalent it was found out from the study that extract possesses in vivo antioxidant activity (Gideon *et al*., 2013).

1.4.9.2 Oral care

The twigs are used as chewed sticks in southern Nigeria and the presence of bioactive compounds which comprise of saponins, tannins, flavonoids, alkaloids is responsible for their hpaste are very efficient, effective and reliable for cleaning teeth. The teeth of chewing stick users are usually strong, clean, fresh and devoid of dental plaque (Okwu and Okeke, 2003).

1.4.9.3 Anti-ulcer activity

Aqueous extract *D.guineense* was evaluated for its anti-ulcer activity using ethanol/HCL and indomethacin as ulcerogens. Its effect on gastric mucous secretion was also investigated. The extract was administered orally at the doses of 100 and 200mg for the experimental groups while the control and reference groups received distilled water (5ml/kg p.o) and cimentidine (32mg/kg p.o) respectively. Reduction in ulcer index was used to determine the extent of healing. The

results showed that the extract significantly *(p<0.05)* reduce the ulcer index from 4.75±0.17 to 0.20± 0.12 and from 3.95±0.19 to 0.14±0.09 I n the ethanol/Hcl and indomethacin induced ulceration respectively. The gastric mucus secretion was increased significantly ($p<0.05$) by the extract.

From the study, it was deduced that aqueous extract of *D.guineense* has anti-ulcer effects which might be due to its ability to increase gastric mucus secretion. The folkloric use of *D.guineense* for the treatment of gastric ulcer was also justified from these findings (Balogun *et al*., 2014). Elucidation of gastro protective mechanism(s) of *D.guineense* plant requires further studies.

1.4.9.4 Antimicrobial activity

In determination of antimicrobial activity of *D.guineense* leaf extract, study was carried out against clinical isolates of 6 biological species (staphylococcus aureus, streptococcus mutans, Escherichia coli, Bacillus cerus, Pseudomonas aeruginosa, klebsielle pneumonia), and fungal species (Candida albicans, Microsporum gypseum, Trichophyton mentagrophytes and Trichophyton rubrum) using agar well diffusion methof of Rath *et al.,* (2002). Inhibition zones formed by the extract were compared with the standards, ciprofloxacin and griseofulvin. At a concentration of 250, 125, 67.5µg/ml, the leaf extract was effective against S.mutans (25.9mm) and P.mirabilis (10.2mm) being the most and lowest sensitive isolates. It was also revealed from one result that significant ($p<0.05$) diameter zones of inhibitions were obtained with 125 and 250Nglml against bacterial isolates when compares with the reference drug. Gram positive bacteria were found to be the most sensitive organisms, followed by the fungi and gram negative bacteria generally.

D.guineense leaf extract demonstrated antimicrobial activity against the organism tested. This study substantiates its popular and wide traditional applications in diverse ailments [Gideon *et al.;* 2013]. In another study, stem bark extract revealed a broad spectrum of activity of *D.guineense* with salmonella typhi and S.aunes showing the greatest zones of inhibition (18.omm). The only fungi tested and was inhibited by the extract is candida albicans (Alajubu *et al*.; 2012).

1.4.9.5 Antibacterial activity

Effects of ethanolic leaf and bark extract of *D.guineense* using agar well diffusion technique against clinical isolates of Klebsiella pneumonia and staphylococcus aureus were carried out. From the results, at varying concentration, the extract exerted antibacterial activity on the test organism. The highest inhibition diameter (18mm) at 0.8g/ml was recorded for cold water leaf extract against staphylococcus aureus and ethanol extract inhibited the growth of the bacterial isolates in the concentration dependent manner with minimum inhibitory concentration(MIC) AT 0.2g/ml (Orji *et al*., 2012).

1.4.9.6 Analgesic activity

In rats, evaluation of the analgesic activity of methanolic stem bark extract of *D.guineense* using 3 anti-nociceptive models, acetic-induced abdominal constriction or writhing, tail immersion and hot plate analgesic models were carried out. Tests of analgesic drugs commonly measure noiception and involve the reaction of animals to painful stimuli (Rang *et al.,* 2003), the stimuli maybe thermal (tail immersion or hot plate tests), chemical (acetic acid-induced writhing or formalin tests) or mechanical (tail or paw pressure tests) (George *et al.,* 2009). Three test doses (250, 500, 100mg/kg) of extract were administered orally via gastric gavages. The activity was

compared and *D.guineense* produced a significant analgesic activity in a dose dependent manner in acetic acid-induced writhing model. All the doses (250, 500, 100mg/kg) had shown a good analgesic activity. In the tail immersion model, the extract at the dose of 1000mg/kg significantly increased the pain reaction time at the doses of 500 and 1000mg/kg (Ezeja *et al., 2011*).

1.4.9.7 Anti-diarrheal activity

Investigation was made on diarrheal-induced rats on the effect of *D.guineense* stem bark extract. The effect of the extract at oral doses of 50-200mg/kg body weight on the castor oil-induced diarrhea, gastrointestinal motility (charcoal meal) and castor oil-induced intestinal fluid accumulation (enterpooling) was examined in rats. The extract produced a dose dependent significant reduction (31.3-80.8%). The extract also doses dependently reduced the small intestine transit time of charcoal meal (28.90%-45.54%) and intestinal fluid volume (46.27-73.88%) in a manner comparable to 5mg/kg each of atropine (58.20%) motility time inhibition and lope amide (76.12%) enterpooling inhibition in a watery nature and frequency of fecal droppings over 4hours, while lope amide gave 85.8% reduction on gastrointestinal motility and enterpooling. Its use as folklore remedies for the treatment of infection such as diarrhea was justified by this finding (Gideon *et al.,* 2013*).*

1.4.9.8 Anti-hepatotoxic activity

The ability of *D.guineense* pulp phenolic extract to protect against aflatoxin B1-induced hepatotoxicity and oxidative stress was investigated in rats from this study. Results from this study showed the aflatoxin B1 mediated elevation in the concentration of oxidative stress bio-markers, malondialdehyde, conjugated diene, lipids hydroperoxides, protein carbonyl, and

percentage DNA fragmentation were significantly lowered by *D.guineense* phenolic extract ($p<0.05$). Likewise, aflatoxin B1 mediated decreases in the activities of reactive oxygen species detoxifying enzymes (superoxide dismutase, catalase, gluthathione peroxidase, gluthathione reductase and glucose 6 phosphate dehydrogenase) was significantly attenuated. *D.guineense* phenolic extract elicited reactive oxygen scavenging and detoxification potential as well as the capability to prevent lipid per oxidation, protein oxidation and DNA fragmentation were suggested in the overall in vivo effects (Abdulwasiu *et al.,* 2014)

1.4.9.9 Anti-vibrio activity

Akinpelu *et al.,* (2011) carried out the determination of anti-vibrio activity of the leaf extract of *D.guineense* on 18 strains of vibrio. It was found to possess bioactivity against 14 out of 18 environmental strains of vibrio species tested at a final concentration of 20mg/ml. Standard antibiotics used ampicilin, which inhibited the growth of 15 out of 18 tested strains of the vibrio species. There was a range between 12 and 20mm of the exhibition by the extract against the tested isolates of the zones of inhibition. Zones of inhibition exhibited by ampicilin against the tested isolates ranged between 7 and 40mm. There was a minimum inhibitory concentration ranging between 0.313 to 5.0mg/ml against vibrio isolates of *D.guineense* leaf extract while the minimum bactericidal concentration exhibited ranged between 0.625 and 10mg/ml by the leaf extract.

1.4.9.10 Anti-plasmodial activity

For this study, twenty volunteer adults infected with malaria parasites were selected. Anti-malaria drug (artesunate) and the plant extract (leaves) (5mg two times daily) were administered separately in some and then co-administered in others for 3 days. After 3 days, the studies of anti-plasmodial effects alone showed only moderate clearance of malaria parasites. Also, after 3 days of treatment, *D.guineense* extract alone showed a moderate anti-plasmodial effect while co-administration of *D.guineense* and artesunate showed that the combination cleared the malaria parasites, suggesting that *D.guineense* has a synergic anti-plasmodial effect (Adumanya *et al.,* 2013). 85 medicinal plants with *Dialium guineense* included were investigated for their potency as anti-malaria in other study. The malaria parasite responsible for the illness, *plasmodium falciparum* was found to be inhibited by *D.guineense* (Bero *et al.,* 2009).

1.4.9.11 Molluscicidal activity

Molluscicidal effect of the fruit and leaves of *D.guineense* have been reported. This was found to be due to glycosides of the triterpenoid oleanic acid. From the fruit three glycosides were isolated and a fourth from the leaves. *D.guineense* is a good candidate for readily available molluscicide in Nigeria villages because of the amount of total saponins (Odukoya *et al.,* 1996).

