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**CERTIFICATION**

I certify that this project was carried out by me, MOHAMMED OLALEKAN PATRICK in the department of biochemistry, faculty of basic medical science ,college of medicine ,university of Ibadan ,Nigeria under my supervisor.

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**DEDICATION**

This project is dedicated to God Almighty in whom I found grace and favour to start and complete this project.

I also dedicate this project work to my parents Mr Mohammed Roberts and Mrs Mohammed Iyabo for their support and prayers

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My utmost thanks is to god almighty for seeing me through the successful completion of this project and my study in the University of Ibadan

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**TABLE OF CONTENT**

Title page

Certification

Dedication

Acknowledgement

Table of content

List of figures

List of tables

Abstract

**Chapter one : introduction and literature review**

1.0 Introduction

1.1 Aims and objectives

1.2 Literature Review

1.2.1 renal toxicity

1.2.2 History

1.2.3 Causes

1.2.4 Diagnosis

1.2.5 Prevention

1.2.6 Treatment

1.2.7 Dimethylnitrosamine (DMN)

1.2.8 Synthesis and medical uses

1.2.9 Mechanism of action of Dimethylnitrosamine

1.2.10 Dimethylnitrosamine induced renal toxicity

1.2.11 Dimethylnitrosamine induced oxidative stress

1.2.12 Rutin

1.2.13 Significance of rutin

1.2.17 The kidney

1.2.18 Function of kidney

1.2.19 Kidney disease

1.2.20 Pathology of DMN induced hepatprenal toxicity

1.2.21 Reactive Oxygen Species and Oxidative Stress

1.2.22 Sources of ROS /RNS

1.2.23 Superoxide Anion

1.2.24 Nitric oxide

1.2.25 Hydrogen Peroxide

1.2.26 Hydroxyl Radical

1.2.27 Antioxidant defense system

1.2.27.1 Enzymatic Antioxidant

1.2.27.2 Superoxide Dismutase (SOD)

1.2.27.3 catalase (CAT)

1.2.27.4 Gluthathione peroxidase (GPX)

1.2.28 Non-Enzymatic Antioxidant

1.2.28.1 vitamin c

1.2.28.2 Gluthathione

1.2.28.3 vitamin E

1.2.28.4 myeloperoxidase

**Chapter Two : Materials and Methods**

2.1 Animal Protocol

2.2 Experimental Drugs

2.3 Grouping of animals

2.4 Sacrifice and preparation of serum

2.5 Preparation of tissues for biochemical assay

2.6 preparation of reagents

2.7 Biochemical Assay

2.7.1

**Chapter three**

3.1 Experiments and results

**Chapter Four : Discussions and recommendation**

4.1 conclusion

4.2 Contribution to knowledge

REFERENCE

LIST OF FIGURES

**CHAPTER ONE**

Figure 1.0: Structure of Dimethylnitorsamine

Figure 1.1: Structure of Rutin

Figure 1.2: Structure of the liver

Figure 1.3: Structure of the kidney

Figure 1.4: Structure of Superoxide Anion

Figure 1.5: Structure of Nitric Oxide

Figure 1.6: Structure of Hydrogen Peroxide

Figure 1.7: Structure of Hydroxyl Radical

Figure 1.8: Structure of Glutathione peroxidase

Figure 1.9: Structure of Vitamin c

Figure 1.10: Structure of Glutathione

Figure 1.11: Structure of Vitamin E

**ABSTRACT**

A potent and rapid inducer of renal toxicity Dimethynitrosamine(DMN) is a nitroso alkyl compound shown to be the cause of oxidative stress and cellular damage and injury due to the enhanced formation of free radicals which are produced during the biotransformation of Dimethynitrosamine(DMN) by a monooxygenase found in the smooth endoplasmic reticulum (CYP450) and the formation of reactive electrophiles such as reactive oxygen species (ROS) and reactive nitrogen species(RNS) .

A total of sixty one wistar rat, ranging between 100-150 g were purchased from physiology department, university of Ibadan and nursed in the experimental facility of the biochemistry department, university of Ibadan. The animals were grouped into five categories, with six animals in each sub group except for group c1/c2 that had 7 animals. Group A was labelled as control and was administered normal saline throughout the course of this study .Group B was administered high dose of Rutin (50mg/kg). Group C were administered 10mg/kg Dimethylnitrosamine (DMN) only three times a week for three weeks. Group D were administered DMN (10mg/kg) and low dose Rutin (25 mg[kg) . Group E were administered Dimethylnitrosamine (DMN) (10mg/kg) and high dose of Rutin (50mg/kg).Rutin was administered orally using cannula for two weeks before administering Dimethylnitrosamine (DMN) via the intraperitoneal route using normal saline as the vehicle .

**CHAPTER ONE**

**1.0 INTRODUCTION AND LITERATURE REVIEW**

The toxico-potency of N –nitroso compound was announced in 1937 when it was reported that Dinitrosamaine (DMN) was one of the causative agent responsible for liver damage in the workers of a chemical factory. In 1978, a teacher in Ulm, Germany, was sentenced to life in prison for trying to murder his wife by poisoning jam with NDMA and feeding it to her. Both the wife and the teacher later died from liver failure.

This announcements were experimentally demonstrated by Barnes and Magee in the year 1954 confirming that indeed, a single oral dose of Dinitrosamaine (DMN) or parental dose (20 to 40 mg/kg body weight) of DMN acts primarily as a liver poison producing resulting in severe liver necrosis in rats ,mice ,rabbits, guinea pigs and dogs . it was further demonstrated that the resulting liver lesion in DMN- poisoned animals is characterized by a sharp line of demarcation between the totally destroyed parenchyma and apparently uninjured liver cell area .

renal diseases are one of the most common malignancies worldwide. renal toxicity is a common reported incidence among hospitalized patient with cirrhosis and ascites. In decompensated cirrhosis, the probability of developing renal toxicity with ascites ranges between 8%-20% per year and increases to 40% at 5 years .

renal disease has been demonsttatively linked with diverse causes, some of which includes excessive consumption of alcohol and hepatitis B and C infection(Kumar et al ,2011). However , due to the high power of liver regenration , renal disease is rarely discovered at the early stage and the treatment has a poor prognosis in most of the cases , which makes it a significant health problem (El- Serag 2007). It is also important to note that the relapse rate of renal disease are very high and the survival rate being very low and this has not improved over the decade.

NDMA is found at low levels in numerous items of human consumption, including cured meat, fish, beer, as well as during use of tobacco products and the inhalation of tobacco smoke.

Generally, Nitrates and nitrites are known to be added to food especially meat and fish for t preservation , colour fixative and as a flouring in which upon their ingestion can result in the endogenous formation of nitroso compound particularly in the presence of nitostable precursor such as a primary amine in the acidic condition of the stomach(Lin et al ,2002). In addition to the previously stated fact,the exposure of man to preformed nitrosoamine occurs due to the use of tobacco product, cosmetics, pharmaceutical product and agricultural chemicals from food is approximately 1mg/day (scanlan 1983)

**1.1 AIMS AND OBJECTIVES**

The aims and objectives of this studies are :

1. To establish on a strong foundation, the toxic effect of Dinitrosamaine (DMN) on the liver and kidney of male wistar rat
2. To demonstratively examine the protective role of the pretreatment with Rutin , an antioxidate in Dinitrosamaine (DMN) induced oxidative damage in the liver and kidney of male wistar rat

**1.2** **LITERATURE REVIEW**

Hepatorenal toxicity refers to the simultaneous impairment of both the liver and the kidneys, often as a result of exposure to toxic substances or certain medications. It is characterized by damage or dysfunction in both organs, leading to a range of symptoms and potential complications.

**1.2.1 Renal Toxicity**

Renal toxicity is a life-threatening complication that compromises the [kidney](https://my.clevelandclinic.org/health/body/21824-kidney" \t "/tmp/wps-patrick/x/_blank) functions, causing prerenal acute [kidney failure](https://my.clevelandclinic.org/health/diseases/17689-kidney-failure" \t "/tmp/wps-patrick/x/_blank), a situation of sudden occurence of [kidney disease](https://my.clevelandclinic.org/health/diseases/15096-kidney-disease-chronic-kidney-disease" \t "/tmp/wps-patrick/x/_blank) or any physical changes to the kidneys themselves. The kidneys might healthy, but they lose the ability to function because their blood supply has been compromised.

**1.2.2 History**

Patients with renal toxicity have occluded and constricted blood vessels in their kidneys in response to liver failure, which reduces blood flow to the kidneys. This progressively slows down kidney functions. renal syndrome requires urgent intervention, and in most cases, a [liver transplant](https://my.clevelandclinic.org/health/treatments/8111-liver-transplantation" \t "/tmp/wps-patrick/x/_blank) is the only cure.

The term "Hepatorenal disease" is used to describe the concurrent dysfunction of the liver and kidneys. However, the concept of renal syndrome (HRS), a specific type of renal disease, has a distinct history.

Hepatorenal syndrome was first recognized and described in the medical literature in the 19th century. In 1877, Friedrich Theodor von Frerichs, a German physician, identified the association between liver disease and kidney dysfunction. He described a condition called "hepatic albuminuria," which referred to the presence of protein in the urine (proteinuria) in patients with liver cirrhosis.

Over the years, further research expanded the understanding of renal syndrome. In 1956, Sheila Sherlock, a renowned hepatologist, classified renal syndrome as a functional renal impairment caused by advanced liver disease. She classified it into two types: Type I, characterized by rapidly progressive renal failure, and Type II, marked by a more stable renal dysfunction.

In the following decades, additional studies focused on elucidating the underlying mechanisms and pathophysiology of renal syndrome. The development of advanced diagnostic techniques, such as Doppler ultrasound and renal angiography, helped improve the diagnosis and understanding of the condition.

In 1996, the International Ascites Club proposed diagnostic criteria for renal syndrome, which included specific criteria for kidney dysfunction in the context of liver disease. These criteria were further refined in subsequent years to enhance accuracy and consistency in diagnosing renal syndrome.

Research efforts also focused on investigating potential treatment options for renal syndrome. In the late 20th century and early 21st century, several therapies, including the use of vasoconstrictor drugs and albumin infusions, showed promise in improving renal function in patients with renal syndrome. Liver transplantation remains the definitive treatment for patients with end-stage liver disease and renal syndrome.

Despite advancements, renal syndrome remains a challenging condition with a poor prognosis. It is associated with high mortality rates, and further research is ongoing to explore novel therapeutic approaches and improve patient outcomes.

It's important to note that renal disease, as a broader term encompassing liver and kidney dysfunction, has a longer history tied to the understanding of liver and kidney diseases individually. However, the specific entity of renal syndrome and its classification and research emerged more recently in the medical literature.

Hepatorenal syndrome (HRS) is a multiorgan condition affecting the kidneys and the liver. It is a cause of acute kidney injury that can be seen in those with acute or chronic liver disease. The first association of renal failure in cirrhosis was observed in the late 1800s. In the mid to late 1900s, further research revealed that renal failure in liver cirrhosis was functional. This was demonstrated in patients with renal syndrome with normal kidney histology in addition to the absence of proteinuria. This was further demonstrated clinically when kidneys from patients with HRS were transplanted into those with chronic kidney disease as well as the improvement of renal function in liver cirrhosis patients who underwent a liver transplant. Further research investigating renal clearance established the association of renal vasoconstriction in HRS.[[1]](https://www.ncbi.nlm.nih.gov/books/NBK430856/" \l "article-22807.r1)

**1.2.3 Causes**

Renal Toxicity affects people who already severe liver disease. It usually occurs in people who have had chronic, progressive liver disease for some time and who are on the threshold of [liver failure](https://my.clevelandclinic.org/health/diseases/17819-liver-failure" \t "/tmp/wps-patrick/x/_blank). Chronic liver disease results in [cirrhosis](https://my.clevelandclinic.org/health/diseases/15572-cirrhosis-of-the-liver" \t "/tmp/wps-patrick/x/_blank), scarring of the liver tissues, which changes its blood supply and stops it from functioning over time. renal Toxicity can also occur with acute liver failure resulting from some sudden cause.

Studies suggest that up to 40% of people with end-stage liver disease will develop renal Toxicity .These people represent all ages and sexes. They are more often in the second half of their lives. Up to 10% of people hospitalized with chronic or acute liver failure will develop it.

**Medications**: Some medications, such as nonsteroidal anti-inflammatory drugs (NSAIDs), certain antibiotics, antiviral drugs, and chemotherapy agents, can cause renal toxicity as a side effect.

**Chemical exposure**: Exposure to certain chemicals, such as heavy metals (lead, mercury), solvents, pesticides, and industrial toxins, can damage both the liver and kidneys, leading to renal toxicity.

**Alcohol abuse**: Chronic and excessive alcohol consumption can cause liver and kidney damage, leading to renal toxicity.

**Infections**: Severe infections, like sepsis, can cause a systemic inflammatory response that affects multiple organs, including the liver and kidneys, leading to renal toxicity.

**1.2.4 Symptoms, Complications and Diagnosis**

Renal toxicity can present with a variety of symptoms, including:

* Jaundice (yellowing of the skin and eyes)
* Abdominal pain
* Nausea and vomiting
* Fatigue
* Decreased urine output
* Swelling in the legs and ankles
* Confusion or altered mental status
* Easy bruising or bleeding

**1.2.4** **DIAGNOSIS**

Hepatorenal syndrome is kidney failure that occurs with advanced liver disease and in the absence of other causes of kidney failure. So, healthcare providers diagnose it by first confirming liver disease and kidney failure, and then ruling out other possible causes of kidney failure. They will use a variety of imagining tests, blood tests and urine tests to evaluate your [liver function](https://my.clevelandclinic.org/health/diagnostics/17662-liver-function-tests" \t "/tmp/wps-patrick/x/_blank)and [kidney function.](https://my.clevelandclinic.org/health/diagnostics/21659-kidney-function-tests" \t "/tmp/wps-patrick/x/_blank)

**1.2.5 PREVENTION**

The best way to prevent Hepatorenal syndrome is to manage liver disease before it progresses to cirrhosis. Chronic liver disease progresses slowly over many years. Those who are aware of it can often slow or reverse it by taking steps such as quitting alcohol use, losing weight and improving their diet. However, many people don’t have symptoms, and may not discover it without a routine checkup.

If you already have cirrhosis, there’s no way to predict or prevent the onset of HRS. But you may be able to prevent spontaneous bacterial peritonitis (SBP), which is the most common “trigger.” SBP in people with cirrhosis precipitates up to 25% of HRS cases. Taking antibiotics preventatively if your provider believes you are at particularly high risk for SBP can reduce the possibility of developing HRS by reducing this precipitating factor.

**1.2.6 TREATMENT**

Kidney failure makes HRS urgent, but liver disease is the root of the problem. renal syndrome is classified as “prerenal” acute kidney failure because it happens when your kidneys are otherwise healthy. In fact, if your kidneys were transplanted into someone else’s body, they would work just fine. By the same token, your kidneys are likely to regain their functionality if you regain liver functionality.

Some people with acute liver failure may recover their liver and kidney functionality. All others will need a liver transplant. They may have needed a transplant eventually, but HRS makes the need more urgent. However, not everyone qualifies for a liver transplant, and those who do may have to wait to get one.

In the meantime, your healthcare provider will work to preserve your kidney function as much as possible and alleviate symptoms and side effects of the condition. These steps may help to improve your overall condition and make you more fit for liver transplant surgery. They may also improve your outcome if you do have liver transplant surgery. But they aren’t curative. Therapies may include:

1. [IV fluids](https://my.clevelandclinic.org/health/treatments/21635-iv-fluids" \t "/tmp/wps-patrick/x/_blank) to treat [electrolyte imbalances](https://my.clevelandclinic.org/health/diagnostics/22358-electrolyte-panel" \t "/tmp/wps-patrick/x/_blank)and support blood flow to your kidneys.
2. Discontinuing certain medications, such as diuretics.
3. Antibiotics to treat any related infections.
4. Paracentesis to remove excess fluid from ascites.
5. Vasoconstrictors, medicines that reduce your abnormally widened blood vessels and increase blood flow to your kidneys.
6. Hemodialysis to support the stressed kidneys.

**1.2.7** **DIMETHYLNITROSAMINE (DMN)**

Dimethylnitrosamine (DMN) is a chemical compound with the molecular formula (CH₃)₂N₂O. It belongs to the class of nitrosamines, which are organic compounds containing the nitroso functional group (NO). Nitrosamines are known to be potent carcinogens, meaning they have the potential to cause cancer.

**1.2.8**  **SYNTHESIS AND MEDICAL USES**

DMN is a highly reactive compound that can be synthesized through various chemical processes. It is not naturally occurring but can be formed as a byproduct of certain industrial processes, such as the production of rubber, pesticides, dyes, and pharmaceuticals. DMN can also be found in tobacco smoke and certain foods, although at much lower levels.

DMN is synthesized endogeneously and also found to be present in tobacco smoke ,alcoholic beverage ,agricultural chemicals ,cosmetics and pharmaceuticals. It is produced from metabolism of certain drugs (sivaramakrishnan et al ). During the processing of food in industries, nitrites are usually added to smoked fish, vegetables and meat for the purpose of inhibiting the growth of bacteria and are usually used as colourants and to enhance flavor. In the process , some of the added nitrites are usually converted to nitrosamine due to the effect of gastric acid and heat . this makes industrially processed food to be the major source of Dimethynitrosamine(DMN)(hill,1988). DMN is among the potent toxin that is described as a potent hepatotoxin in experimental animals after a course of repeated admistration.

**1.2.9** **MECHANISM OF ACTION OF DIMETHYLNITROSAMINE**

NDMA and NDEA are metabolized by a microsomal enzyme system that requires NADPH and oxygen. This metabolism leads to an unstable product which decomposes to yield a reactive alkylating species. This species is too reactive chemically to influence significantly organs other than those in which it was generated. Alkylation of cellular components, particularly DNA, is a critical event in the initiation of tumours by these carcinogens. The greatest capacity to metabolize these nitrosamines to alkylating agents is found in the liver, but other organs, including the oesophagus, lung and kidney, are also capable of activation. These organs may be more susceptible to alkylation than the liver because they have a lesser ability to catalyse the removal of 06-alkylguanine from their DNA. However, orally administered doses of NDMA and the NDMA formed by nitrosation reactions within the gastrointestinal tract are rapidly absorbed from the upper part of the small intestine and carried to the liver in the portal blood supply. When small doses are given in this way, the capacity of the liver to metabolize the carcinogen is sufficient that the nitrosamine is effectively cleared in a 'first-pass' effect, leaving very little to interact with other organs. This has two important consequences: firstly, levels of NDMA found in peripheral blood may be significantly lower than those expected on the basis of total dietary exposure because of the rapid metabolism and effective clearance of the carcinogen by the liver; secondly, physiological factors leading to reduction of the metabolic activation in the liver may result in more of the carcinogen being metabolized other tissues and in a greater risk of cancer developing in those tissues.

**1.2.10**  **DIMETHYLNITROSAMINE INDUCED RENAL TOXICITY**

There have been many experimental evidence to demonstrate the close relationship between the exposure of DMN and the damage to the liver and kidney

**1.2.11** **DIMETHYLNITROSAMINE INDUCED OXIDATIVE STRESS**

Dimethylnitrosamine (DMN) exposure has been shown to induce oxidative stress in various tissues and organs. Oxidative stress occurs when there is an imbalance between the production of reactive oxygen species (ROS) and the ability of the body's antioxidant defenses to neutralize them. ROS, such as superoxide radicals, hydrogen peroxide, and hydroxyl radicals, are highly reactive molecules that can damage cellular components, including lipids, proteins, and DNA.

When DMN is metabolized in the body, it generates ROS as a byproduct. The excessive production of ROS overwhelms the antioxidant defense mechanisms, leading to oxidative stress. Here are some ways in which DMN-induced oxidative stress can occur:

Generation of ROS: DMN can undergo metabolic activation in the liver by enzymes called cytochrome P450, resulting in the production of reactive intermediates and ROS. These ROS can cause damage to cellular structures and initiate a cascade of oxidative events.

Lipid Peroxidation: ROS can attack polyunsaturated fatty acids in cell membranes, leading to lipid peroxidation. This process generates lipid peroxides, which further propagate oxidative damage and disrupt cell membrane integrity.

Protein Oxidation: ROS can oxidize amino acids in proteins, altering their structure and function. This can impair enzymatic activity, disrupt cellular signaling pathways, and lead to protein dysfunction.

DNA Damage: ROS can directly damage DNA, causing modifications to the DNA bases and DNA strand breaks. If not properly repaired, DNA damage can lead to mutations and potentially contribute to the development of cancer.

Antioxidant Depletion: DMN-induced oxidative stress can deplete the levels of endogenous antioxidants, such as glutathione, which are essential for neutralizing ROS. Reduced antioxidant capacity further exacerbates the damaging effects of ROS.

The consequences of DMN-induced oxidative stress depend on the extent and duration of exposure, as well as individual susceptibility. Prolonged oxidative stress can contribute to the development of various diseases, including liver damage, kidney dysfunction, and cancer.

To counteract the harmful effects of oxidative stress, antioxidant defenses are crucial. These include endogenous antioxidants, such as superoxide dismutase, catalase, and glutathione peroxidase, as well as exogenous antioxidants obtained from the diet, such as vitamins C and E, carotenoids, and flavonoids.

Understanding the role of oxidative stress in DMN toxicity has paved the way for research on potential protective strategies, including the use of antioxidants and other compounds that can mitigate oxidative damage. However, it's important to note that further studies are needed to fully understand the mechanisms and develop effective therapeutic interventions in the context of DMN-induced oxidative stress.

**1.2.12** **RUTIN**

Rutin (3,3′,4′,5,7-pentahydroxyflavone-3-rhamnoglucoside, Fig. 1) is a flavonol, abundantly found in plants, such as passion flower, buckwheat, tea, and apple. Rutin is a plant pigment that is found in certain [fruits and vegetables](http://www.webmd.com/food-recipes/ss/slideshow-exotic-fruits). Buckwheat, Japanese pagoda tree, and Eucalyptus are sources of rutin. Rutin is also found in lime tree flowers, elder flowers, hawthorn, rue, St. John's Wort, Ginkgo, apples, and other [fruits and vegetables](http://www.webmd.com/food-recipes/ss/slideshow-fun-facts-fruits-vegetables). Rutin might have antioxidant and anti-inflammatory effects. It might also offer some protection against cancer and other diseases.  
  
Rutin is commonly used for autism, aging [skin](http://www.webmd.com/skin-problems-and-treatments/picture-of-the-skin), airway infections caused by [exercise](http://www.webmd.com/fitness-exercise/ss/slideshow-7-most-effective-exercises), and many other purposes, but there is no good scientific evidence to support any of these uses.

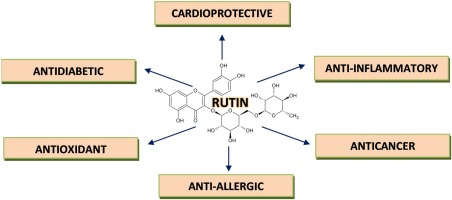


Fig 1.1 Structure of the structure of Rutin

**1.2.13**  **SIGNIFICANCE OF RUTIN**

Intervention strategies using plant-derived bioactive compounds have been offered as a form of treatment for these debilitating conditions, as there are currently no remedies to prevent, reverse, or halt the progression of certain liver and kidney injury. Rutin, a glycoside of the flavonoid quercetin, is found in many plants and fruits, especially buckwheat, apricots, cherries, grapes, grapefruit, plums, and oranges. Pharmacological studies have reported the beneficial effects of rutin in many disease conditions, and its therapeutic potential in several models of renal toxicity which has created considerable excitement. Here, we have summarized the current knowledge on the hetaorenalprotective mechanisms of rutin in various experimental models of renal toxicity. The mechanisms of action reviewed in this article include reduction of proinflammatory cytokines, improved antioxidant enzyme activities, activation of the mitogen-activated protein kinase cascade, downregulation of mRNA expression of PD-linked and proapoptotic genes, upregulation of the ion transport and antiapoptotic genes, and restoration of the activities of mitochondrial complex enzymes. Taken together, these findings suggest that rutin may be a promising neuroprotective compound for the treatment of NDs. Similarly, there is currently an increase in the usage of natural compounds/products as potential neuroprotective agents. Examples include, curcumin, bilobalide, chitosan, and apigenin, all known to have potent protective effects on neurons. Recently, bioflavonoids have found use in the healthcare system owing to their wide range of biological activities, low cost, and significantly high safety margins. Rutin (3,3′,4′,5,7-pentahydroxyflavone-3-rhamnoglucoside, [Figure 2](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6040293/figure/fig2/" \t "/tmp/wps-patrick/x/figure)) also called sophorin, rutoside, and quercetin-3-rutinoside is a polyphenolic bioflavonoid, largely extracted from natural sources such as oranges, lemons, grapes, limes, berries, and peaches. Rutin is a vital nutritional component of plants and its name originates from the plant *Ruta graveolens*, which also contains rutin . Chemically, it is a glycoside comprising of flavonol aglycone quercetin along with disaccharide rutinose . Some studies suggest that rutin has a potential protective role in renal damage due to its beneficial effects as a potent antioxidant . Hence, this review presents an outline of the scientific literature regarding the potential protective role of rutin in renal damage

**1.2.17**  **THE KIDNEY**

The kidneys are two bean-shaped organs, each about the size of a fist. They are located just below the rib cage, one on each side of your spine.