1.4.10 Mineral composition of *dialium guineense*

1.4.10.1 Fruit

It was revealed that the high performance liquid chromatography (HPLC) analysis of *D.guineense* fruits has high glucose and fructose content (90.8% of total soluble sugar)/ total quantity of proteins (5.3%) containing all essential amino acids, lipids (3.1%) and essential

vitamins such as ascorbic acid, B-carotene and tocopherol in trace were found to be relatively low by comparison according to Nicholas *et al.;*(2014). The mineral composition includes phosphorus, potassium, zinc, calcium, manganese. It is also a potential source of iron(4.8-8.4mg/100g) and would contribute towards meeting the recommended daily allowances of these micronutrients in combating malnutrition especially in sub-saharan Africa (Nicholas *et al.,* 2014)

1.4.10.2 Seed and pulp

This was done according to method described by Association of Official Analytical Chemist (AOAC) and carried out in duplicate. From whole seed and pulp, values for the approximate analysis were obtained, these values moisture (10.135 and 10.53%), dry matter (90.15% and 88.40%), ash (2.55% and 12.50%), organic matter (12.60% and 41.55%), crude fat (35.33% and 5.34%), crude fiber (13.52% and 1.05%), carbohydrate (943.90% an d 58.65%), protein (17.44% and 3.94%), and total nitrogen free extract (2.79% and 0.65%) respectively. The proximate mineral composition was: magnesium (0.16mg/I and 0.40mg/I), sodium (2.42md/I and 2.88mg/I), iron (0.91mg/I and 1.43mg/I), calcium (0.54mg/I and 0.35mg/I) and potassium (0.3mg/I and 1.21mg/I) (Folake *et al.;* 2013)

1.4.11 Nutritive value of d. Guineense fruit (Nicholas *et al.; 2014*)

CONSTITUENTS	**D. GUINEENSE FRUIT**
Carbohydrates	58.65%
Protein	3.94%
Crude fat	5.34%
Iron	14.3mg/100g

Magnesium	4.0mg/100g
Sodium	28.8mg/100g
Calcium	3.5mg/100g
Potassium	12.1mg/100g
Moisture	10.53%
Ash	12.50%
Crude fiber	1.05%

1.4.12 Phytochemistry of *Dialium guineense*

Crude extract of stem bark revealed the presence of bioactive compounds comprising cardiac glycosides, tannins, phlobtannins, saponins, terpenoids, resins, steroids, triterpenes, alkaloids, flavonoids, reducing sugars and carbohydrates from Gideon and Raphael (2013) findings, while phytochemicals identified in the leaf extract are tannins, alkaloids, flavonoids, saponins, steroids and cardiac glycosides (David *et al., 2011;* Ogu and Amiebenemo, 2012). Standard procedures described by Harbone (1984) and Trease and Evans (1984) was used for phytochemical screening. Most of the effects observed with extracts of *D.guineense* may be attributed to the constituent compounds of phenols which play important roles in health in addition to enhancing antimicrobial activity in this plant. *D.guineense* has medicinal value that lies in these chemical substances that produce a definite physiological action in human body. Tannins, flavonoids, saponins, cardiac glucosides among others are phenolic compounds. They are known to be biologically active and their presence has been reported for several activities like antibacterial(Orji *et al.;* 2012), molluscicidial (Odukoya *et al.;*1996),anti-plasmodial (Bero *et al.;*2009, Adumanya *et al.;* 2013), anti-microbial (Gideon *et al.;* 2012), anti-vibrio(Akinpelu *et*

al.; 2011), analgesic (Ezeja *et al.;* 2011), anti-hepatotoxic (Abdulwasiu *et al.;*2014), anti-ulcer(Balogun *et al.;*2013), anti-microbial(Gideon *et al.;* 2013), anti-hemorrhoid(Odukoya *et al.;* 2009), oral care (Okwu and Okeke, 2003). The active ingredients in the plant that could be responsible for these activities are not yet known.

1.5 Metformin

Since the approval in the United Kingdom in 1958 and in United States in 1995, metformin has become one of the most widely used drugs in the treatment of type 2 diabetes mellitus (T2DM) with doses ranging from 500 to 2500mg/day. According to the American Diabetes Association/European Association for study of diabetes guidelines, metformin is the first-line therapy for patients with T2DM (Inzucchi *et al.,* 2012). It works by decreasing intestinal glucose absorption, improving peripheral glucose uptake, in reduction of blood glucose concentrations without causing over hypoglycemia (Grzybowska *et al.,* 2011). Additionally, with the activation of AMP-activated protein kinase(AMPK), metformin can inhibit gluconeogenesis (Greten and Matthaei, 1991). In the regulation of energy metabolism, AMPK is an important player which plays a key role in diabetes and related metabolic diseases. It is demonstrated that AMPK is required for maintaining glucose homeostasis (Zhang *et al.,* 2009). In diabetic patients with liver and kidney dysfunction (Salpeter*.,* 2006), metformin has few adverse effects, the most common adverse effect being gastrointestinal symptoms (incidence rate 20%-30%) including nausea and vomiting (Diabetes care, 2012), and the most serious adverse effect being lactic acidosis (incidence rate 1/30000).

Numerous studies concerning other potential indications have emerged since metformin's worldwide spread for over 50 years, which showed that metformin can also be used as an

anticancer agent (Gandini*.,* 2014), antiaging agent, (Bannister *et al.,* 2014) a cardiovascular protective agent (Hong J *et al.,* 2013), a neuroprotective agent (Cheng *et al.,* 2014), or an optional drug for polycystic ovary syndrome (PCOS) (Patel R. and Shah G, 2017).

1.6 Streptozotocin (STZ)

A medication that causes diabetes permanently is streptozotocin (STZ). It is produced by a strain of the gram-positive bacterium Streptomyces achromogenes, which has a wide range of antibacterial activities. Streptozotocin is a unique aminoglycoside with a nitrosoamino group that was first identified as an antibiotic in 1959 and is currently marketed as a generic medication. The metabolite can function as a nitric oxide (NO) donor because of the nitrosoamino group. The body uses nitric oxide as a vital messenger molecule in a variety of physiological and pathological activities. Streptozotocin (STZ) has four crucial biological qualities that are demonstrated by its antibacterial, -cell (beta)-cytotoxic, oncolytic, and oncogenic activities. It is frequently used to induce diabetes in mouse models by inhibiting -cell O-GlcNAcase. Antineoplastic antibiotic, this medication is mostly used to treat pancreatic (islet cell) cancers. Malignant insulinomas are treated with it. Because of the unique toxicity it has in relation to pancreatic beta-cells, streptozotocin is now used mostly as an exploratory medication in diabetes research. Streptozotocin is transported into the cell through the low affinity glucose transporter GLUT2 of -cells, which results in DNA alkylation and irreversible necrosis of cells. Streptozotocin inhibits DNA synthesis in both human and bacterial cells. Both insulin-dependent (IDDM) and non-insulin-dependent diabetic mellitus are often induced with streptozotocin (NIDDM). The antibiotic and anticancer drug streptozotocin causes diabetes mellitus in

pancreatic beta-cells by reducing nicotinamide adenine dinucleotide (NAD+) in vivo (Busineni et al., 2015).

1.6.1 Structural features of streptozotocin

Using a chromatographic approach, streptozotocin (2-deoxy-2-[3-methyl-3-nitrosourea] 1-D-glucopyranose) may be distinguished into two anomeric forms, and (HPLC). It appears as a crystalline powder that is light yellow or off-white. The chemical formula for streptozotocin is C8H15N3O7, and it has a molecular weight of 265 g/mol. The chemical structure of streptozotocin is identical to that of 2-deoxy-D-glucose with the exception that an N-methyl-N-nitrosourea group, which is the cytotoxic component of STZ in harming beta cells, has been substituted at position C2. A glucose molecule and a methyl group are connected to one end of the glucosamine nitrosourea complex known as streptozotocin (Busineni et al., 2015).

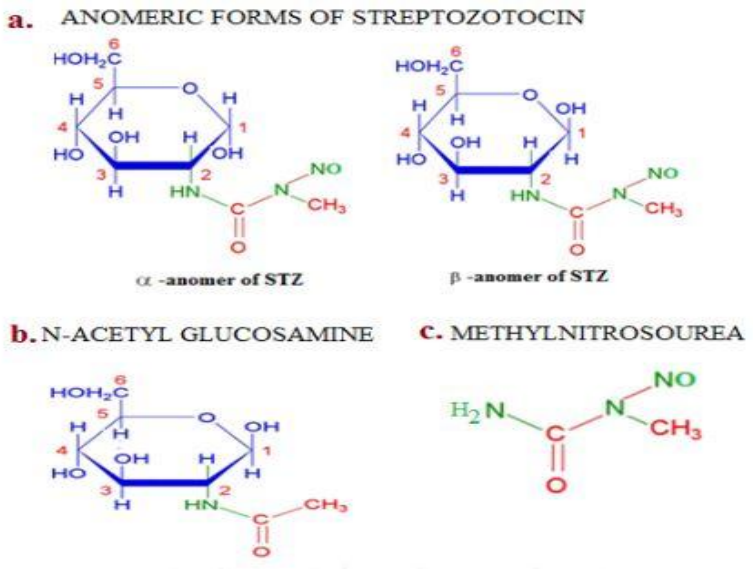


Figure 1.1: Chemical structure of STZ (Busineni *et al*., 2015)**.** A schematic diagram representing - a) α and β Anomeric forms of STZb) structural analog of STZ - N-acetyl glucosamine c) cytotoxic moiety of STZ- N-methyl-Nnitrosourea

1.6.2 Administration and dose schedule of streptozotocin

There have been numerous reports of using different dose regimens and administration methods to cause diabetes in rats. Streptozotocin is most delivered by one of two routes, intraperitoneal (IP) or intravenous (IV), although other methods including subcutaneous, intracardiac, and intramuscular delivery have been used in rodents. Even though intraperitoneal injection is rapid and simple, particularly for trials requiring several doses of the medication, unintentional drug transport into the colon or subdermal region may raise morbidity or lessen the diabetogenic impact. Additionally, other studies have reported that IV administration of streptozotocin produces a more stable and reproducible model of diabetes than IP administration; the two most common protocols of dose schedule are intraperitoneal injection of a single high dose or multiple low doses. Streptozotocin is commonly administered as a single intravenous dosage to adult rats to induce IDDM. However, greater doses are also employed. Streptozotocin is similarly effective when administered intraperitoneally at a similar or greater dose; however, a single dose of less than 40 mg/kg body weight may not be as effective (Businenі et al., 2015).

1.7 Oxidative stress

Oxidative stress is an imbalance between oxidants and antioxidants in the cell or body. Normal processes in the body generate oxidants, for instance. Superoxide is formed in a reaction between molecular oxygen and electrons that leak out in the electron transport chain (ETC). Reactive oxygen species such as superoxide, hydrogen peroxide and free hydrogen radicals are also generated from macrophages in other to eliminate bacteria by the respiratory burst. Oxidative stress generated can lead to oxidative damage of lipid, proteins and DNA. Multiple ways have been developed by cells to remove reactive oxygen species and thus protect themselves against

their deleterious effects and this is done by employing antioxidants enzymes such as Catalase, glutathione peroxidase and superoxide dismutase which help to scavenge these radicals by terminating the chain reaction that result to oxidative damage of lipids, proteins and DNA. The pentose phosphate pathway plays a crucial role in preventing oxidative damage by producing a ready supply of NADPH. NADPH serves as an antioxidant enzymes cofactor and helps to convert glutathione to its reduced form.