Healthy kidneys filter about a half cup of blood every minute, removing wastes and extra water to make [urine](https://www.niddk.nih.gov/Dictionary/U/urine). The urine flows from the kidneys to the bladder through two thin tubes of muscle called ureters, one on each side of your bladder. Your bladder stores urine. Your kidneys, ureters, and bladder are part of your [urinary tract](https://www.niddk.nih.gov/health-information/urologic-diseases/urinary-tract-how-it-works). You have two kidneys that filter your blood, removing wastes and extra water to make urine.Your kidneys remove wastes and extra fluid from your body. Your kidneys also remove acid that is produced by the cells of your body and maintain a healthy balance of water, salts, and minerals—such as [sodium](https://www.niddk.nih.gov/Dictionary/S/sodium), [calcium](https://www.niddk.nih.gov/Dictionary/C/calcium), [phosphorus](https://www.niddk.nih.gov/Dictionary/P/phosphorus), and [potassium](https://www.niddk.nih.gov/Dictionary/P/potassium)—in your blood.Without this balance, nerves, muscles, and other tissues in your body may not work normally.Your kidneys also make hormones that help control your [blood pressure](https://www.niddk.nih.gov/Dictionary/B/blood-pressure) and make [red blood cells](https://medlineplus.gov/ency/anatomyvideos/000104.htm)

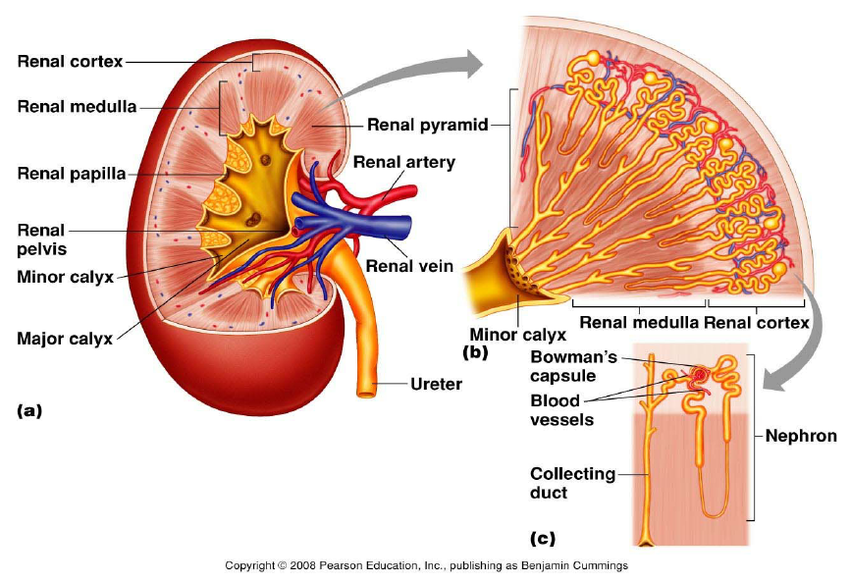


Fig 1.3 Structure of the kidney

**1.2.18 Functions of the kidney**

The kidneys are vital organs with numerous important functions in the body. Here are some key functions performed by the kidneys:

**Filtration of Waste Products**:

The kidneys filter waste products, toxins, and excess substances from the bloodstream. They selectively remove substances like urea, creatinine, uric acid, and various metabolic byproducts while retaining essential nutrients and molecules.

**Regulation of Fluid and Electrolyte Balance:**

The kidneys help maintain the balance of fluids and electrolytes in the body. They regulate the concentration of ions, such as sodium, potassium, calcium, and phosphate, in the blood by reabsorbing or excreting them as needed. This function is crucial for maintaining proper hydration, blood pressure, and pH balance.

**Acid-Base Balance**:

The kidneys play a crucial role in maintaining the body's acid-base balance. They regulate the excretion of hydrogen ions and bicarbonate ions to help keep the blood pH within a narrow range, ensuring the proper functioning of enzymes and physiological processes.

**Blood Pressure Regulation**:

The kidneys contribute to the regulation of blood pressure by adjusting the volume of blood and the constriction or relaxation of blood vessels. They release a hormone called renin, which initiates a cascade of events that ultimately help control blood pressure.

**Erythropoiesis Regulation**:

The kidneys produce and release a hormone called erythropoietin, which stimulates the production of red blood cells in the bone marrow. This hormone plays a critical role in maintaining adequate oxygen-carrying capacity in the blood.

**Vitamin D Activation**: The kidneys are involved in the conversion of inactive vitamin D (calcidiol) to its active form (calcitriol). Active vitamin D helps regulate calcium and phosphate levels, promoting proper bone health and supporting various physiological processes.

**Toxin and Drug Metabolism**:

The kidneys participate in the metabolism and elimination of certain drugs and toxins. They help filter these substances from the bloodstream and facilitate their excretion in the urine, preventing their accumulation in the body.

**Regulation of Water Balance**:

The kidneys adjust the excretion or retention of water to maintain proper water balance in the body. This mechanism helps regulate overall fluid volume, blood pressure, and concentration of solutes in the blood.

**1.2.19** **KIDNEY DISEASE**

Kidney diseases, also known as renal diseases or nephropathies, encompass a wide range of conditions that affect the structure and function of the kidneys. These diseases can have various causes, including infections, autoimmune disorders, genetic abnormalities, systemic diseases, medications, and other factors. Some common kidney diseases include:

**Chronic Kidney Disease (CKD)**:

CKD is a progressive condition in which the kidneys gradually lose their ability to function properly over time. It is often caused by conditions such as diabetes, high blood pressure, glomerulonephritis, and polycystic kidney disease. CKD can lead to end-stage renal disease (ESRD) if left untreated, requiring dialysis or kidney transplantation.

**Acute Kidney Injury (AKI):**

AKI refers to a sudden loss of kidney function, usually occurring over a short period. It can result from severe infections, dehydration, drug toxicity, urinary tract obstruction, or reduced blood flow to the kidneys. Prompt medical intervention is essential to prevent complications and restore kidney function.

**Glomerulonephritis**:

Glomerulonephritis is a group of diseases that involve inflammation of the glomeruli, the tiny filtering units within the kidneys. It can be caused by infections, autoimmune disorders, certain medications, or other underlying conditions. Glomerulonephritis can lead to proteinuria, hematuria, reduced kidney function, and impaired urine filtration.

**Polycystic Kidney Disease (PKD)**:

PKD is an inherited disorder characterized by the growth of multiple cysts within the kidneys. These cysts can gradually enlarge, affecting kidney function and leading to complications such as hypertension, kidney stones, and kidney failure.

**Urinary Tract Infections (UTIs)**:

UTIs can occur in any part of the urinary system, including the kidneys. Bacterial infections of the kidneys (pyelonephritis) can cause inflammation and impair kidney function. Prompt treatment with antibiotics is necessary to prevent further complications.

**Kidney Stones**:

Kidney stones are hard mineral and salt deposits that form within the kidneys. They can cause severe pain, blood in the urine, and obstruction of urine flow. Treatment may involve medications, increased fluid intake, and, in some cases, surgical intervention.

**Nephrotic Syndrome**:

Nephrotic syndrome is a condition characterized by increased excretion of protein in the urine (proteinuria), low blood protein levels, high cholesterol levels, and edema (swelling). It can be caused by various kidney diseases, including glomerulonephritis and diabetic nephropathy.

**Renal Failure**:

Renal failure refers to the significant loss of kidney function, resulting in the inability to adequately filter waste products and maintain fluid and electrolyte balance. It can be acute or chronic and may require dialysis or kidney transplantation for management.

Early detection, proper management, and lifestyle modifications are important for treating and slowing the progression of kidney diseases. Treatment approaches may include medications, dietary changes, blood pressure control, immune system modulation, and addressing underlying causes. Regular monitoring and follow-up with healthcare professionals are crucial for individuals with kidney diseases.

**1.2.21** **REACTIVE OXYGEN SPECIES AND OXIDATIVE STRESS**

In recent times, there has been keen interest in the role of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in clinic medicine (Halliwell and Gutheridge, 1999). By definition, free radicals are reactive compounds having a single unpaired electron in the outer orbit (Halliwell, 2011). This unpaired electron usually generates highly reactive free radicals through reactions with adjacent molecules, such as proteins lipids, carbohydrate and nucleic acid. (Rahman, 2007). ROS/RNS are known to play a dual role in biological systems, because they can be harmnful or beneficial to living system(Valkro et al., 2004). The deleterious effects and biological damage consequence by action ofROS and RNS is termed oxidative and nitrosative stress respectively (Valko et al., 2007). These effects of ROS are balanced by the antioxidant enzymes and supported by antioxidant action of non-enzymatic antioxidants (Halliwell, 1996) It has been proposed that the presence of antioxidant defence system act against oxidative damage from ROS, which accumulates during lifecycle and radical related damage to DNA, proteins, and lipids has also been implicated in the progress of age dependent diseases such as cancer, arterioscleroses, arthritis, neurodegenerative diseases and other conditions (Halliwell and Gutheridge. 1999). Useful effects of ROS involve physiological roles in cellular responses to noxia such as defence against infectious agents and in a function of cellular signalling system.On the contrary, at high concentrations, ROS can be a basic mediator of damage to cell structures, including lipids and membranes, proteins and nucleic acid; this damage is known as oxidative stress (Poli et al., 2004).

**1.2.21** **SOURCES OF ROS/RNS**

The generation of ROS/ RNS depends on a variety of factors, but can mainly be divided into exogenous and endogenous (khansari et al., 2009), Endogenous sources include mitochondria, cytochrome P450 and inflammatory cells activation (Inoue et al., 2003).

**1.2.23** **SUPEROXIDE ANION**

Superoxide anion (02) is a reduced form of molecular oxygen created by one electron reduction of oxygen.mitochondrial electron transport endoplasmic reticulum but the main source of superoxide anion is the mitochondria electron transport chain. Superoxide anion can also be produced by the activation of Oxygen by physical irradiation, radiolysis of water by ionizing radiation (Klaunig et al., 2010). Other cellular source of superoxide radical is the enzyme xanthine oxidase (XO). It is widely distributed and catalyses the hydroxylation of purines (Harrison, 2002). Superoxide anion can be generated endogenously from chain, immune cells, enzymatic activities and

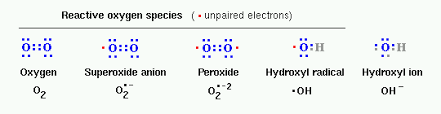


Fig 1.4 some reactive oxygen species

**1.2.24** **NITRIC OXIDE**

Nitric oxide is generated in biological tissue by specific nitric oxide synthases (NOSS) which metabolizes arginine to citrulline with the formation of NO via five electron oxidative reaction (Ghafourifar and Cadenas, 2005). Cells of the immune system produce both the superoxide anion and nitric oxide during the oxidative burst triggered during inflammatory processes.Under these conditions, nitric oxide and the superoxide anion may react together to produce significant amounts of much more oxidatively active molecule, peroxynitrite anion (ONOO), which is an oxidizing free radical that can cause DNA fragmentation

and lipid oxidation (Carr et al.2000).

**NO + O2 ===> ONOO+**

NO has effects on neuronal transmission as well as synaptic plasticity in the central

nervous system. Nitrosative stress may lead to nitrosylation reactions that can alter the structure of proteins and alter their normal function (Valko et al, 2006).

`

**1.2.25** **HYDROGEN PEROXIDE**

Hydrogen peroxide though not a radical is formed in the mitochondria and it is an ROS precursor in superoxide, the dismutation of O2 is catalysed by superoxide dismutase (Lee et: al, 2004). Other endogenous sources of hydrogen peroxide are peroxisomes and Phagocytes

**1.2.26**  **HYDROXYL RADICAL**

Hydrogen peroxide is the precursor of hydroxyl radical OH. Hydroxyl radical is formed by the Fenton reaction from hydrogen peroxide in the presence of ferrous ions.

**H202+ Fe\*→ OH+ OH+ Fe2**

Or by the Haber-Weiss reaction from hydrogen peroxide and superoxide radical

**02 + H202→ OH + OH + O:**

The hydroxyl radical is highly reactive with a half-life in aqueous solution of less than a nanosecond. Thus when produced in vivo, it reacts close to its site of formation (Valko et.; al 2006).

**1.2.27** **ANTIOXIDANT DEFENSE SYSTEM**

The effect of reactive oxygen species is balanced by the help of antioxidant enzymes (Valko et.; al, 2006). An ideal antioxidant should be easily absorbed, counteract free radical and chelate redox metals at physiologically relevant levels. It should also work in both aqueous and or membrane domains and have a positive effect on gene expression. Antioxidants can be grouped into endogenous and exogenous antioxidants. Endogenous antioxidants play a crucial role in maintaining optimal cellular function (Rahman, 2007). Endogenous antioxidants can be further grouped into enzymatic and non-enzymatic antioxidants. The most important enzymatic antioxidant involves catalase, glutathione peroxidase and superoxide dismutase (Mates, 1999). Non enzymatic antioxidants involve Vitamin C, Vitamin E, carotenoids, thiol antioxidants, natural flavonoids and other compounds (McCall, 1999).