1.7.1 Reactive oxygen species (ROS) and oxidative damage

Oxygen is a basic element for life to carry out basic biological functions such as catabolism of proteins, carbohydrate and lipids in order to generate energy (ATP) for growth and other activities (Valko *et al*., 2007). It has also been discovered that oxygen has a role in toxic agent for living tissues. In the synthesis of various types of reactive oxygen species, oxygen is used though not hazardous by itself (Valko *et al.,* 2007)

1.7.2 Reactive oxygen species and free radicals

Reactive oxygen species or an oxidant is a collective term that includes all reactive forms of oxygen, including both radical and non-radical species that participate in the initiation and/or propagation of chain reaction. ROS play beneficial or even crucial roles as regulatory mediators in signaling or defense processes, including the erytropoietin production, promotion of apoptosis, angiogenesis, endothelium-dependent vasorelaxation, and destruction of bacteria and other foreign substances by macrophages at physiological levels (Valko *et al.,* 2007). In both aging and a wide spectrum of human diseases including inflammatory disorders such as hypertension, diabetes and cardiovascular diseases, a growing body of evidence implicates oxidative stress

(Cui *et al.,* 2004, Sedelnikova *et al.,* 2010). During cellular metabolism, ROS may be produced in a regulated manner in the form of free radicals, but in an unregulated manner they arise by metabolic dysfunction and by exogenous stresses (Valko *et al.,* 2007). Thus free radicals represent a class of highly reactive intermediate chemical entities whose reactivity is derived from the presence of unpaired electrons in their structure, and for a very brief period of time they exist independently (Halliwell, 2001).

1.7.3 Types and sources of ROS

Normal metabolic processes in living system produce a number of free radicals or ROS. These are form by either two ways by electron transfer reactions: (1) enzymatic reaction involving xanthine oxidase (XO), NADPH oxidases and lipoxygenases; (2) non-enzymatic sequence of reactions such as the catalytic action of free transition metals (for example iron and copper), toxic actions of some chemicals for instance doxorubicin, primaquine, attacks of electrons leaked from the mitochondrial electron transport chain and effect of radiation including UV light and radon (Ra) gas. Free and non-free radical species make up different types of reactive species generated in biological system (Bakonyi and Radak, 2004; Cui *et al.,* 2004). Some of these species are much less reactive than others, e.g O2- and NO* which react directly with molecules in the human body, while OH* react with anything and when generated in vivo, reacts at its site of formation. The end products of free radical attack on fatty acid chains and lipid molecules are alkyl, alkoxyl, alkperoxyl radicals and lipid peroxide (R*, RO*, ROO* AND LOOH). Superoxide anion (O2*-) though the reduced form of oxygen plays an important role in the formation of powerful radicals like H2O2 and nitric oxide radicals while itself is not damaging and also for transition metals it also acts as an oxidant or reductant (Kumar, 2011). In cells at

mitochondrial, endoplasmic reticulum, and cell cytoplasm membranes, superoxide radical production takes place. Two enzymatic sites have been fully identified in the mitochondrial in addition to other enzymes of the electron transfer chain, as a major source for one-electron reduction of oxygen, ubiquinone-cytochrome C reductase involve in auto-oxidation of ubisemiquinone, NADH dehydrogenase and semi-flavin cofactor (Halliwell, 2001). The oxy-complex of cytochrome P450 and action of NADH cytochrome P450 reductase produce O2*- inside the membrane of endoplasmic reticulum (Henderson and Chappel, 1996). Through the enzyme electron transfer chain reaction, NADPH oxidase is involved in the generation of O2*- radicals by the transfer of one electron to molecular oxygen (Pithon-Curi *et al.,* 2002).

In reperfused tissues, it has been shown from studies (Pithon-Curi *et al.,* 2012) that Xanthine oxidase is responsible for a significant source of O2*-. Molecular oxygen is used by xanthine oxidase as an acceptor and produce superoxide anion.

1.7.4 Adverse effect of ROS

Free radicals can easily interact with various biomolecules inclusive of proteins, lipids, carbohydrates and DNA because of high reactivity. Following reaction with biomolecules, ROS lead to local injury and eventual organ dysfunction. Aging and related degenerative processes are also accelerated. In pathogenesis of various clinical conditions involving almost all organ system ROS are also involved

1.7.5 Effects of ROS on proteins, lipids and DNA

The presence of polyunsaturated fatty acids (PUFA) leads to the vulnerability of biological membranes to peroxidation. The C-H bond of the adjoining carbon atom (allylic carbons) is weaken by the presence of double bonds in PUFA and facilitates the hydrogen abstraction step, this in turn initiates peroxidation reactions (John and Fisher, 1994). Varieties of PUFA are contained in cell membranes such as arachidonic, linoleic and linolenic acids, principally in the form of esters with triglycerides, phospholipids or with cholesterol. Multiple fatty acid side chains are converted into lipid peroxides by an attack of one reactive free radical on PUFA molecule, which makes the membrane leaky and eventually cause breakdown of the membrane (Cheeseman and Slater, 1993; Schafer *et al.,* 2000). Nucleic acids and proteins are generally less susceptible to free radical attack than PUFAs and hence have less possibility part of involvement in the progression of chain reactions. This happens if radicals are allowed to accumulate or if the damage is focused on a particular site of protein (Standtman and Oliver, 1991; Leeuwenburgd *et al.,* 1998).

In living cells, deoxyribonucleic acid is a sensitive target for free radicals-mediated damage. Specific sites of DNA can be damage by free radical leading to rupturing of strands, or it might delay the repair before replication occurs leading to mutation (Cheeseman and Slater, 1993). ROS may be involved in cell death or sub-lethal injuries such as chromosomal aberrations, carcinogenesis and mutations by damaging DNA and DNA repair processes. These studies were made by Van Rensburg *et al.,* 1992; Aust and Eveleigh, 1999.

1.7.6 Mechanism of action of ROS

Initiation, propagation and termination are three processes involve in oxidative process of free radical mediated reactions. Initiation starts with the abstraction of hydrogen atom to form biomolecule. For exsmple, fatty acid (LH) can be converted into radicals. PUFAs are oxidized by free radicals such as hydroxyl (OH*), alkperoxyl (ROO*), and alkoxyl (RO*). Peroxyl radicals (LOO*) that propagate the reaction by initiating a new chain of oxidation with the formation of lipid peroxide (LOOH*) is generated by extremely rapid addition of oxygen to the fatty acid radicals. This chain reaction continues till its interrupted by antioxidant through scavenging the radicals in the termination step (John and Fisher, 1994; Leeuwenburgh *et al.,* 1998).

1.8 Antioxidant enzymes

Cells protect themselves against their deleterious effects by developing multiple ways to remove oxygen species. Nature has created antioxidant defense system comprising of a group of compounds and enzymes to remove free radicals before causing any tissue damage in other to defend against the destructive action of ROS.

1.8.1 Types of antioxidants

Antioxidants are categorized into two which are endogenous and exogenous antioxidants. Our body produces endogenous antioxidants, while exogenous are derived from food source which are obtained naturally or produced synthetically. On the basis of localization, these antioxidants can be classified into four groups: system antioxidants, circulating antioxidants, cytosol antioxidants and membrane antioxidants (Cemeli *et al*, 2009; Corneli, 2009).

1.8.2 Exogenous dietary antioxidants

Vitamins, polyphenols or even synthetic antioxidants are form in which dietary antioxidants exist. Good sources of exogenous antioxidants are vitamins such as vitamin C (a water soluble antioxidants) and E (a lipid soluble antioxidant) which can easily be obtained from dietary supplements. Both are powerful antioxidants although they dissolve in different sources. In the prevention of protein, lipid and DNA oxidation, vitamin C serves as electron donor while during lipid oxidation vitamin E prevents the production of ROS. Cardiovascular diseases, stroke and cancer are prevented by both (Kris-Etherton *et al.,* 2004; Padayatty *et al.,* 2003).

Apart from obtaining antioxidants from natural sources, in food industry synthetic antioxidants such as butylhydroxyanisole (BHA) and butylhydroxytoluene (BHT) are widely used. However, it has been shown that these synthetic antioxidants possess toxic effects (Anagnostopoulou *et al.,* 2006), volatile and easily decomposable at high temperature (Hinneburg *et al.,* 2006). Consumption of antioxidants from natural sources such as plants has become crucial due to these reasons.

1.8.3 Endogenous antioxidants

Endogenous antioxidants can either be hormone, enzymes or shock adsorber. In other to neutralize unstable oxidative species present as a result of cellular metabolic processes, the system naturally produces antioxidants enzymes which work as our first line of defense. Catalase, superoxide dismutase and glutathione peroxidase are the three most common among the cellular antioxidant enzymes. Conversion of toxic oxidative species into less or non-toxic end-products is the main activity of these enzymes. Depending on the ion couple to superoxide dismutase an enzyme found in the cytosol, it is divided into few types. In our body, the major superoxide

dismutases found are copper-zinc superoxide dismutase (Cu-Zn SOD) and manganese superoxide dismutase (Mn SOD). In the lysosomes and nucleus, the Cu-Zn SOD is present while in the mitochondria the Mn SOD is present. In conversion of oxidative molecules such as superoxide anions into oxygen and hydrogen peroxide, SOD works as an enzyme which helps in this conversion. Glutathione can be found in tissues such as kidney and liver. Lipid hydroperoxide is reduced to alcohol by glutathione peroxidase when it acts as a good antioxidant towards lipid peroxidation. It also reduces hydrogen peroxide to water and oxygen. Catalase is highly active in peroxisome and is present in most organs. It has the highest turnover value and therefore considered as most important endogenous antioxidant which catalyzes the reduction of hydrogen peroxide to water and oxygen. In a second, millions of hydrogen peroxide can be converted to water and oxygen by a molecule of Catalase (Cemeli *et al.,* 2009).

Hormones such as melatonin and oestrogen can also function as endogenous antioxidants outside enzymes. Unlike antioxidants enzymes, melatonin does not undergo redox reaction. As its free radical scavenging capability it captures up to 10 ROS by donating hydrogen atoms from its amine group. Regulation of antioxidants enzymes and cellular mRNA expression of these enzymes is another function of melatonin (Rogdriguez *et al.,* 2004). Oestrogen has a cardio protective effect. Level of interleukin-6 (IL-6) which is related to diseases such as cancer, atherosclerosis and diabetes is lowered by oestrogen. By reducing oxidative stress and inhibiting the formation of superoxide anions and hydrogen peroxide, it functions as an antioxidant.

Other proteins and molecules can also function as antioxidants just like enzymes and hormones. ROS can be scavenge by plasma proteins such as albumin, transferin, and lactoferrin. Albumin a major plasma protein acts as an antioxidant either through specific or non-specific binding. Albumin binds to the free copper found in physiological system in specific binding. Production

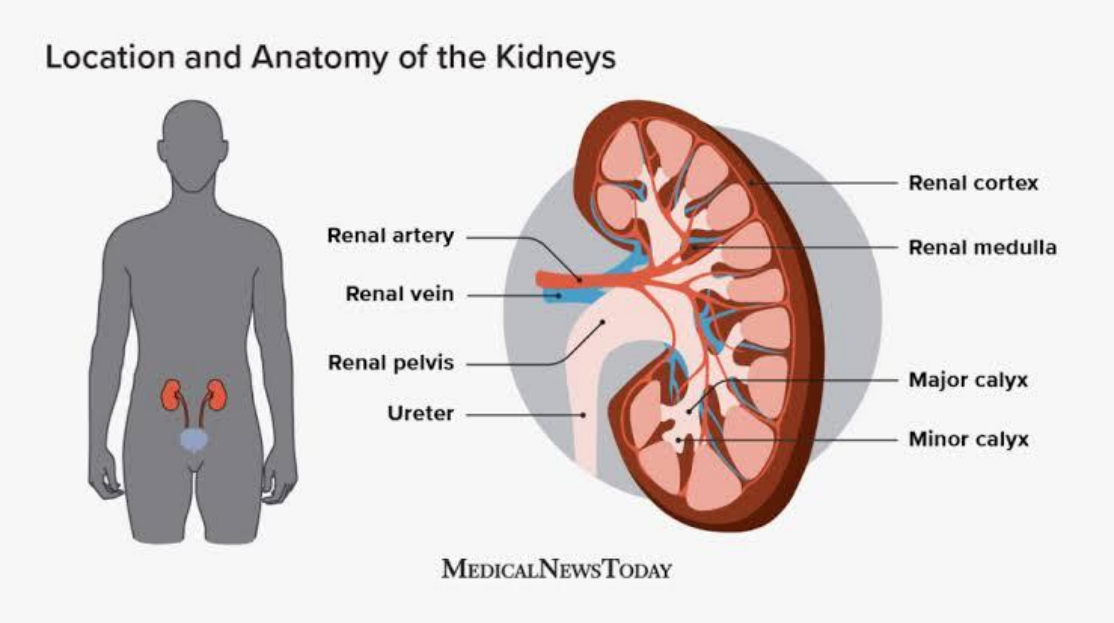
of free radicals is catalyzed by free copper. This reaction is stopped by the binding of albumin. In the presence of thiol groups albumin scavenges ROS in the non-specific binding (Kouoh *et al.,* 1999). A major iron transporting protein is transferring. It regulates cellular iron uptake by functioning as a cell surface receptor. Cellular iron homeostasis leads to cellular oxidative damage by ROS. Lactoferrin is an iron-binding protein that functions in protection from cell lysis. Lipid- peroxidation that causes breakage of membrane especially in erythrocytes is initiated by iron. Occurrence of erythrocytes membrane lipid peroxidation is reduced by the binding of lactoferrin to erythrocytes as unsaturated lactoferrin still possess iron-binding capacity. Uric acid in serum is also an important antioxidant other than proteins. Uric acid is shown to be effective in free radical scavenging even though high concentration of uric acids is associated with increased cardiovascular risk. It can effectively capture superoxide, hydroxyl and peroxynitrite radicals as it can prevent lipid peroxidation (Waring, 2002).

1.9 THE KIDNEY

The kidneys are two organs that resemble beans and measure about the size of a fist. A hard, fibrous renal capsule surrounds each kidney, supporting the delicate tissue inside by acting as support. The kidney is divided into several pyramid-shaped lobes, each of which has an inner renal medulla and an outside renal cortex. These portions are connected by nephrons. The glomerulus, a filter found in each of these nephrons, cleans the blood before it enters the kidney by the renal arteries and exits through the renal vein. Additionally, nephrons have tubules. The tubule's job is to remove waste products from the urine and restore essential components to the

circulation. Through the ureter, the kidneys expel urine (a tube that leads to the bladder). (Adam Bernstein, 2021).

Figure 1.2: Structure of the Kidney



The kidneys carry out a number of crucial tasks, such as filtering, excreting metabolic waste products (urea and ammonium), regulating electrolytes, acid-base balance, and fluid, and promoting the creation of red blood cells. By way of the renin-angiotensin-aldosterone pathway, they are engaged in limiting water absorption and preserving intravascular volume. Additionally, the kidneys reabsorb glucose and amino acids and regulate hormones by activating erythropoietin, calcitriol, and vitamin D. (Charbel. E.C. *et al*., 2017).

The three phases in the production of urine are filtration, reabsorption, and secretion. Unwanted compounds are removed from the circulation by the kidneys, which also create urine to do so. Urine is produced in three stages: glomerular filtration, reabsorption, and secretion. These procedures make sure that the body is solely expelled of waste and extra water.

1.9.1 The Glomerulus

The glomerulus filters blood as part of the process. There are around 1 million minuscule nephrons in each kidney. Each nephron has a glomerulus, which is where blood is filtered. The glomerular capsule (also known as Bowman's capsule) surrounds the capillary network that makes up the glomerulus. Blood pressure forces water and other solutes from the capillaries into the capsule through a filtering membrane when the blood passes through the glomerulus. The process of urine production starts with this glomerular filtration Blood pressure forces fluid from capillaries into the glomerulus and passes through a specific layer of cells into the glomerular capsule. The filtration barrier in this layer prevents blood cells and big proteins from passing, but it enables water and other tiny solutes to flow. These substances continue to circulate in the blood. The filtrate, or fluid that has traversed the membrane, travels farther into the nephron from the glomerular capsule. In addition to trash, the filtrate also includes nutrients the body requires, such as glucose, amino acids, and smaller proteins. The filtrate enters the renal tubule, a duct in the nephron, after leaving the glomerulus. The required materials, along with some water, are reabsorbed into nearby capillaries via the tube wall as it travels. The second phase in the production of urine is the reabsorption of essential elements from the filtrate.

The renal tubule is where nutrients and water from the filtrate that the glomerulus absorbed are reabsorbed into capillaries. Hydrogen ions and waste ions both move simultaneously from the

capillaries into the renal tubule. Secretion is the term for this action. Urine is created when the released ions interact with the leftover filtrate. The nephron tubule releases the urine into a collecting duct. It exits the kidney via the renal pelvis, travels via the ureter, and then descends to the bladder. The percentage composition of salts, ammonia, urea, water and other components of urine.

1.9.2 The Nephron

First, the renal corpuscle and renal tubule are the two primary components of the nephron. Let's start with the renal corpuscle's structures. It consists of the renal capsule (also known as Bowman's capsule), which envelops the glomerulus, and the glomerulus, which is a tiny knot of capillaries. The filtering procedure takes place here. High pressure blood enters the glomerulus, and the glomerulus' walls are more porous than most capillaries, which makes for an ideal environment for filtering. In essence, plasma is expelled from the circulation at the glomerulus and then caught by Bowman's capsule, which then directs that fluid into the renal tubule. Filtrate is the term used to describe the fluid that was removed from the blood. Water, electrolytes, nutrients, and waste products are all present in this filtrate, just like they are in plasma. Red blood cells, white blood cells, and proteins shouldn't be present in the filtrate since they can't pass through the gaps in the glomerulus' walls to be filtered out. Our kidneys generate around 180 liters of filtrate every day. We must now discuss what happens in the following regions of the nephron to alter that filtrate and transform it into urine because we most certainly do not create 180 liters of pee every day.

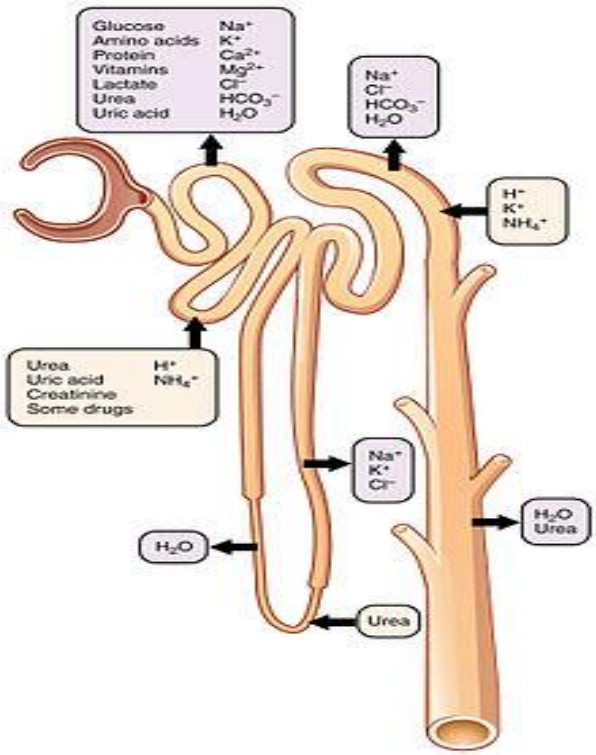


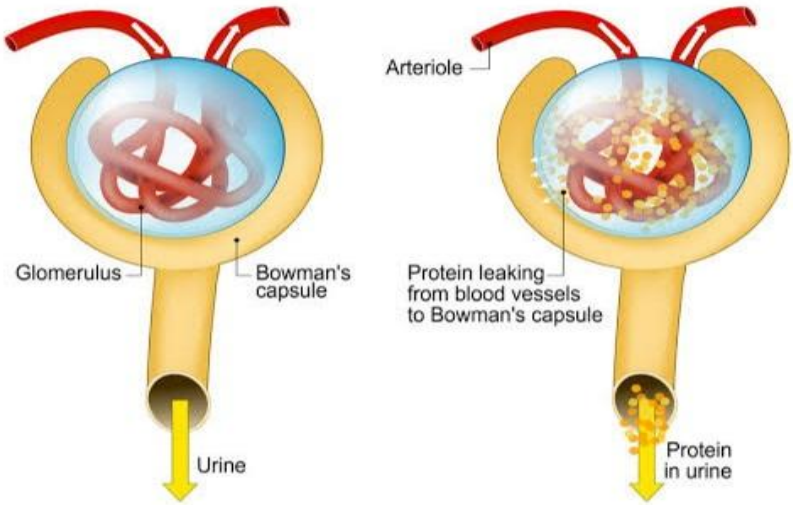
Figure 1.3: Structure of the Nephron

1.9.3 Diabetes Mellitus and kidney Damage

Nephrons, which are millions of these small filters, make up each kidney. Diabetes-related elevated blood sugar over time can harm kidney blood vessels and nephrons, impairing their ability to function as they should. Any kidney injury would stop all of the functions. Uncontrolled hyperglycemia is the main cause of kidney impairment. High blood pressure is a common complication of diabetes, and it can also harm kidneys. Diabetes that is not properly managed over time might harm blood vessel clusters in your kidneys that filter waste from your blood. High blood pressure and renal damage may result from this by putting more strain on the kidneys' sensitive filtration mechanism. High blood pressure can exacerbate existing kidney disease.