**1.2.27.1** **ENZYMATIC ANTIOXIDANTS**

**1.2.27.2** **SUPEROXIDE DISMUTASE (SOD)**

One of the most efficient intracellular enzymatic antioxidants is the superoxide dismutase (SOD). Superoxide dismutase is the antioxidant enzyme that catalyses the dismutation of superoxide anion into less reactive form (Valko et al.2009)

**20 + 2H ==> H02 + O2**

Superoxide dismutase exists in some isoformns. In the human body there are three forms of SOD; cytosolic Cu-Zn-SOD, mitochondrial Mn-SOD and extracellular SOD.

**1.2.27.3** **CATALASE (CAT)**

Catalase is present in the peroxisomes of aerobic cells and plays a major role in the conversion of hydrogen peroxide to water and molecular oxygen. Catalase has one of the highest turnover rates for all approximately 6 million molecules of hydrogen peroxide to water and oxygen each minute (Rahman 2007). enzymes; one molecule of catalase can convert

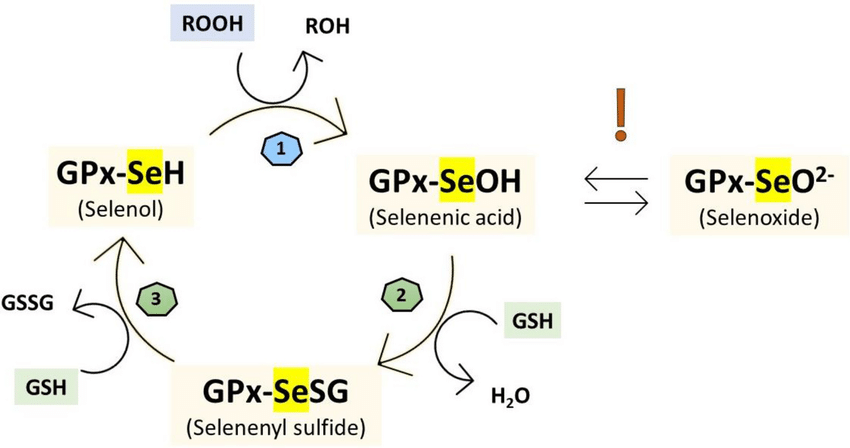
**2H202 ==> 2H2O**

**1.2.27.4 GLUTATHIONE PEROXIDASE (GPX)**

Glutathione peroxidase has two forms, one of which is selenium independent (Glutathione-S- transferase) while the other is selenium dependent (GPx) (Mates, 1999). Humans have four different Se-dependent glutathione peroxidases (Mates, 1999). All GPx enzymes are known to add two electrons to reduce peroxides by forming selenoles (Se-OH). GPx catalyzes the conversion of H,0; to water and it requires glutathione (GSH) which is a tripeptide as a substrate. It can also reduce other peroxides to alcohol (e.g lipid peroxides) while simultaneously oxidizing GSH. It also competes for H202 as a substrate and is the major source of protection against low level of oxidative stress (Chaudiere and Ferrari-1lou, 1999).w

**ROOH + 2GSH + H02 → ROH + GSSG+H20**

GPx is found in the cytosol and mitochondria and mitochondria matrix and it requires selenium as a cofactor for its optimum.



**1.2.27.5** **NON-ENZYMATIC ANTIOXIDANT**

**1.2.27.16** **VITAMINC**

Vitamin C is considered the most important water-soluble antioxidant in extracellular fluids. It is found in fruits (mainly citrus), vegetables, cereals, beef, poultry, fish, etc. It is helpful in preventing some of the DNA damage caused by free radicals, which may contribute to the aging process and the development of diseases, such as cancer, heart disease, and arthritis. It is also capable of neutralizing ROS in the aqueous phase before lipid peroxidation it is initiated. In stressful situations adrenal glands react by releasing hormones that trigger the "fight or flight" reaction. It has been indicated that 200mg of vitamin C a day may reduce the levels of stress hormones. Stress suppresses the immune system. Mega doses of vitamin C increase the levels of antibody that fights against germs and viruses in both stressed and unstressed rats, with greater antibody increase in the unstressed rats Block (1999).

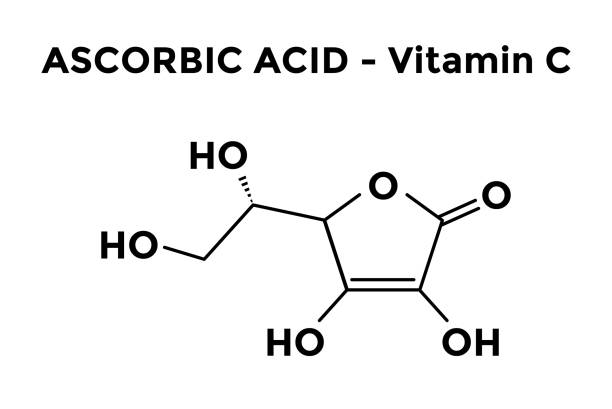


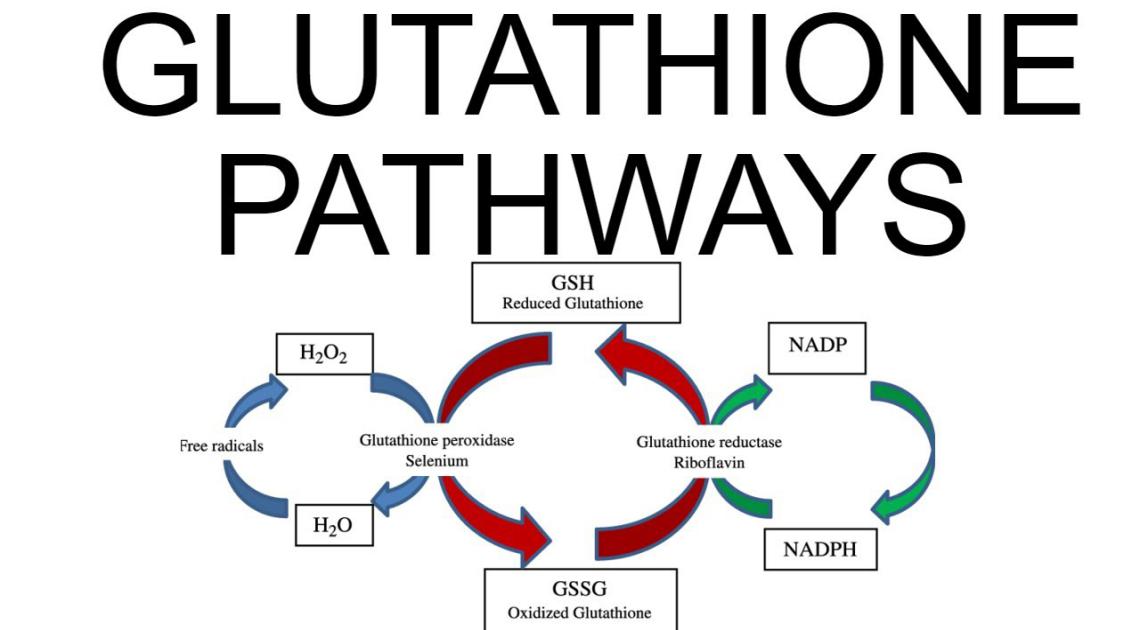
Fig 1.9 Structure of vitamin c

**1.2.27.7**  **GLUTATHIONE**

Glutathione (GSH) is an important antioxidant in plants, animals, fungi, and some bacteria and archaea, preventing damage to important cellular components caused by reactive oxygen species such as free radicals and peroxides (Pompella, 2003). It is a tripeptide with a gamma peptide linkage between the carboxyl group of the glutamate side-chain and the amine group of cysteine (which is attached by normal peptide linkage to a glycine Thiol groups are reducing agents, existing at a concentration around 5 mM in animalcells. Glutathione reduces disulfide bonds formed within cytoplasmic proteins to cysteine by serving as an electron donor. In the process, glutathione is converted to its Oxidized form, glutathione disulfide (GSSG), lso called L-(-)-glutathione. Once oxidized, glutathione can be reduced back by glutathione reductase, using NADPH as an electron donor (Couto et al, 2013).The ratio of reduced glutathione to oxidized glutathione within cells is often used as a measure of cellular toxicity(Pastore et. ;al 2003). Glutathione is a multifunctional intracellular non-enzymatic antioxidant. It is considered to be the major thiol-disulphide redox buffer of the cell (Masella et.; al, 2005).

Glutathione is highly abundant in the cytosol (1-1lmM), nuclei (3-15mM), and mitochondria (5-11MM) and is the major soluble antioxidant in these cell compartments. The reduced formn of glutathione is GSH, glutathione, and the oxidized form is GSSG, glutathione disulphide. GSH in the nucleus maintains the redox state of critical protein sulphydryls that are necessary for DNA repair and expression. Oxidised glutathione is accumulated inside the cells and the ratio of GSH/GSSG is a good measure of oxidative stress of an organism (Jones et al, 2000). Too high a concentration of GSSG may damage many enzymes oxidatively.

The main protective roles of glutathione against oxidative stress are (Masella et al.2005) glutathione is a cofactor of several detoxifying enzymes against oxidative stress, e.gglutathione peroxidase (GPx), ghutathione-s-transferase and others; (ii)GSH participates in a mino acid transport through the plasma membrane; (iii) GSH scavenges hydroxyl radical and singlet oxygen directly, detoxifying hydrogen peroxide and lipid peroxides by the catalytic action of glutathione peroxidase; (iv) glutathione is able to regenerate the most important antioxidants, Vitamins C and E, back to their active forms; glutathione can reduce the tocopherol radical of Vitamin E directly, or indirectly, via reduction of semidehydroascorbate to asortbate. The capacity of glutathione to regenerate the most important antioxidants is linked with the redox state of the glutathione disulphide- glutathione couple (GSSG/2GSH) (Pastore et.;al. 2003).



**1.2.27.8**  **VITAMIN E**

Vitamin E, a major lipid-soluble antioxidant, is the most effective chain-breaking antioxidant within the cell membrane where it protects membrane fatty acids from lipid peroxidation. It has about eight different forns such as a-, P-, Y, and ő-tocopherol and a-,B-, Y-, and ð tocotrienol, In its function as a chain-breaking antioxidant, vitamin E rapidly transfers its phenolic H-atom to a lipid peroxyl radical, converting it into a lipid hydroperoxide and a vitamin E radical Bashir et al. (2004). Vitamin E scavenges peroxyl radical Unsaturated Fatty Acid (PUFA) present in cell membrane and density lipoprotein (LDL), against lipid peroxidation Vivek and Surendra (2006). The various functions of vitaminE include maintaining the normal conditions of cells, healthy skin and tissues, Protects red blood cells, antioxidation and enhancing immunity. The important sources of vitamin E include wheat germ, nuts, seeds, whole grains, green leafy vegetables, vegetable oil and fish-liver oil.

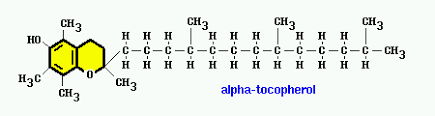


Fig 1.11 Structure of vitamin E

**CHAPTER TWO**

**MATERIALS AND METHODS**

**MATERIALS**

* **Dimethyl nitrosamine**
* **Rutin,Thiobarbituric acid (TBA)**
* **Trichloroacetic acid (TCA)**
* **Normal saline**
* **Phosphate buffer**
* **Phosphoric acid, sodium hydroxide**
* **Sodium chloride**
* **Reduced gluthathione**
* **Disodium hydrogen phosphate**
* **Sodium dihydrogen phosphate**
* **Sulphosalicylic acid**
* **Hydrogen peroxide**
* **Potassium iodide**
* **Commasiac blue**
* **Chloro1 -2,4-dinitrobenzene(CDNB)**
* **Gluthathione peroxidase**
* **Sodium azide**
* **Bovine serum alb**umin .

**2.1**  **ANIMALS**

Sixty one wistar rat, between 100-150 g were purchased from physiology department, university of Ibadan and housed in the experimental facility of the biochemistry department, university of Ibadan. They were fed with rat pellet which was purchased from and unlimited clean water supply.

**2.2 EXPERIMENTAL DRUGS**

Rutin that was used during the course of the experiment was gotten from the laboratory at drug metabolism unit, biochemistry department, university of Ibadan

Dimethylnitrosamine that was used to induce the condition in question

**2.3**  **GROUPING OF ANIMALS**

The animals were grouped into five, with six animals in each sub group excep.t for group c1/c2 having 7 animals. Group A served as control and was administered normal saline throughout the course of the experiment .Group B was administered high dose of Rutin (50mg/kg). Group C were administered 10mg/kg Dimethylnitrosamine (DMN) only three times a week for three weeks. Group D were administered DMN (10mg/kg) and low dose Rutin (25 mg[kg) . Group E were administered Dimethylnitrosamine (DMN) (10mg/kg) and high dose of Rutin (50mg/kg).Rutin was administered orally using cannula for two weeks before administering Dimethylnitrosamine (DMN) via the intraperitoneal route using normal saline as the vehicle .