A syndrome known as diabetic nephropathy (DN), also known as diabetic kidney disease, is characterized by the loss of glomerular filtration rate (GFR) in diabetics as well as the presence of pathological levels of urine albumin excretion. Type 1 diabetes (autoimmune -cell destruction and absolute insulin deficiency), type 2 diabetes (relative insulin deficiency and resistance), and other types of diabetes can all be classified (eg, pancreatic disease). Albumin, which was previously utilized to pass past the filters, can seep into the urine as a result of damaged kidneys. The symptoms of advanced kidney disease include edema in the hands, feet, and ankles, frothy, bubbly urine (from albumin in the urine), blood in the urine, shortness of breath, nausea, and on-going weariness.

Figure 1.4: Diabetes Nephropathy



1.9.4 Screening for Diabetic Nephropathies

The majority of screening recommendations call for using spot urine samples from either first morning (recommended) or random specimens to measure the albumin/creatinine ratio (ACR; normal, 30 mg/g creatinine). For consistency, an aberrant result is repeated once or twice over a few months. In order to stage chronic kidney disease, this is combined with an evaluation of renal function using the Modification of Diet in Renal Disease or Chronic Kidney Disease Epidemiology Collaboration formulae for estimated GFR (eGFR) (CKD). Screening starts when type 2 diabetes is diagnosed and typically takes place 5 years after type 1 diabetes first manifests. It is also possible to use timed samples, which will average out diurnal changes in albumin excretion (normal, 20 g/minute).

CHAPTER TWO

2.0 MATERIALS AND METHODS

2.1 Materials

- Clean plain containers
- Spatula
- Micro pipette (Labline stock centre)
- Pasteur pipette (Avon Healthcare)
- Water bath(HH-W)
- Centrifuge (80.2 Techmel and techmel USA)
- Spectrophotometer (search Tech.721G)
- Refrigerator
- Distilled water
- Normal saline (Sino pharmaceutical)
- Analytical weighing balance (OHAUS Corp pine , USA)
- Test tubes racks
- Test tubes (Pyrex , England)
- Pipette (Pyrex , England) o Beaker (Pyrex ,England)
- Porcelain crucible (Pyrex , England)
- 10% Formal saline
- Large bowls for extracts
- Picric acid
- Wooden cages

- Ceramic plate
- Gavage
- Measuring cylinder (Pyrex)
- Gloves
- Scissors
- Dissecting kit (Atico Medical Pvt.Ltd)
- Cotton wool
- Syringes(2ml and 5ml, Atico Medical Pvt.Ltd)
- Lithium Heparin container
- Accucheck Glucometer
- Glucose strips
- Drug sachet
- Chloroform
- Ice
- Carbon paper
- Foil paper

2.2 Chemicals

- TCA
- TBA
- HCL
- Pyrogallol
- Phosphate buffered H_2O_2

- Hydrogen tetraoxosulphate vi acid (H_2SO_4)
- $KMNO_4$
- ELMA'S reagent
- Phosphate buffer
- Diazo reagent

2.3 Plant Sample Collection and Identification

Fresh velvet tamarind (*Dialium guineense*) stem bark was obtained from Benin City , Edo State, Nigeria. Specimen of the stem bark was identified by Dr. Aigbokhan E.l., of the Department Plant Biology and Biotechnology

2.4 Experimental Animals

Male adult Wistar rats weighing between 150-180g were obtained from the Department of Biochemistry, University of Benin, Benin City. They were handled with the international, national and institutional guidelines for care and use of laboratory animals in Biochemical research as promulgated by that Canadian Council of Animal Care (1984).

They were housed in cages and allowed to acclimatize under stands laboratory conditions for fifty two (52) days. During this period, the rats were given access to rat feed and water ad libitum. After acclimatization, some rats were transferred into metabolic cages. The rats were fed with standard rat feed, water and kept in a cage at the animal house in the department of Biochemistry throughout the period of the experiment. They were kept in well ventilated room at ambient temperature of 28+2^{o}C under twelve (12) hour light/dark cycle. Generally, the study was conducted in accordance with the recommendations from the declaration of Helsinki (Adopted by 8th World Medical Assembly, Helsinki, 1964; revised by 29th Medical Assembly, Tokyo, 1975; Venice, 1983 and Hong Kong, 1989) on guiding principles in care and use of animals.

The animals were marked at different parts of their bodies using picric acid so that they can easily be identified during administration. At the onset of the administration, the rats were randomized into 5 groups of 4 rats each. Group 1 served as normal control group, Group 2 as diabetic control group, Group 3 administered standard drug metformin, 4 and 5 as the treatment groups (administered ethanol extract) 100mg/body weight and 200mg/body weight respectively.

2.5 Measurement of Body Weights

The weight of each rat was measured weekly after acclimatization. This was done to ascertain the effect of the various feed constituents on their body weights.

2.6 Induction of Diabetes

Diabetes mellitus was induced by injecting 50mg of freshly prepared streptozotocin (STZ) which was diluted in 10 ml of normal saline after a 12hour fast.

Four days after the rats with blood glucose concentration above 190mg/dL were considered diabetic; this was done by measuring the blood sugar level of the rats induced using Accucheck glucose test kit.

After stable diabetic status was established, the rats were treated with the ethanol extract of *Dialium guineense* for 14 days.

2.7 Administration of Plant Extract

The rats in group 4 and 5 were orally administered 200mg/kg body weight and 300mg/kg body weight respectively of the methanol fraction of ethanol extract of *Dialium guineense* stem bark mixed in distilled water for 14 days.

2.8 Reagents

Distilled water	Nomangbon Pharmaceuticals
Formaldehyde	BDH chemicals Ltd, Pools England
Total Protein	Randox Laboratories Ltd, U.K
Streptozotocin (STZ)	Pyrex

2.9 Experimental Design

The Albino wister rats were shared into group of five with each groups having four rats each. These rats were grouped into five different cages. Group 4 and 5 was treated with ethanol extract

of stem bark of *Dialium guineense*. Group 3 was treated with the standard drug Metformin while group 2 is the diabetic control and group 1 is the normal control with rats that were not induced with STZ and thus didn't have diabetes.

Group 1

This serves as the normal control. STZ was not induced and administration was not given.

Group 2

This serves as the diabetic control. STZ was induced and administration was not given.

Group 3

This was the standard drug metformin group; 50mg of metformin per Kg body weight was administered to this group.

Group 4

200mg/kg body weight of ethanol extract of *Dialium guineense* was administered to this group.

Group 5

300mg/kg body weight of ethanol extract of *Dialium guineense* was administered to this group.

2.10 Blood Sample Collection

This was done at the end of every week by collection of blood through the tail of each rats used in the experiment. The glucose level of the rats was determined using a glucometer, and this was done every week.

2.11 Sacrifice of Animals

After 14 days of administration, the rats were sacrificed. Cotton wool was soaked in chloroform and placed in a transparent bucket that has a lid. The chloroform was used as a mild anaesthetic. The rats which were placed in the bucket were brought out and an incision was cut through the rats with dissecting tools. Blood was collected from the abdominal aorta and the heart using 5ml syringe. The blood was collected and put into a labeled sample container. The bottles containing anticoagulants were obtained from the whole blood after centrifugation for 10 minutes. The serum and plasma were stored in a laboratory refrigerator at a temperature of 4°C for preservation.

2.12 Antioxidant Assays

2.12.1 Determination of Concentration of Plasma Total Protein

Principle

Cupric ions, in an alkaline medium, interact with protein peptide bonds resulting in the formation of a coloured complex.

Assay Procedure

Biuret reagent (2.5 mL) was added to 0.05 mL of plasma and 0.05 mL of standard. The blank contained 2.5 mL of Biuret and 0.05 mL of distilled water. The solution in each tube was incubated for 10 min at 37 ^{o}C, and the absorbance was read at 546 nm against the reagent blank.

Calculations

- When measurements are taken at 546 nm, total protein concentration may be calculated as follows:

$$Total\ Protein\ (g/L) = 190 \times A_{Sample}$$

$$Total\ Protein\ (g/dL) = 19 \times A_{Sample}$$

- When using a standard

$$Total\ Protein\ Concentration = \frac{A_{Sample}}{A_{Standard}} \times Standard\ Conc.$$

Concentration of plasma globulin was calculated as shown in Equation 27:

Globulin Concentration = Total Protein – Albumin

2.12.2 Determination of Superoxide Dismutase (SOD) Activity

Principle

The activity of SOD was assessed based on the method of Misra and Fridovich (1972). Adrenaline auto-oxidizes rapidly in aqueous solution to adrenochrome whose concentration can be determined spectrophotometrically at 420 nm. The auto-oxidation depends on the presence of superoxide anions (O^{2-}). Superoxide dismutase (SOD) inhibits this auto-oxidation by catalyzing the breakdown of superoxide anions. The degree of inhibition is thus a measure of SOD activity. The amount of enzyme producing 50 % inhibition is defined as one unit of the enzyme activity.