**2.4**  **SACRIFICE AND PREPARATION OF SERUM**

The animals were fasted and sacrificed 12 hours after the last dosage of Rutin was administered .

Blood samples was collected by ocular puncture using capiliary tubes into non–herparin centrifuge tube and allow to clot for an hour .

The clotted samples were centrifuged at 3000 rpm for 15 minutes .

The supernatant was collected and stored to be used for enzymes assays .

The animals were sacrificed by cervical dislocation then the liver and kidney were carefully excised with a dissecting scissors and forceps , harvested and rinsed in a rinsing buffer and stored .

**2.5**  **PREPARATION OF TISSUES FOR BIOCHEMICAL ASSAY**

After the animals were sacrificed the liver and kidney samples were excised and rinsed in ice cold 1.15% kcl buffer solution.

The liver and kidney samples were chopped into smaller pieces and homoginsed in 5 volume of homogenizing buffer (0.1 M pH 7.4), using Teflon homogenizer.

The resulting homogenate was centrifuges at 10,000rpm for ten minutes to obtain the post mitochondrial fraction (PMF).

The supernatant was collected and stored at 4C for biochemical assay.

### **PREPARATION OF REAGENTS**

**i. Potassium chloride (1.15%)**

1.15g of potassium chloride (KCl) was dissolved in 60ml of distilled water and the volume made up to 100ml. The prepared potassium chloride was used as a rinsing buffer for the harvested organs.

**ii. Formalin (10%)**

10mls of formaldehyde was dissolved in distilled water and made up to 100mls with distilled water.

**iii. Homogenizing buffer (0.1M Phosphate buffer, pH 7.4)**

3.497 g of dipotassium hydrogen phosphate trihydrate and 1.318 g of potassium dihydrogen phosphate were dissolved in 200 ml of distilled water, the pH adjusted to 7.4 and the volume made up to 250 ml with distilled water.

### **2.6 DETERMINATION OF TOTAL BODY AND RELATIVE ORGAN WEIGHTS**

The total body weight of each rat was determined using a digital balance before and after the experimental period. Weights recorded before the commencement of treatments were tagged initial body weights whereas weights recorded after the experimental period were tagged final body weights. Thereafter, the mean body weights for each group was calculated. Changes in weights were also expressed as percentage weight increase.

Percentage weight increase was calculated from the formula:



Where Wx = Initial mean total body weight

Wy = Final mean total body weight

The weights of harvested organs of respective rats was measured with a digital balance and presented as percentage liver weight per total body weight. This was calculated from the formula:

****

### **2.7 BIOCHEMICAL ASSAYS**

**Kidney Function Test**

* **Urea**
* **Creatinine**
* **Total Protein**
* **Superoxide dismutase (SOD)**
* **Catalase**
* **Glutathione –S- Transferase (GST)**
* **Glutathione Peroxidase (GPx)**
* **Glutathione (GSH)**
* **Xanthine Oxidase (XO)**
* **Total Sulfhydryl (TSH)**
* **Inflammatory Biomarkers**
* **Nitric oxide (NO) determination**
* **Myeloperoxidase (MPO)**
* **Oxidative Stress Indices**
* **Reactive Oxygen and Nitrogen Species (RONS)**
* **Lipid peroxidation**

#### 

#### **2.7.2 Kidney Function Test**

##### **2.7.2.1 Measurement of Urea Activity**

Urea is the principal nitrogenous waste product of metabolism and is generated from protein breakdown. It is eliminated from the body almost exclusively by the kidneys in urine. Serum/plasma urea concentration reflects the balance between urea production in the liver and urea elimination by the kidneys, in urine; so increased plasma/serum urea can be caused by increased urea production, decreased urea elimination, or a combination of the two. By far the highest urea levels occur in the context of reduced urinary elimination of urea due to advanced renal disease and associated marked reduction in glomerular filtration rate (GFR). The limitation of urea as a test of renal function is that in some circumstances plasma urea is not a sufficiently accurate reflection of GFR. For example, urea is an insensitive indicator of reduced GFR; GFR must be reduced by around 50 % before serum/plasma urea increases above the upper limit of the reference range. Furthermore, urea may be raised despite a normal GFR (i.e. normal renal function) so as a test of renal function, urea lacks specificity.

**Principle**

Urea is first degraded into ammonia and carbon dioxide, which further reacts with an alkaline developer to produce a blue-green coloured product that can be measured with a standard spectrophotometric plate reader at an optical density between 580-630 nm. The level of urea in the serum was measured according to the method of Fawcett *et al.,* 1960.

Urea + H20 → 2NH3 + CO2

NH3 + Hypochloric + Phenol → Indophenol (Blue compound)

Urea + H20 → 2NH3 + CO2

**Reagents**

* **Reagent 1**
* 116mmol/l of EDTA
* 6mmol/l of Sodium nitroprusside
* 1g/l of Urease
* **Reagent 2**
* 120mmol/l of Phenol (Diluted)
* **Reagent 3**
* 27mmol/l of Sodium hypochlorite (diluted)
* 0.14N of Sodium Hydroxide
* **Cal**
* 13.4mmol/l (80mg/dl) of Standard

**Table 2.7: Procedure for determination of Urea level in serum samples.**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | **Reagent blank(µl)** | | **Standard(µl)** | **Sample(µl)** |
| Sample | --------- | | ---------- | 20 |
| Standard (Cal) | --------- | | 20 | --------- |
| Reagent 1 (R1) | 50 | | 50 | 50 |
| Distilled water | 20 | | --------- | ------- |
|  | | Thoroughly mix and incubate for 10 minutes at 37°C | | |
| Reagent 2 (R2) | 1.25ml | | 1.25ml | 1.25ml |
| Reagent 3 (R3) | 1.25ml | | 1.25ml | 1.25ml |
|  | | Mix, allow to stand for 15 minutes at 37°C | | |

Absorbance of samples (Asample) and standard (Astandard) were read against blank with a microplate reader at 546nm.

**Calculation:**

Urea concentration= Asample  x 50(mmol/l)

Astandard  1

##### 

##### **2.7.2.2 Measurement of Creatinine Activity**

The kidneys play a vital role in the excretion of waste products and toxins such as urea, creatinine and uric acid, regulation of extracellular fluid volume, serum osmolality and electrolyte concentrations, as well as the production of hormones like erythropoietin and 1, 25 dihydroxyvitamin D and renin. The functional unit of the kidney is the nephron which consists of the glomerulus, proximal and distal tubules, and collecting duct. Assessment of renal function is important in the management of patients with kidney disease or pathologies affecting renal function. Tests of renal function have utility in identifying the presence of renal disease, monitoring the response of kidneys to treatment, and determining the progression of renal disease. The most commonly used endogenous marker for assessment of glomerular function is creatinine. Creatinine is a waste product produced by muscles from the breakdown of a compound called creatine. For the most part, creatinine is cleared from the blood entirely by the kidneys, which filter almost all of it from the blood and release it into the urine. Decreased clearance by kidney results in an increased blood creatinine. Creatinine test measures the amount of creatinine in the blood and/or urine. The amount of creatinine produced per day depends on muscle bulk, and thus, there is a difference in creatinine ranges between males and females with lower creatinine values in children and those with decreased muscle bulk.

**Principle**

Creatinine in alkaline solution reacts with picric acid to form a coloured complex. The amount of the complex formed is directly proportional to the creatinine concentration. The activity of creatinine was determined following the principle described by Bartels *et al.,* 1972.

**Reagents:**

* **Reagent 1a**
* 35mmol/l of Picric Acid
* **Reagent 1b**
* 0.32mol/l of Sodium hydroxide
* **Cal**
* 181 (µmol/l) of Standard

**Table 2.8: Procedure for determination of Creatinine level in serum samples.**

Wavelength: 492nm

|  |  |  |  |
| --- | --- | --- | --- |
|  | **Reagent blank(µl)** | **Standard(µl)** | **Sample(µl)** |
| Sample | --------- | ---------- | 20 |
| Standard (Cal) | --------- | 20 | --------- |
| Working Reagent | 200 | 200 | 200 |
| Distilled water | 20 | ------ |

Mix and read Absorbance (A1) of samples and standard after 30 seconds. Exactly 2 minutes later, read absorbance A2 of standard and sample.

**Calculation:**

A2-A1 = Δ Asample or Δ Astandard

Creatinine concentration = ΔAsample  x Standard conc. (µmol/l)

ΔAstandard

#### **2.7.3 Determination of Total Protein Concentration**

The protein concentration of the various homogenates was determined by means of the Lowry method described by (Lowry *et al.,* 1951).

**Principle**

The principle behind the Lowry method of determining protein concentrations lies in the reactivity of the peptide nitrogen(s) with copper (ii) ions under alkaline conditions and the subsequent reduction of the Folin-Ciocalteau phosphomolybdic phosphotungstic acid to heteropolymolybdenum blue by the copper-catalysed oxidation of aromatic acids. The Lowry method is sensitive to pH changes and therefore the pH of assay solution should be maintained at 10-10.5.

****

**Reagents**

**a) 2% Na2CO3 in 0.1M NaOH**

500ml of 0.1M NaOH was prepared by dissolving 2g of NaOH in distilled water and making up to 500ml. 10g of Na2CO3 was then dissolved in the 500ml of 0.1M NaOH and the solution was labelled reagent A.

**b) 2% Na-K Tartrate**

0.2g of Na-K Tartrate was dissolved in 10ml of distilled H2O and labelled as reagent B.

**c) 1% CuSO₄.5H2O**

0.1g of CuSO₄.5H2O was dissolved in 10ml of distilled H2O and labelled as reagent C.

**d) Alkaline CuSO₄ Solution**

Alkaline CuSO₄.5H2O was prepared by mixing reagent A, B and C in the ratio 100:1:1. To a clean 500ml volumetric flask, 490.19mls of reagent A, 4.9mls of reagent B and 4.9mls of reagent C were mixed, giving 500mls of alkaline CuSO₄ solution.

**e) Folin-C** A solution of 1ml/5ml of Folin-C was prepared. This was done by adding 40mls of water to 8mls of Folin-C, to give 48mls of Folin-C solution.

**Standard protein (BSA) curve**

2mg/ml stock solution of Bovine Serum Albumin was prepared. Serial dilutions of the solutions were placed in 5 different test tubes into 1ml of each protein standard solution in a test tube was added 3ml of alkaline CuSO₄. The mixture was allowed to stand at room temperature for 10minutes after which 0.3ml of Folin-C was added and allowed to stand at room temperature for 30 minutes. The optical densities of the resulting solutions were read in a spectrophotometer 750nm against a blank of 1ml of distilled water and 3ml of biuret reagent. A curve of absorbance against protein concentration was plotted.

**Table 2.9: Protocol for protein standard curve preparation**

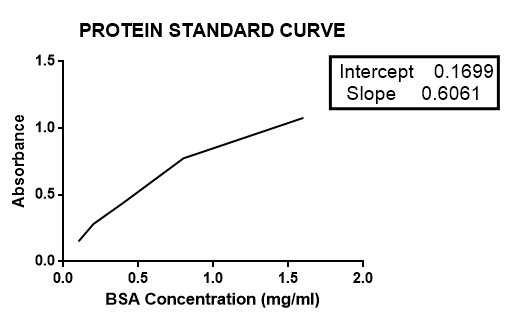
|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Test tubes No.** | **1** | **2** | **3** | **4** | **5** |
| Stock BSA (ml) | 0.05 | 0.10 | 0.20 | 0.40 | 0.80 |
| Distilled water (ml) | 0.95 | 0.90 | 0.80 | 0.60 | 0.20 |
| Alkaline CuSO ₄ (ml) | 3.00 | 3.00 | 3.00 | 3.00 | 3.00 |
| Folin-C reageant (ml) | 0.30 | 0.30 | 0.30 | 0.30 | 0.30 |

**Procedure for sample preparation**

7µl of sample was added to 23µl of distilled water in a microplate, to which 150µl of alkaline CuSO₄ solution was added and left to incubate at room temperature for 10 minutes. 15 µl of folin-c solution was then added and the mixture was incubated at room temperature for 30 minutes, after which the absorbance was read at 750nm using the reagent blank.

**Calculation**

The total protein levels in liver homogenates was extrapolated from a standard curve of the values below



**Figure 2.1: Protein Standard Curve****.**

#### **2.7.4 ANTIOXIDANT ASSAYS**

##### **2.7.4.1 DETERMINATION OF SUPEROXIDE DISMUTASE (SOD) ACTIVITY**

The activity of SOD was determined by the method of Misra and Fridovich (1972).