Assay Procedure

Liver homogenate (0.2 mL) was added to 2.5 mL of 0.05 M carbonate buffer (p^H 10.2) and allowed to equilibrate. The reaction was initiated by the addition of 0.3 mL of freshly prepared 0.03 mM adrenaline as substrate. The solution was mixed by inversion. The reference tube contained 2.7 mL of carbonate buffer and 0.3 mL of adrenaline, while the blank contained 2.5 mL of carbonate buffer, 0.2 mL of distilled water and 0.3 mL of 0.03 mM adrenaline. The increase in absorbance at 420 nm due to the formation of adrenochrome was monitored every 30

sec for 120 sec. One unit of SOD activity was taken as the amount of SOD necessary to cause 50 % inhibition of the oxidation of adrenaline to adrenochrome within 120 sec.

Calculation

$$\%\ Inhibition = \frac{O.D_{test} - O.D_{reference}}{O.D_{test}} \times \frac{100}{1}$$

$$Enzyme\ Activity\ (units/mg\ protein) = \frac{\%\ inhibition}{50 \times Y}$$

Where Y = mg of protein in the volume of sample.

A unit of SOD activity was taken as the amount of SOD required to cause 50 % inhibition of the auto-oxidation of adrenaline to adrenochrome per minute.

2.12.3 Determination of MDA Concentration

The concentration of MDA was determined according to the method of Guttridge and Wilkins (1982), a modification of the procedure used by Hunter, *et al*., (1963). The principle that underlies this assay is that MDA – a product of lipid peroxidation when heated with thiobarbituric acid (TBA), in the presence of an acid, forms a pink or reddish complex that is measured spectrophotometrically at 532 nm. The table below clearly illustrates the procedure adopted in the determination of the level of malondialdehyde.

Assay Procedure

Aliquot of the liver homogenate was added to 3.0 mL of TCA – TBA – HCl reagent and mixed thoroughly by swirling. The solution was heated for 15 min in a boiling water bath. After cooling,

the flocculent precipitate was removed via centrifugation at 1000 g for 10 min. The absorbance of the clear supernatant was measured against a reference blank at 535 nm.

Calculation

$$\frac{O.D \times V_t \times 1000}{a \times V \times L \times Y}$$

where,

O.D = Absorbance of sample test at 535 nm

V_t = Total volume of the reaction mixture = 3.6 mL

a = Molar extinction coefficient of product = $1.56 \times 10^5\ M^{-1}cm^{-1}$

L = Light path = 1.0 cm

V = Volume of sample homogenate used = 0.6 mL

Y = mg of tissue in the sample used

The unit of MDA is moles/mg wet tissue

2.12.4 Determination of Catalase Activity

Principle

This is based on the method of Cohen, *et al.,* (1970). This estimation is based on the measurement of the rate of decomposition of hydrogen peroxide (H_2O_2), after the addition of the material containing the enzyme.

Catalase catalyses the reaction: $2H_2O_2 \longrightarrow 2H_2O + O_2$

The quantity of hydrogen peroxide decomposed is directly proportional to the concentration of the enzyme in the sample. The hydrogen peroxide produced in tissues is measured by reacting it with excess potassium permanganate ($KMNO_4$) and then measuring the residual $KMNO_4$ spectrophotometrically at 480 nm.

Assay Procedure

Liver homogenate (0.5 mL) was placed in ice – cold test tubes, the blank contained 0.5 mL distilled water. Cold phosphate-buffered H_2O_2 (30 mM, 5 mL) was added to both blank and sample tubes at fixed intervals, and were mixed by inversion. After 3 min, the reaction was stopped by rapid addition of 1 mL of 6 M H_2SO_4. The tubes were mixed thoroughly by inversion after which 7 mL of 0.01 M $KMNO_4$ was added. Absorbance was read at 480 nm within 3 min.

Calculation

The activity of catalase in each sample is calculated thus:

$$\frac{O.D/min \times Vt \times 1000}{M \times V \times L \times Y}$$

where,

O.D = Absorbance of sample test at 480 nm

Vt = Total volume of the reaction mixture = 13.5 mL

M = Molar extinction coefficient of H_2O_2 = $43.6M^{-1}\ cm^{-1}$

L = Light path = 1.0 cm

V = Volume of sample homogenate used = 0.5 mL

Y = mg of protein in tissue used

2.12.5 Determination of Glutathione Peroxidase Activity

Glutathione peroxidase (GPx) activity was measured according to the method described by Nyman (1959).

Principle

This is based on the oxidation of pyrogallol to purpuragallin by peroxidase, resulting to a deep brown colouration, which is read at 430 nm.

Procedure

To an aliquot of plasma (0.2 mL), 5 mL of phosphate-buffered H_2O_2, and 1.5 mL of pyrogallol were added. The reaction mixture was allowed to stand for 30 min at room temperature. A deep colour was formed, which was read at 430 nm.

Calculation

$$Enzyme\ Activity = \frac{OD/min \times Vt \times Df}{E \times Vs \times Y}$$

where OD = Absorbance of test

Vt = Total volume of reaction mixture

Df = Dilution factor

E = Molar extinction coefficient (12/M/cm)

Vs = Volume of sample

Y = mg of protein used

2.12.6 Determination of Plasma Concentration of Reduced Glutathione

The plasma concentration of reduced glutathione (GSH) was determined using the method described by Ellman (1959).

Reagents

5, 5[1]-dithiobis-2-nitrobenzoic acid (DTNB), sodium citrate, and trichloroacetic acid (TCA)

Procedure

To 1.0 mL of plasma, 2.5 mL of 10 % TCA was added and centrifuged at 3000 g for 10 min. Then, 1.0 mL of the supernatant was treated with 0.5 mL of Ellman's reagent (0.0189 % DTNB and 1 % sodium citrate) and 3.0 mL of 0.3 M phosphate buffer (pH 8.0). The yellow colour developed was read immediately at 412 nm and expressed as µM GSH/g plasma.

Calculation

$$Concentration\ of\ GSH = \frac{A_{test} \ x \ Conc.\ of\ Standard}{A_{standard}}$$

$$\%\ Glutathione\ Reduced = \frac{(A_0 - A_1) \ \times 100}{A_0}$$

where A_0 = Absorbance of reference sample

A_1 = Absorbance of sample

Redox Status = GSH/GSSG

2.12.7 Determination of Plasma Concentration of Nitric Oxide (NO)

Nitric oxide (NO) was assayed using the method described by Marcocci *et al*. (1994).

Principle

When sodium nitroprusside is dissolved in aqueous solution, NO is spontaneously generated from it at physiological pH (7.2), and interacts with oxygen to produce nitrite ions that can be estimated by the use of Greiss reagent. The absorbance of the pink colour formed is read at 540 nm.

Procedure

To 0.5 mL of plasma, 0.5 mL sodium nitroprusside prepared in 10 mM potassium phosphate buffer (pH 7.4) was added and incubated at 25 °C for 15 min. At the end of incubation, the absorbance was read, and the samples were allowed to react with 1.0 mL of Greiss reagent containing equal volumes of solutions A (solution of 2 % sulfanilamide and 4 % H_3PO_4) and B (0.2 mL of naphthylethylene diamine dihydrochloride). The absorbance of the chromophore formed during the diazotization of nitrite with sulfanilamide and subsequent coupling with naphthylethylenediamine was read at 540 nm. The concentration of NO was extrapolated from standard calibration curve for NO. The % NO scavenged was calculated as shown in Equation 55:

$$\% \text{ NO Scavenged} = \frac{A_0 - A_1}{A_0} \times 100)$$

A_0 = Absorbance before reaction with Greiss reagent

A_1 = Absorbance after reaction with Greiss reagent

CHAPTER THREE

3.0 RESULTS

3.1 Activities of Antioxidant Enzymes and Concentration of MDA, NO and GSH in Diabetic Rats Treated with Ethanol Extract of *D. guineense* Stem Bark

There was a significant ($p < 0.05$) increase in the concentration of renal total protein, MDA and NO sequel to the intraperitoneal induction of STZ to the male rats when compared with the normal control ($p < 0.05$). When the concentration of renal total protein, MDA and NO of the diabetic control group was compared to the extract-treated groups, it was observed that the elevated concentration was reduced in a dose dependent manner ($p < 0.05$). Furthermore, treatment of diabetic animals with metformin significantly reduced the concentrations of renal total protein, MDA and NO when compared to the diabetic control group ($p < 0.05$).

Activities of renal antioxidant enzymes (SOD, catalase, and GPx) were significantly reduced in the diabetic control group when compared to the normal control ($p < 0.05$). In the same manner, the concentration of GSH was significantly reduced in the group induced with diabetic but not treated when compared with the normal control group ($p < 0.05$). However, treatment with graded doses of the extract and metformin, significantly elevated the activities of the renal antioxidant enzymes when compared to the diabetic control group ($p < 0.05$). Furthermore, the concentration of renal GSH was elevated in response to treatment with the extract and metformin respectively when compared to the diabetic control group ($p < 0.05$).

Table 3.1: Effect of Methanol Fraction of Ethanol Extract of Dialium guineense (MEDG) Stem Bark on Renal Total Protein and Glutathione Level

Group	T.P (mg/ml)	GSH (µM)
Normal Control	38.65 ± 1.30	1.53 ± 0.09
Diabetic Control	57.90 ± 0.52[a]	0.36 ± 0.04[a]
Metformin	39.48 ± 0.75[b]	1.25 ± 0.08[b]
Extract (low dose)	51.55 ± 2.62	1.16 ± 0.04[b]
Extract (high dose)	41.73 ± 1.83[b]	1.27 ± 0.03[b]

Data are pancreatic total protein and GSH levels and are expressed as mean ± standard error of mean (SEM).