**Principle**

The ability of SOD to inhibit the autoxidation of epinephrine at pH 10.2 makes this reaction a basis for a simple assay for this dismutase. Superoxide radical causes the oxidation of epinephrine to adrenochrome and the yield of adrenochrome produced per superoxide radical introduced increases with increasing pH and concentration of epinephrine.

**Reagents**

1. **0.05M Carbonate buffer (pH 10.2)**

1.573 g of Na2CO3.10H2O and 0.588 g of NaHCO3 were dissolved in 200 ml of distilled water. The pH was adjusted to 10.2 and then made up to 250 ml with distilled water.

**2. 0.3M Epinephrine**

0.05 g of epinephrine was dissolved in 200 ml of distilled water containing 0.5 ml of concentrated HCl (37%).

**Procedure**

50 µl of sample was added to 2.5 ml of 0.05M carbonate buffer (pH 10.2) and 0.3 ml of epinephrine in a cuvette, mixed by inversion and change in absorbance monitored every 30 sec for 2 minutes at 480 nm. The reference cuvette was the same as for the samples with water replacing the samples.

**Calculation**

% inhibition = 100 – (100 × Increase in absorbance per min for sample)

Increase in absorbance per min for blank

1 unit of SOD activity was given as the amount of SOD necessary to cause 50% inhibition of the autooxidation of epinephrine.

##### **2.7.4.2 DETERMINATION OF CATALASE ACTIVITY**

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

**Principle**

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

**Reagents**

1. **Phosphate buffer (0.05 M, pH 7.4)**

Dipotassium hydrogen phosphate trihydrate (0.696 g) and potassium dihydrogen phosphate (0.265 g) were dissolved in 90 ml of distilled water, the pH adjusted to 7.4 and the volume made up to 100 ml with distilled water.

**2. Hydrogen peroxide (19 mM)**

194 µl of 30% H2O2 was added to 50 ml of 0.05 M phosphate buffer, pH 7.4 and the volume made up to 100 ml with the same.

**Procedure**

Hydrogen peroxide (2.95 ml of 19 mM solution) was pipetted into a 1 cm quartz cuvette and 50 µl of sample added. The mixture was rapidly inverted to mix and placed in a spectrophotometer. Change in absorbance was read at 240 nm every minute for 5 min.

**Calculation**

Catalase activity = ΔA240/min × reaction volume × dilution factor

0.0436 × sample volume × mg protein/ml

= µmole H2O2/min/mg protein

##### **2.7.4.3 DETERMINATION OF GLUTATHIONE S-TRANSFERASE**

##### **ACTIVITY**

Glutathione S-transferase activity was determined according to Habig, Pabst and Jacoby (1974).

**Principle**

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

**Reagents**

**1. 1-Chloro-2,4-dinitrobenzene (20 mM)**

16.85 mg of 1-chloro-2,4-dinitrobenzene (CDNB) was dissolved in 5 ml of absolute ethanol.

**2. Reduced Glutathione (0.1 M)**

30.73 mg of glutathone (GSH) was dissolved in 1 ml of 0.1 M phosphate buffer (pH 6.5).

**3. Phosphate buffer (0.1 M, pH 6.5)**

Dipotassium hydrogen phosphate trihydrate (0.381 g) and potassium dihydrogen phosphate (1.134 g) were dissolved in 90 ml of distilled water, the pH adjusted to 6.5 and the volume made up to 100 ml with distilled water.

**Procedure**

The medium for the estimation was prepared as shown in the table below and the reaction was allowed to run for 3 min with readings taken every 60 seconds against the blank at 340 nm.

**Table 2.9.1: Glutathione S-Transferase Assay Medium**

|  |  |  |
| --- | --- | --- |
| Reagent | Blank | Test |
| CDNB (20 mM) | 10 μl | 10 μl |
| Reaction mixture (20mls phosphate buffer, 0.5mls Reduced glutathione and 10.5mls distilled water) | 170 μl | 170 μl |
| Sample | - | 20 μl |

**Calculations**

The extinction coefficient of CDNB at 340 nm = 9.6 mM-1cm-1

GSH S-transferase activity = ΔA340/min × reaction volume × dilution factor

9.6 × sample volume × mg protein/ml

= µmole/min/mg protein

##### **2.7.4.4 DETERMINATION OF GLUTATHIONE PEROXIDASE ACTIVITY**

Glutathione peroxidase (GPX) activity was measured according to the procedure of Rotruck *et al*., (1973) with some modifications.

**Principle**

Glutathione peroxidase is allowed to conjugate hydrogen peroxide to glutathione for a fixed period of time after which the reaction is quenched. The remaining glutathione is reacted with Ellman’s reagent and the GSH consumed is then used as a measure of enzyme activity.

**Reagents**

1. **Sodium azide (10 mM)**

3.25 mg of sodium azide was dissolved in 50 ml of distilled water.

1. **Reduced glutathione (4 mM)**

12.3 mg of GSH was dissolved in 10 ml of 0.1 M phosphate buffer, pH 7.4.

3. **Hydrogen peroxide (2.5 mM)**

14 µl of 30% hydrogen peroxide was added to distilled water and the volume made up to 50 ml with the same.

4. **Trichloroacetic acid (10%)**

2 g of TCA was dissolved in distilled water and the volume made up to 20 ml with the same.

5. **Dipotassium hydrogen orthophosphate (0.3 M)**

4.11 g of K2HPO4.3H2O was dissolved in distilled water and the volume made up to 60 ml with the same.

6. **Ellman’s reagent (DTNB)**

19.8 mg of DTNB was dissolved in 50 ml of 0.1 M phosphate buffer, pH 7.4.

7. **Phosphate buffer (0.1 M, pH 7.4)**

Dipotassium hydrogen phosphate trihydrate (1.399 g) and potassium dihydrogen phosphate (0.527 g) were dissolved in 90 ml of distilled water, the pH adjusted to 7.4 and the volume made up to 100 ml with distilled water.

**Procedure**

To 50µl of phosphate buffer in a test tube was added 10µl of NaN3, 20µl of GSH, 10µl of H2O2 and 50µl of sample (added last). The reaction mixture was incubated for 3 min at 37˚C after which 50µl of TCA was added and the final mixture centrifuged at 3000 rpm for 5 min. To 50µl of the supernatants, 100µl of K2HPO4 and 50µl of DTNB were added and the absorbance read against a reagent blank of 50µl distilled water, 100µl of K2HPO4 and 50µl of DTNB at 412 nm.

**Calculation**

GSH consumed = initial GSH amount (129.39 µg) – GSH remaining (µg/ml × 4 ml)

GPX activity = GSH consumed/mg protein

= µg GSH/mg protein

##### 

##### **2.7.4.5 DETERMINATION OF REDUCED GLUTATHIONE (GSH) ACTIVITY**

The method of Beutler *et al*. (1963) was followed in estimating the level of reduced glutathione (GSH).

**Principle**

This method is based upon the development of a relatively stable yellow coloured product when 5, 5′–dithiobis-2-nitrobenzoic acid (DTNB; Ellman’s reagent) is added to sulfhydryl compounds of which glutathione comprises the bulk in tissues. The resulting chromophoric product possesses maximum absorbance at 412 nm.

**Reagents**

1. **GSH stock solution**

40 mg of GSH was dissolved in 0.1 M phosphate buffer, pH 7.4 and made up to 100ml with the same.

**2. Phosphate buffer (0.1 M, pH 7.4)**

Dipotassium hydrogen phosphate trihydrate (2.098 g) and potassium dihydrogen phosphate (0.791 g) were dissolved in 120 ml of distilled water, the pH adjusted to 7.4 and the volume made up to 150 ml with distilled water.

**3. Ellman’s Reagent**

60 mg of Ellman’s reagent was in dissolved in 0.1 M phosphate buffer, pH 7.4 and made up to 150 ml with the same.

**4. Sulphosalicylic Acid (4% solution)**

0.8 g of sulphosalicylic acid was dissolved in 20 ml of distilled water.

**Procedure**

80µl of sample was added to 80µl of precipitating solution which was vortexed and centrifuged at 4000 rpm for 5 minutes. Thereafter, 50µl of the supernatant was added to 150µl of Ellman’s reagent. The absorbance of the reaction mixture was read at 412 nm against a reagent blank using a plate reader.

**GSH Standard Curve Preparation**

Serial dilutions of the GSH stock solution were prepared as shown in the table below. The absorbance of the yellow colour formed upon the addition of Ellman’s reagent was read within 30 min at 412 nm against a blank of 1.5 ml of Ellman’s reagent and 0.5 ml phosphate buffer. A plot of absorbance against concentration of reduced GSH was then plotted.

**GSH Standard Curve Protocol**

**Table 2.9.2: Method for the preparation of serial dilutions of the GSH working standard**

|  |  |  |  |
| --- | --- | --- | --- |
| **GSH Stock (ml)** | **Phosphate Buffer (ml)** | **Ellman’s Reagent (ml)** | **GSH Conc. (µg/ml)** |
| 0.01 | 0.49 | 1.5 | 1 |
| 0.03 | 0.47 | 1.5 | 3 |
| 0.05 | 0.45 | 1.5 | 5 |
| 0.10 | 0.40 | 1.5 | 10 |
| 0.15 | 0.35 | 1.5 | 15 |
| 0.20 | 0.30 | 1.5 | 20 |

Figure 2.2: Standard curve for Reduced Glutathione level

##### **2.7.4.6 DETERMINATION OF XANTHINE OXIDASE ACTIVITY**

The activity of xanthine oxidase was assessed by the method of Bergmeyer *et al*., (1974).

**Principle**

The assay is based on the measurement of uric acid absorbance at 290 nm as it is produced from xanthine by the action of xanthine oxidase.

**Reagents**

1. **Phosphate buffer (0.05 M, pH 7.5)**

Dipotassium hydrogen phosphate trihydrate (0.760 g) and potassium dihydrogen phosphate (0.227 g) were dissolved in 90 ml of distilled water, the pH adjusted to 7.5 and the volume made up to 100 ml with distilled water.

1. **Xanthine solution (0.15 mM)**

2.3 mg of xanthine was dissolved in a few drops of 0.1 M NaOH, 90 ml of distilled water added, the solution adjusted to pH 7.5 with dilute acid or base, and the solution made up to 100 ml.

**Procedure**

Into a micro plate, 150µl of phosphate buffer, 80µl of xanthine solution and 8µl of sample were pipetted. The mixture was mixed and absorbance was taken every minute for 3 minutes at 290 nm. A blank was made by replacing 8µl of sample with distilled water.

**Calculation**

The extinction coefficient of uric acid at 290 nm = 12.1 mM-1cm-1

Xanthine oxidase activity = ΔA290/min × reaction volume × dilution factor

12.1 × sample volume × mg protein/ml

= µmole/min/mg protein

##### **2.7.4.7 DETERMINATION OF TOTAL SULFHYDRL ACTIVITY**

Thiols are a class of organic compounds that contain a sulfhydryl group (-SH), also known as a thiol group, usually composed of a sulfur atom and a hydrogen atom attached to a carbon atom. Sulfhydryl groups have great and varied reactivity. They oxidize easily, with the formation of disulfides and sulfenic, sulfinic, or sulfonic acids, and they readily undergo alkylation, acylation, and thiol-disulfide exchange. They form mercaptides upon reacting with the ions of heavy metals, and they form mercaptals and mercaptols upon reacting with aldehydes and ketones, respectively. Sulfhydryl groups play an important role in several biochemical processes. The importance of sulfhydryl groups is seen in coenzyme A, lipoic acid, and 4’-phosphopantotheine, where sulfhydryl groups play vital roles in the formation and transfer of acyl residues involved in lipid and carbohydrate metabolism. The importance of sulfhydryl groups is also seen in glutathione, as they aid in the neutralization of foreign organic compounds and the reduction of peroxides. In proteins, amino acids such as cysteine possess sulfhydryl groups. Sulfhydryl groups are also found as components of the active sites of several enzymes, where they participate in the catalytic effect of the enzymes and in the binding of substrates, coenzymes, and metal ions. The total thiol status in the body, especially thiol (-SH) groups present on protein are considered as major plasma antioxidants in vivo and most of them are present over albumin (Prakash *et al.,* 2004), and they are the major reducing groups present in our body fluids (Monod *et al.,* 1965).

**Principle**

The evaluation of total thio groups was determined according to the method of Hu & Dillard (1994) using dithionetrobenzoic acid (DTNB). DTNB reacts with thiol functional groups and creates a yellow compound which is absorbed at 412nm.

**Reagents**

**1. Phosphate Buffer (0.1 M, pH 7.4)**

Dipotassium hydrogen phosphate trihydrate (2.098 g) and potassium dihydrogen phosphate (0.791 g) were dissolved in 120 ml of distilled water, the pH adjusted to 7.4 and the volume made up to 150 ml with distilled water.

**2. Ellman’s Reagent**

37.6mg of Ellman’s reagent was in dissolved in 0.1 M phosphate buffer, pH 7.4 and made up to 10 ml with the same.

**Procedure**

Into an eppendorf tube, 150µl of sample, 100µl of phosphate buffer and 250µl of distilled water were pipetted. The mixture was then allowed to stand. Into a microplate, 15µl of Ellman’s reagent was pipetted, to which 230µl of the reaction mixture was added and allowed to stand for 2 minutes. The absorbance was then read at 412nm.

#### 

#### **2.7.5 INFLAMMATORY BIOMARKERS ASSAYS**

##### **2.7.5.1 DETERMINATION OF NITRIC OXIDE (NO) LEVEL**

The NO. Radical plays an important role as a physiological messenger. NO is formed from L-arginine (Palmer *et al.*, 1988) by NO synthase, which exists in several isoforms (Griffith and Stuehr, 1995). Constitutive calcium-dependent isoforms (cNOS) modulate the control of vascular tone in endothelial cells or the neurotransmission in neurons, whereas inducible calcium-independent isoforms (iNOS) are located in macrophages, chondrocytes and hepatocytes and are induced by cytokines and endotoxin (Bredt and Snyder, 1994; Nathan, 1992). Pathological conditions associated with increased release of cytokines and endotoxin, e.g. inflammation or sepsis can therefore increase NO production. Upon coming into the blood stream, nitrite reacts immediately with oxy-haemoglobin to form methaemoglobin. The level of nitric oxide was determined by the method of Green *et al.,* (1982).

**Principle**

Nitrite mediates the nitrosative modification of sulphanilic acid which then reacts with N-naphthylethylenediamine dihydrochloride forming a pink orange coloured products with maximum absorbance at 550nm.

**Reagent**

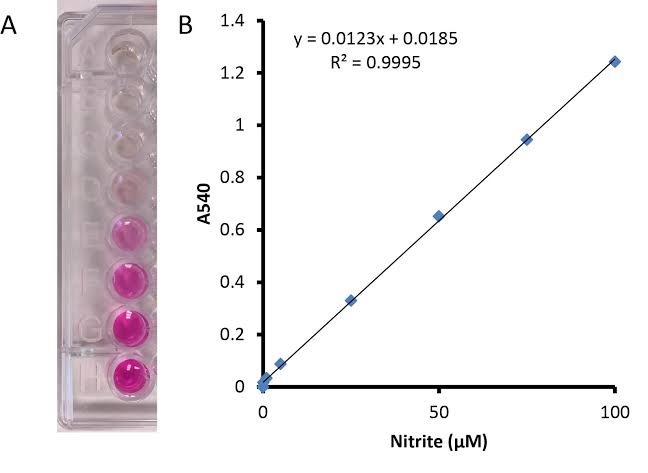
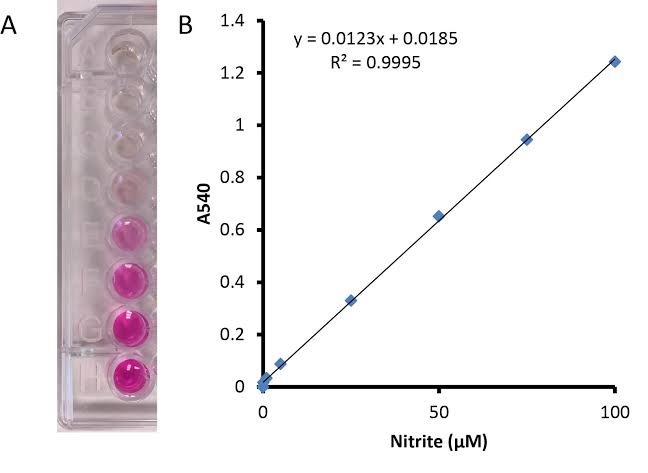
Griess Reagent [0.1% N-(1-naphthyl)ethylenediamine dihydrochloride; 1% sulphanilamide in 5% phosphoric acid 1:1].

**Procedure**

The amounts of nitrite in supernatants or in serum were measured following the Griess reaction by incubating a 100µL of sample with 100µL of Griess reagent [0.1% N-(1-naphthyl) ethylenediamine dihydrochloride; 1% sulfanilamide in 5% phosphoric acid; 1:1 purched a] at room temperature for 20 min. The absorbance at 550 nm (OD 550) was measured spectrophotometrically. Nitrite concentration was calculated by comparison with the OD 550 of a standard solution of known sodium nitrite concentrations.

**Calibration curve**

Calibrator at various concentrations was prepared by diluting stock 20 mmol/L solutions of NaNO2 with distilled water. The nitrate calibrator was diluted with glycine buffer just as the serum samples were. Calibration curve was made over a linear range of nitrate between 0 and 100 µmol/L.



**Figure 2.3: NO calibration curve.**

##### **2.7.5.2 DETERMINATION OF MYELOPEROXIDASE (MPO) ACTIVITY.**

Myeloperoxidase (MPO) is a lysosomal enzyme present in the azurophilic granules of polymorphonuclear leukocytes (PMNs) and is unique to neutrophils and monocytes. However, monocytes contain only one-third of the MPO found in PMNs. MPO utilizes H2O2 produced by the neutrophils to oxidize a variety of aromatic compounds to give substrate radicals for bacterial activity (Hampton *et al.*, 1998). This enzyme is unique however in that it can oxidize chloride ions to produce a strong non-radical oxidant, hypochlorous acid (HOCl). HOCl is the most powerful bactericidal compond produced by neutrophils. Excessive production of these radicals can cause oxidative stress leading to oxidative tissue injury. Myeloperoxidase (MPO) activity, an indicator of polymorphonuclear leukocyte accumulation, was determined by the modification of the method described by Trush et al.*,* (1994).

**Principle**

The activity of MPO was measured spectrophotometrically using o-dianisidine (Sigma-Aldrich) and hydrogen peroxide. In this presence of H2O2 as an oxidizing agent, MPO catalyses the oxidation of o-dianisidine yielding a brown coloured product, oxidized O-dianisidine, with a minimum absorbance at 470 nm, according to the following overall reaction:

2H2O2 + O-dianisidine Oxidized O-dianisidine + 4H2O

**Procedure**

200 µl of O- dianisidine mixture [containing 16.7 mg of o-dianisidine dihydrochloride (3,3- Dimethoxybenzidine, Fast Blue B, C14 H16 N2 O2 Mol. Wt 244.3) in 100 ml of 50 mM phosphate buffer, pH=6.0, plus 50 µl of dilute H2O2 (4 µl of 59% H2O2 diluted in 96 µl of dH2O)] was added to 7 µl of tissue homogenate (in triplicate). Absorbance readings were taken at 30 seconds interval for 4 minutes at 460 nm using a spectrophotometer. The MPO activity is in unit (U) of MPO/mg tissue, where one unit of MPO is defined as the amount needed to degrade 1 µmol of H2O2 per minute at room temperature. Considering that one unit (U) of MPO = 1 µmol of H2O2 split and that 1 µmol of H2O2 gives a change of absorbance of 1.13 x 10-2 nm/min, units of MPO in each sample is determined as change in absorbance, that is: [ΔAbs (t2 – t1)]/ Δmin x (1.13 x 10-2)].

To get unit mg of tissue, the tissue: buffer ratio is used. For example, if a tissue: buffer ratio of 50 mg/ml is used, in 7 µl (0.007 ml) of homogenate, there is 0.35 mg of tissue. Therefore, to get units per mg tissue, the units MPO divided by 0.35.

**Reagents**

1. **0.5 M Phosphate buffer (pH 6.0)**

Dipotassium hydrogen phosphate trihydrate (K2HPO4.12H2O (0.052g)) and Potassium dihydrogen phosphate (KH2PO4.2H2O (0.64g)) were dissolved in 100 ml of distilled water, pH was adjusted to 6.0

1. **Hydrogen peroxide**
2. µl of 59% H2O2 diluted in 96 µl distilled water.

**3. O-Dianisidine**

16.7 mg of o-dianisidine was dissolved in 100 ml 0.5M phosphate buffer.

**NOTE:** Add a drop of HCl to obtain clear mixture, if working with O-dianisidine instead of O-dianisidine dihydrochloride. However, do not calibrate pH back to 6.0 after dissolving O-Dianisidine with HCl in buffer.

**Procedure**

200 µl of combined solutions (buffered O-dianisidine and H2O2) and 7 µl of sample were pipetted into a microplate and absorbance was measured at 30 seconds interval for 4 minutes at 460 nm.

**Calculation**

MPO activity = ΔAbs (t2-t1) / min x volume of mixture x dilution factor

(11.3 x 10-3) x volume of sample x mg protein

#### 

#### **2.7.6 OXIDATIVE STRESS ASSAYS**

##### **2.7.6.1 DETERMINATION OF REACTIVE OXYGEN/NITROGEN SPECIES LEVEL**

**Principle**

The RONS production was quantified according to established protocol which is based on the RONS-dependent oxidation of 2', 7’-dichlorodihydrofluorescin diacetate (DCFH-DA) to DCF   
(Adedara *et al.,* 2016).

**Reagent**

**1. Phosphate Buffer (0.1 M, pH 7.4)**

Dipotassium hydrogen phosphate trihydrate (1.399 g) and potassium dihydrogen phosphate (0.527 g) were dissolved in 90 ml of distilled water, the pH adjusted to 7.4 and the volume made up to 100 ml with distilled water.

**2. Mother Solution (DCFH-DA)**

0.025g of 2', 7’-dichlorodihydrofluorescin diacetate (DCFH-DA) was dissolved in 10mls of absolute ethanol.

**3. Working Solution**

A working solution was prepared by adding 8µl of the mother solution to 2mls of absolute ethanol.

**Procedure**

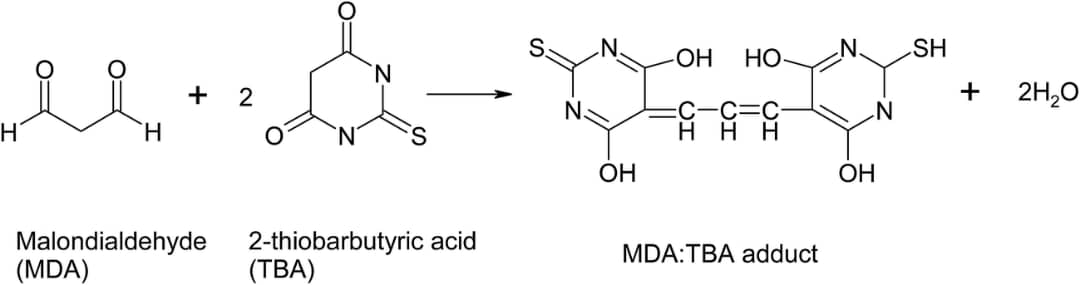
Briefly, the reaction mixture consisted of 10 µL of the sample, 150 µL of 0.1M potassium phosphate buffer (pH 7.4), 35 µL of distilled water and 5 µL of DCFH-DA (200 µM, final concentration 5 µM). The fluorescence emission of DCF resulting from DCFHDA oxidation was analyzed for 10 min (30s intervals) at 488 nm excitation and 525 nm emission wavelengths using a SpectraMax plate reader (Molecular Devices, CA, USA). The rate of DCF formation was expressed in percentage of control group (Adedara *et al*., 2016).

##### **2.7.6.2 DETERMINATION OF LIPID PEROXIDATION**

Lipid peroxidation was determined by measuring the formation of thiobarbituric acid reactive substances (TBARS) present in the test sample according to the method of Varshney and Kale (1990).

**Principle**

Under acidic conditions, malondialdehyde (MDA) produced from the peroxidation of fatty acids reacts with the chromogenic reagent 2-thiobarbituric acid to yield a pink coloured complex with maximum absorbance at 532 nm and is extractable into organic solvent such as butanol. Malondialdehyde is often used to calibrate this test and thus the result is expressed as the amount of free MDA produced.



**Figure 2.4: Chemical reaction for lipid peroxidation**

**Reagents**

**1. 30% Trichloroacetic acid (TCA)**

4.5 g of TCA (CCl3COOH) was dissolved in distilled water and made up to 15ml with the same.

**2. 0.1M Hydrochloric acid (HCl)**

13 µl of concentrated HCl (36.5-38%) was added to distilled water and the volume made up to 15 ml with the same.

**3. 0.75% Thiobarbituric acid (TBA)**

0.1125 g of TBA was dissolved in 0.1 M HCl and made up to 15 ml with the same. Dissolution was aided by stirring in a hot water bath (50˚C).

**4. 0.15 M Tris-KCl buffer (pH 7.4)**

0.559 g of KCl and 0.909 g of Tris base were dissolved in 45 ml of distilled water, the pH was then adjusted to 7.4 with HCl and the volume made up to 50 ml with the same.

**Procedure**

An aliquot of 40µl of the test sample was mixed with 160µl of Tris-KCl buffer to which 50µl of 30% TCA was added. Then 50µl of 0.75% TBA was added and placed in a water bath for 45 minutes at 800C. This was then cooled in ice to room temperature and centrifuged at 3000 rpm for 10 min. 200µl of the clear supernatant was collected and absorbance measured against a reference blank of distilled water at 532 nm using a microplate reader.