Table 3.2: Effect of MEDG on renal antioxidant indices in diabetic male Wistar rats

Group	Catalase (U/mg protein)	SOD (U/mg protein)	MDA (mole/mg protein)
Normal Control	14.62 ± 2.72	4.15 ± 0.15	10.05 ± 0.05
Diabetic Control	7.23 ± 0.80[a]	1.02 ± 0.07[a]	31.85 ± 1.65[a]
Metformin	12.90 ± 2.78[b]	3.97 ± 0.01[b]	13.05 ± 1.95[b]
Extract (low dose)	10.20 ± 5.39[b]	3.26 ± 0.04[b]	18.25 ± 4.05[b]
Extract (high dose)	15.21 ± 2.39[b]	3.98 ± 0.17[b]	16.45 ± 8.25[b]

Data are markers of oxidative stress and are expressed as mean ± SEM

Table 3.4: Effect of MEDG Stem Bark on Pancreatic NO Level

Group	NO Concentration (µmole/L)	GPx (U/mg protein)
Normal Control	10.63 ± 0.00	2.04 ± 0.06
Diabetic Control	30.64 ± 0.03[a]	0.14 ± 0.02[a]
Metformin	15.82 ± 0.03[b]	1.31 ± 0.01[b]
Extract (low dose)	20.76 ± 0.05[b]	1.42 ± 0.02[b]
Extract (high dose)	17.26 ± 0.04[b]	1.55 ± 0.04[b]

Data are pancreatic NO concentrations and GPx are expressed as mean ± SEM

Key: Extract (low dose) = 200mg/kg body weight of methanol fraction of ethanol extract of *Dialium guineense*; Extract (high dose) = 300mg/kg body weight of methanol fraction of ethanol extract of *Dialium guineense*; T.P = Total protein; GSH = Reduced glutathione; GPx = Glutathione peroxidase; SOD = Superoxide dismutase; MDA = Malondiadehyde; NO = Nitric

oxide. Values are represented as mean ± SEM. Values with superscript 'a' differ significantly from the normal control and 'b' differ significantly from the diabetic control ($p < 0.05$).

3.2 BLOOD GLUCOSE

Table 3.3 Body Weights and Blood Glucose Levels

Group	Weight Change (g)	% Weight Change	FBG (mg/dL)	Glycemic Change (mg/dL)	% Glycemic Change
Normal Control	-		-	-	-
Diabetic Control	-		> 800	-	-
Metformin	20.35	12.16	> 800	399.00	49.88
Extract (200 mg/kg bwt)	16.00	11.19	427.00	311.00	71.61
Extract (300 mg/kg bwt)	27.00	15.68	467.67	394.33	78.19

Data are weight and FBG parameters and are expressed as mean ± SEM (n = 5).

Group	Weight of Kidney (g)	Organ/Body Weight Ratio x 10^{-3}
Normal Control	0.73 ± 0.05	3.95 ± 0.41
Diabetic Control	0.44 ± 0.02	2.71 ± 0.17
Metformin	0.60 ± 0.30[b]	4.08 ± 0.22[b]
Extract (low dose)	0.49 ± 0.04[b]	2.92 ± 0.08[b]
Extract (high dose)	0.63 ± 0.03[b]	4.44 ± 0.11[b]

Values with superscript "a" are significantly different from the normal control group.

Values with superscript "b" are significantly different from the diabetic control group.

Induction of diabetes mellitus using STZ significantly increased the blood glucose concentrations of the rats, but it reduced the weight of rat kidney significantly ($p < 0.05$). However, treatment of the diabetic rats with the extract markedly reduced the FBG of rats, while increasing kidney weight as well as organ/body weight ratio ($p < 0.05$).

CHAPTER FOUR

4.0 DISCUSSION ND CONCLUSION

4. 1 Discussion

Medicinal plants are plants that contain substances that could be used for therapeutic purposes, or which are precursors for the synthesis of useful drugs (Abolaji *et al.,* 2007). A plant becomes medicinal only when its biological activity has been ethnobotanical reported or scientifically established (Elujoba, 1997). Traditional systems of medicine continue to be widely practiced on many accounts. As a result of population rise, inadequate supply of drugs, the prohibitive cost of treatments, side effects of several synthetic drugs, and development of resistance to currently used drugs for infectious diseases. There have been increased emphases on the use of plant materials as a source of medicine for a wide variety of human activities.

Plant extract contains bioactive compounds comprising cardiac glycosides, tannins, phlobatannins, saponins, terpenoids, resins, steroids, triterpenes, alkaloids, flavonoids, reducing sugars, and carbohydrates from Gideon and Raphael (2013). These components of the plant are generally known as secondary metabolites. Phenolic compounds are one of the major aromatic plant secondary metabolites which possess mainly free radical scavenging ability and also have several biological properties such as anti-apoptosis, anti-aging, anti-cancer, anti-inflammation, antioxidant, and antiatherosclerosis (Wei *et al.,* 2010) due to their antioxidant activities. It has been reported that the antioxidant activity of plant phenolics is due to their redox properties, hydrogen donor and singlet oxygen quencher (Banerjee and Bonde, 2011). These phytochemicals could therefore contribute to the overall antioxidant potential observed in the extracts. Plants rich in secondary metabolites, including phenolics, flavonoids and carotenoids,

have antioxidant activity due to their redox properties and chemical structures (Geetha *et al.,* 2003).

The kidney plays a significant role in regulating the electrolyte/fluid balance, pH buffer system, and the removal of by-products (Wei *et al*., 2010). Chemical and drug-induced nephrotoxicity is a leading cause of acute kidney injury which may be due to altered intra-glomerular hemodynamic, rhabdomyolysis, inflammation, microangiopathy, and tubular cell toxicity and are being recognized as the main causes of mortality and morbidity (Shahbazi *et al.,* 2012). Kidneys are highly vulnerable to damage caused by reactive oxygen species (ROSs) likely due to oxidative stress by polyunsaturated fatty acids in the composition of renal lipids (Ozbek, 2012).

Streptozotocin (STZ) has four crucial biological qualities that are demonstrated by its antibacterial, -cell (beta)-cytotoxic, oncolytic, and oncogenic activities. It is frequently used to induce diabetes in mouse models by inhibiting -cell O-GlcNAcase. Because of the unique toxicity it has in relation to pancreatic beta-cells, streptozotocin is now used mostly as an exploratory medication in diabetes research. Streptozotocin is transported into the cell through the low affinity glucose transporter GLUT2 of -cells, which results in DNA alkylation and irreversible necrosis of cells. Streptozotocin inhibits DNA synthesis in both human and bacterial cells.

This study also evaluated the antioxidant potential of *D. guineense*. It was shown that after induction of diabetes that the rate of oxidative stress increased which is evident among the diabetic control groups. The oxidative stress caused the activity of the anti-oxidant enzyme of the diabetic control group to decrease; such anti-oxidant enzyme includes superoxide dismutase, catalase, and glutathione peroxidase. Oxidative stress can lead to cell damage because of increased level of free radicals and most plants show their medicinal properties by having a high

anti-oxidant activity. The level of malondialdehyde which is a serum biomarker for oxidative stress was measured and it was very high among the diabetic control group. However, administration of the extract was able to reduce the malondialdehyde to the level comparable to the normal control group. In addition, the extract increased the activity of anti-oxidant enzymes. These established that the extract of *D. guineense* reduced oxidative stress and also served as a good source of anti-oxidants by increasing the activities of superoxide dismutase and glutathione peroxidase which scavenge for free radicals and stop peroxidation reactions. This result is in agreement with the report of Merghem *et al*. (2019) who reported the *in vivo* antioxidant activity of *Ruta montana* L. extract. Again, the result is consistent with the report of Suganya *et al*. (2016) where they reported that *Hemidesmus indicus* had high superoxide dismutase activity, glutathione peroxidase activity and also reduced malondialdehyde (the biomarker of oxidative stress). The result is also consistent with that reported by Fatmawaty *et al*. (2019) who reported that *Persea americana Mill* and *Piper crocatum Ruiz* and *Pav* leaf extracts have been successfully utilized as antioxidant agents *in vitro* and *in vivo*.

4.2 CONCLUSION

It can be concluded that the methanol fraction of ethanol extract of *Dailium guineense* possesses an ameliorative potential against STZ induced oxidative stress.

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APPENDICES

APPENDIX 1 (SOD)

Descriptives

VAR00002

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
1	3	66.2500	12.31107	7.10780	35.6676	96.8324	56.25	80.00
2	4	65.0000	6.12372	3.06186	55.2558	74.7442	57.50	70.00
3	2	77.5000	5.30330	3.75000	29.8517	125.1483	73.75	81.25
4	2	1.0812E2	9.72272	6.87500	20.7698	195.4802	101.25	115.00
5	2	85.6250	.88388	.62500	77.6836	93.5664	85.00	86.25
Total	13	77.0192	17.19631	4.76940	66.6276	87.4109	56.25	115.00

Multiple Comparisons

Dependent Variable:VAR00002

	(I) VAR00001	(J) VAR00001	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
LSD	1	2	1.25000	6.26950	.847	-13.2075	15.7075
		3	-11.25000	7.49349	.172	-28.5300	6.0300
		4	-41.87500*	7.49349	.001	-59.1550	-24.5950
		5	-19.37500*	7.49349	.032	-36.6550	-2.0950
	2	1	-1.25000	6.26950	.847	-15.7075	13.2075
		3	-12.50000	7.10895	.117	-28.8933	3.8933
		4	-43.12500*	7.10895	.000	-59.5183	-26.7317
		5	-20.62500*	7.10895	.020	-37.0183	-4.2317
	3	1	11.25000	7.49349	.172	-6.0300	28.5300
		2	12.50000	7.10895	.117	-3.8933	28.8933
		4	-30.62500*	8.20870	.006	-49.5543	-11.6957
		5	-8.12500	8.20870	.351	-27.0543	10.8043

	4	1	41.87500*	7.49349	.001	24.5950	59.1550
		2	43.12500*	7.10895	.000	26.7317	59.5183
		3	30.62500*	8.20870	.006	11.6957	49.5543
		5	22.50000*	8.20870	.025	3.5707	41.4293
	5	1	19.37500*	7.49349	.032	2.0950	36.6550
		2	20.62500*	7.10895	.020	4.2317	37.0183
		3	8.12500	8.20870	.351	-10.8043	27.0543
		4	-22.50000*	8.20870	.025	-41.4293	-3.5707

*. The mean difference is significant at the 0.05 level.

APPENDIX 2 (Catalase)

Descriptives

VAR00002

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
1	3	5.3791	.20124	.11619	4.8792	5.8790	5.16	5.55
2	4	5.5392	.21805	.10903	5.1922	5.8862	5.31	5.75
3	4	5.8578	.21202	.10601	5.5205	6.1952	5.67	6.16
4	4	5.6029	.15050	.07525	5.3635	5.8424	5.43	5.76
5	4	5.7794	.40132	.20066	5.1408	6.4180	5.45	6.29
Total	19	5.6450	.28157	.06460	5.5093	5.7807	5.16	6.29

Multiple Comparisons

Dependent Variable:VAR00002

	(I) VAR00001	(J) VAR00001	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
LSD	1	2	-.16013	.19468	.425	-.5777	.2574
		3	-.47876*	.19468	.028	-.8963	-.0612
		4	-.22386	.19468	.269	-.6414	.1937

		5	-.40033	.19468	.059	-.8179	.0172
	2	1	.16013	.19468	.425	-.2574	.5777
		3	-.31863	.18024	.099	-.7052	.0679
		4	-.06373	.18024	.729	-.4503	.3229
		5	-.24020	.18024	.204	-.6268	.1464
	3	1	.47876*	.19468	.028	.0612	.8963
		2	.31863	.18024	.099	-.0679	.7052
		4	.25490	.18024	.179	-.1317	.6415
		5	.07843	.18024	.670	-.3081	.4650
	4	1	.22386	.19468	.269	-.1937	.6414
		2	.06373	.18024	.729	-.3229	.4503
		3	-.25490	.18024	.179	-.6415	.1317
		5	-.17647	.18024	.344	-.5630	.2101
	5	1	.40033	.19468	.059	-.0172	.8179
		2	.24020	.18024	.204	-.1464	.6268
		3	-.07843	.18024	.670	-.4650	.3081
		4	.17647	.18024	.344	-.2101	.5630

*. The mean difference is significant at the 0.05 level.

APPENDIX 3 (GPx-)

Descriptives

VAR00002

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
1	3	6.6718E2	23.55070	13.59701	608.6763	725.6827	640.00	681.54
2	4	6.4865E2	52.30156	26.15078	565.4304	731.8773	573.08	693.08
3	4	6.9115E2	15.49323	7.74661	666.5007	715.8071	670.00	703.85
4	4	6.7596E2	8.22214	4.11107	662.8783	689.0448	668.46	687.69
5	4	6.6038E2	16.77677	8.38839	633.6890	687.0802	636.15	673.08
Total	19	6.6874E2	29.06071	6.66698	654.7381	682.7518	573.08	703.85

Multiple Comparisons

Dependent Variable:VAR00002

	(I) VAR00001	(J) VAR00001	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
LSD	1	2	18.52568	21.48928	.403	-27.5643	64.6156
		3	-23.97437	21.48928	.283	-70.0643	22.1156
		4	-8.78205	21.48928	.689	-54.8720	37.3079
		5	6.79490	21.48928	.757	-39.2950	52.8848
	2	1	-18.52568	21.48928	.403	-64.6156	27.5643
		3	-42.50005	19.89521	.051	-85.1710	.1709
		4	-27.30773	19.89521	.191	-69.9787	15.3633
		5	-11.73077	19.89521	.565	-54.4018	30.9402
	3	1	23.97437	21.48928	.283	-22.1156	70.0643
		2	42.50005	19.89521	.051	-.1709	85.1710
		4	15.19232	19.89521	.458	-27.4787	57.8633
		5	30.76927	19.89521	.144	-11.9017	73.4403
	4	1	8.78205	21.48928	.689	-37.3079	54.8720
		2	27.30773	19.89521	.191	-15.3633	69.9787
		3	-15.19232	19.89521	.458	-57.8633	27.4787
		5	15.57695	19.89521	.447	-27.0940	58.2479
	5	1	-6.79490	21.48928	.757	-52.8848	39.2950
		2	11.73077	19.89521	.565	-30.9402	54.4018
		3	-30.76927	19.89521	.144	-73.4403	11.9017
		4	-15.57695	19.89521	.447	-58.2479	27.0940

APPENDIX 4 (GSH)

Descriptives

VAR00002

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
1	2	1.0244E2	7.76091	5.48779	32.7100	172.1680	96.95	107.93
2	2	77.2866	23.92953	16.92073	-137.7117	292.2849	60.37	94.21
3	3	79.2683	22.81701	13.17340	22.5877	135.9489	62.20	105.18
4	2	70.4268	.00000	.00000	70.4268	70.4268	70.43	70.43
5	2	73.1707	.00000	.00000	73.1707	73.1707	73.17	73.17
Total	11	80.4047	17.24008	5.19808	68.8226	91.9867	60.37	107.93

Multiple Comparisons

Dependent Variable:VAR00002

	(I) VAR00001	(J) VAR00001	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
LSD	1	2	25.15242	16.70372	.183	-15.7201	66.0250
		3	23.17073	15.24834	.179	-14.1406	60.4821
		4	32.01218	16.70372	.104	-8.8604	72.8847
		5	29.26828	16.70372	.130	-11.6043	70.1408
	2	1	-25.15242	16.70372	.183	-66.0250	15.7201
		3	-1.98170	15.24834	.901	-39.2930	35.3296
		4	6.85976	16.70372	.696	-34.0128	47.7323
		5	4.11585	16.70372	.814	-36.7567	44.9884
	3	1	-23.17073	15.24834	.179	-60.4821	14.1406
		2	1.98170	15.24834	.901	-35.3296	39.2930
		4	8.84145	15.24834	.583	-28.4699	46.1528
		5	6.09755	15.24834	.703	-31.2138	43.4089
	4	1	-32.01218	16.70372	.104	-72.8847	8.8604
		2	-6.85976	16.70372	.696	-47.7323	34.0128

		3	-8.84145	15.24834	.583	-46.1528	28.4699
		5	-2.74390	16.70372	.875	-43.6164	38.1286
	5	1	-29.26828	16.70372	.130	-70.1408	11.6043
		2	-4.11585	16.70372	.814	-44.9884	36.7567
		3	-6.09755	15.24834	.703	-43.4089	31.2138
		4	2.74390	16.70372	.875	-38.1286	43.6164

APPENDIX 5 (MDA)

Descriptives

VAR00002

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
1	3	.2713	.14141	.08164	-.0800	.6226	.12	.40
2	4	.1817	.13766	.06883	-.0373	.4008	.05	.32
3	3	.2696	.20512	.11843	-.2399	.7792	.11	.50
4	4	.3648	.15979	.07990	.1105	.6190	.24	.59
5	4	.2112	.06494	.03247	.1078	.3145	.15	.30
Total	18	.2585	.14324	.03376	.1873	.3298	.05	.59

Multiple Comparisons

Dependent Variable:VAR00002

	(I) VAR00001	(J) VAR00001	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
LSD	1	2	.08959	.11012	.431	-.1483	.3275
		3	.00171	.11772	.989	-.2526	.2560
		4	-.09343	.11012	.412	-.3313	.1445
		5	.06015	.11012	.594	-.1777	.2981
	2	1	-.08959	.11012	.431	-.3275	.1483
		3	-.08788	.11012	.439	-.3258	.1500

		4	-.18302	.10195	.096	-.4033	.0372
		5	-.02944	.10195	.777	-.2497	.1908
	3	1	-.00171	.11772	.989	-.2560	.2526
		2	.08788	.11012	.439	-.1500	.3258
		4	-.09514	.11012	.403	-.3330	.1428
		5	.05845	.11012	.605	-.1795	.2963
	4	1	.09343	.11012	.412	-.1445	.3313
		2	.18302	.10195	.096	-.0372	.4033
		3	.09514	.11012	.403	-.1428	.3330
		5	.15358	.10195	.156	-.0667	.3738
	5	1	-.06015	.11012	.594	-.2981	.1777
		2	.02944	.10195	.777	-.1908	.2497
		3	-.05845	.11012	.605	-.2963	.1795
		4	-.15358	.10195	.156	-.3738	.0667

APPENDIX 6 (Total Protein)

Descriptives

VAR00002

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
1	3	99.9642	5.66160	3.26873	85.9000	114.0283	96.02	106.45
2	4	78.7903	8.40104	4.20052	65.4224	92.1583	66.67	85.05
3	4	90.4301	14.89808	7.44904	66.7239	114.1363	76.56	105.81
4	4	80.5108	5.39022	2.69511	71.9337	89.0878	76.24	87.74
5	4	97.3925	12.22545	6.11273	77.9391	116.8459	79.35	106.24
Total	19	88.8625	12.54472	2.87796	82.8161	94.9088	66.67	106.45

Multiple Comparisons

Dependent Variable:VAR00002

	(I) VAR00001	(J) VAR00001	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
LSD	1	2	21.17383*	7.84550	.017	4.3469	38.0008
		3	9.53404	7.84550	.244	-7.2929	26.3610
		4	19.45340*	7.84550	.026	2.6265	36.2803
		5	2.57167	7.84550	.748	-14.2553	19.3986
	2	1	-21.17383*	7.84550	.017	-38.0008	-4.3469
		3	-11.63979	7.26353	.131	-27.2185	3.9389
		4	-1.72043	7.26353	.816	-17.2991	13.8583
		5	-18.60216*	7.26353	.023	-34.1809	-3.0235
	3	1	-9.53404	7.84550	.244	-26.3610	7.2929
		2	11.63979	7.26353	.131	-3.9389	27.2185
		4	9.91935	7.26353	.194	-5.6594	25.4981
		5	-6.96237	7.26353	.354	-22.5411	8.6163
	4	1	-19.45340*	7.84550	.026	-36.2803	-2.6265
		2	1.72043	7.26353	.816	-13.8583	17.2991
		3	-9.91935	7.26353	.194	-25.4981	5.6594
		5	-16.88173*	7.26353	.036	-32.4604	-1.3030
	5	1	-2.57167	7.84550	.748	-19.3986	14.2553
		2	18.60216*	7.26353	.023	3.0235	34.1809
		3	6.96237	7.26353	.354	-8.6163	22.5411
		4	16.88173*	7.26353	.036	1.3030	32.4604

*. The mean difference is significant at the 0.05 level.