**Calculation**

MDA (units/mg protein) = Absorbance x Volume of mixture

E532nm x Volume of Sample x mg Protein

**CHAPTER THREE**

EFFETS OF RUTIN ON XANTHINE OXIDE ACTIVITY IN DMN-INDUCED LIVER DAMAGE IN MALE WISTAR RATS



Fig 3.12

Values are expressed as mean ± standard error of mean where n = 8

1. Significant as compared with Control : p < 0.05
2. Significant as compared with Rutin group: p < 0.05
3. Significant as compared with DMN group: p < 0.05

**Fig 3.1** Shows significant increase (p 0.05)in the level of XO activity in animals treated with DMN alone as compared with control.

There is also a significant increase (p 0.05) in the level of XO in those animals treated with 10mg/kg of DMN and 25mg/kg of rutin and those treated with 10mg/kg DMN and 50mg/kg of Rutin as compared with those animals treated with DMN only .There is no significant difference animals treated with Rutin as compared to control.

EFFETS OF RUTIN ON MPO ACTIVITY IN DMN-INDUCED LIVER DAMAGE IN MALE WISTAR RATS



Values are expressed as mean ± standard error of mean where n = 8

1. Significant as compared with Control : p < 0.05
2. Significant as compared with Rutin group: p < 0.05
3. Significant as compared with DMN group: p < 0.05

**Fig 3.12** Shows significant increase (p 0.05)in the level of MPO activity in animals treated with DMN alone as compared with control.

There is also a significant decrease (p 0.05) in the level of MPO activity in those animals treated with 10mg/kg of DMN and 25mg/kg of rutin and those treated with 10mg/kg DMN and 50mg/kg of Rutin as compared with those animals treated with DMN only .There is a significant difference in animals treated with Rutin as compared to control.

Conclusion : Rutin is an extra line of defense against inflammation, hence, in the presence of an antioxidant, MPO activity is reduced as rutin takes over the immunity process

EFFETS OF RUTIN ON GSH LEVEL IN DMN-INDUCED LIVER DAMAGE IN MALE WISTAR RATS



**Fig 3.12**

Values are expressed as mean ± standard error of mean where n = 8

1. Significant as compared with Control : p < 0.05
2. Significant as compared with Rutin group: p < 0.05
3. Significant as compared with DMN group: p < 0.05

Fig 3.12 Shows significant increase (p 0.05)in the level of GSH activity in animals treated with DMN alone as compared with control.

There is also a significant decrease (p 0.05) in the level of GSH activity in those animals treated with 10mg/kg of DMN and 25mg/kg of rutin and those treated with 10mg/kg DMN and 50mg/kg of Rutin as compared with those animals treated with DMN only .There is a significant difference in animals treated with Rutin as compared to control.

**EFFECTS OF RUTIN ON HYDROGEN PEROXIDE LEVEL IN DMN-INDUCED LIVER DAMAGE IN MALE WISTAR RATS**



**Fig 3.13**

Values are expressed as mean ± standard error of mean where n = 8

1. Significant as compared with Control : p < 0.05
2. Significant as compared with Rutin group: p < 0.05
3. Significant as compared with DMN group: p < 0.05

Fig 3.13 Shows significant increase (p 0.05)in the level of H2O2 activity in animals treated with DMN alone as compared with control.

There is also a significant decrease (p 0.05) in the level of H2O2 activity in those animals treated with 10mg/kg of DMN and 25mg/kg of rutin and those treated with 10mg/kg DMN and 50mg/kg of Rutin as compared with those animals treated with DMN only .There is a significant increase in animals treated with Rutin as compared to control.

EFFETS OF RUTIN ON NO LEVEL IN DMN-INDUCED LIVER DAMAGE IN MALE WISTAR RATS



**Fig 3.14**

Values are expressed as mean ± standard error of mean where n = 8

1. Significant as compared with Control : p < 0.05
2. Significant as compared with Rutin group: p < 0.05
3. Significant as compared with DMN group: p < 0.05

Fig 3.14 Shows significant increase (p 0.05)in the level of NO level in animals treated with DMN alone as compared with control.

There is also a significant decrease (p 0.05) in the level of NO activity in those animals treated with 10mg/kg of DMN and 25mg/kg of rutin and those treated with 10mg/kg DMN and 50mg/kg of Rutin as compared with those animals treated with DMN only .There is a significant decrease in animals treated with Rutin as compared to control.

EFFETS OF RUTIN ON TSH LEVEL IN DMN-INDUCED LIVER DAMAGE IN MALE WISTAR RATS



Values are expressed as mean ± standard error of mean where n = 8

1. Significant as compared with Control : p < 0.05
2. Significant as compared with Rutin group: p < 0.05
3. Significant as compared with DMN group: p < 0.05

Fig 3.15 Shows significant decrease (p 0.05)in the level of TSH level in animals treated with DMN alone as compared with control.

There is also a significant decrease (p 0.05) in the level of NO activity in those animals treated with 10mg/kg of DMN and 25mg/kg of rutin and those treated with 10mg/kg DMN and 50mg/kg of Rutin as compared with those animals treated with DMN only .There is a significant increase in animals treated with Rutin as compared to control.

EFFETS OF RUTIN ON GPx ACTIVITY IN DMN-INDUCED LIVER DAMAGE IN MALE WISTAR RATS



Fig 3.16

Values are expressed as mean ± standard error of mean where n = 8

1. Significant as compared with Control : p < 0.05
2. Significant as compared with Rutin group: p < 0.05
3. Significant as compared with DMN group: p < 0.05

Fig 3.16 Shows significant increase (p 0.05)in the level of GPx activity in animals treated with DMN alone as compared with control.

There is also a significant decrease (p 0.05) in the level of NO activity in those animals treated with 10mg/kg of DMN and 25mg/kg of rutin and those treated with 10mg/kg DMN and 50mg/kg of Rutin as compared with those animals treated with DMN only .There is no significant increase in animals treated with Rutin as compared to control.

**EFFECTS OF RUTIN ON LPO LEVEL IN DMN-INDUCED LIVER DAMAGE IN MALE WISTAR RATS**



Fig 3.17

Values are expressed as mean ± standard error of mean where n = 8

1. Significant as compared with Control : p < 0.05
2. Significant as compared with Rutin group: p < 0.05
3. Significant as compared with DMN group: p < 0.05

Fig 3.17 Shows significant decrease (p 0.05)in the level of TSH level in animals treated with DMN alone as compared with control.

There is also a significant decrease (p 0.05) in the level of NO activity in those animals treated with 10mg/kg of DMN and 25mg/kg of rutin and those treated with 10mg/kg DMN and 50mg/kg of Rutin as compared with those animals treated with DMN only .There is a significant increase in animals treated with Rutin as compared to control.

**EFFECTS OF RUTIN ON SERUM CREATININE LEVEL IN DMN-INDUCED LIVER DAMAGE IN MALE WISTAR RATS**



Fig 3.18

Values are expressed as mean ± standard error of mean where n = 8

1. Significant as compared with Control : p < 0.05
2. Significant as compared with Rutin group: p < 0.05
3. Significant as compared with DMN group: p < 0.05

Fig 3.18 Shows significant decrease (p 0.05)in the level of TSH level in animals treated with DMN alone as compared with control.

There is also a significant decrease (p 0.05) in the level of NO activity in those animals treated with 10mg/kg of DMN and 25mg/kg of rutin and those treated with 10mg/kg DMN and 50mg/kg of Rutin as compared with those animals treated with DMN only .There is a significant increase in animals treated with Rutin as compared to control.

**EFFECTS OF RUTIN ON THE LEVEL OF SOD ACTIVITY IN DMN-INDUCED LIVER DAMAGE IN MALE WISTAR RATS**



Fig 3.19

Values are expressed as mean ± standard error of mean where n = 8

1. Significant as compared with Control : p < 0.05
2. Significant as compared with Rutin group: p < 0.05
3. Significant as compared with DMN group: p < 0.05

Fig 3.19 Shows significant decrease (p 0.05)in the level of SOD activity in animals treated with DMN alone as compared with control.

There is also a significant decrease (p 0.05) in the level of SOD activity activity in those animals treated with 10mg/kg of DMN and 25mg/kg of rutin and those treated with 10mg/kg DMN and 50mg/kg of Rutin as compared with those animals treated with DMN only .There is a significant increase in animals treated with Rutin as compared to control.

**EFFECTS OF RUTIN ON GST ACTIVITY IN DMN-INDUCED**

**LIVER DAMAGE IN MALE WISTAR RATS**



**Fig 3.20**

Values are expressed as mean ± standard error of mean where n = 8

1. Significant as compared with Control : p < 0.05
2. Significant as compared with Rutin group: p < 0.05
3. Significant as compared with DMN group: p < 0.05

Fig 3.20 Shows significant increase (p 0.05)in the level of GST activity in animals treated with DMN alone as compared with control.

There is also a significant decrease (p 0.05) in the level of NO activity in those animals treated with 10mg/kg of DMN and 25mg/kg of rutin and those treated with 10mg/kg DMN and 50mg/kg of Rutin as compared with those animals treated with DMN only .There is a significant decrease in animals treated with Rutin as compared to control.

**CHAPTER 4**

4 CHAPTER 4

4.1 DISCUSSION AND CONCLUSION

4.1.1 DISCUSSION

As shown from the copius evidences, Dimethylnitrosamine (DMN) is a highly toxic and potent carcinogenic compound belonging to the class of N-nitrosamines. A proven potent renal toxicant and is associated with the development of kindney abnormalities in humans and animals. DMN has been extensively studied for its carcinogenic properties, particularly its role in kidney damage

Being classed as a Group 2A carcinogen by the International Agency for Research on

Cancer (IARC), it’s mutagenicity to humans (IARC,2012) has been proven. The primary route of exposure to DMN is through ingestion, although it can also enter the body through inhalation and dermal contact (ATSDR, 2019).

Researches have shown that DMN induces DNA mutation procedures and tumor formation via the formation of reactive intermediates that can alkylate DNA and proteins (Stiborova et. al, 2018) and this ultimately result in the development of cancer (Poirier, 2005)

The kidney is one of the primary target organ for DMN-induced toxicity. Animal studies have demonstrated that chronic exposure to DMN leads to the development of renocellular carcinoma (HCC) (IRAC, 2012) Additionally, DMN has been shown to promote liver fibrosis and cirrhosis diseases of the liver, further increasing the risk of renal carcinomer (Chen and Senthilkumar, 2019).

Strangely, DMN can also be synthesised in the human body via endogenous

Pathways which involves Nitrosation of secondary amines, such as dimethylamine, in the presence of nitrite under acidic conditions, leading to the formation of DMN (Mirvish, 1995).

To minimize the risk of exposure to DMN, government policies have established limit its presence in various products. For example, the World Health Organization

(WHO) has set a provisional tolerable daily intake (PTDI) of 0.03 μg/kg of body weight for DMN (WHO, 2000).

On the other hand, rutin has been shown to have a gigantic array of pharmacological applications due to its protective properties including antioxidant, anti-inflammatory, cardiovascular, neuroprotective, antidiabetic, and anticancer activities (Al-Dhabi et al, 2015).Under normal condition, cell possess enzymatic and non-enzymatic defenses to cope with free radicals, such as reduced glutathione (GSH). Oxidative stress normally occurs when there is an imbalance between oxidants and anti-oxidative mechanisms favoring the proliferation of oxidants and free radicals, thus, leading to the oxidative damage. Hitherto, free radicals induced oxidative damage has been implicated in the part to genesis of several diseases (Freeman and Crapo, 19827).

In this our research, each animal's organ weight was divided by the body weight to get the relative organ weight which was then plotted, analysed, and interpreted. It was observered there was an increase in the kidney weights which may be associated with the induction of inercased incidences of renalcellular neoplasia

and hydronephrosy respectively or accumulation of fat or glycogen in the organs. The exposure of animals to liver enzyme inducers and toxicants may increase the liver and kidney weights which go in line with Alin's research in 2018 on the causes of weight gain in organs (Alin, 2018).

AST, ALT and GGT are important biomarkers in accessing the status or the health of liver. Increased levels or activities of these enzymes act an indicative of liver dysfunction. In our study,

Rutin and dimethylnitrosamine increased the levels of ALT and AST and decreased the levels of GGT which shows that these compounds are hepatotoxic.

An antioxidant may be defined as a substrate which, when present at low concentration compared to an oxidizable substrale, such as fats, protein carbohydrates or DNA significantly delays or prevents the oxidation of the substrate (Halliwel, 1990), They are important in regulating cell signaling. In the presence of a toxicant, antioxidant levels might repress resulting in increase in reactive oxygen species which drives different kinds of organ damage or malfunction (Bakhiya,

1. .

However, distortion in the system of the kidney can be due to the presence of a known toxicant. In this study, Rutin and DMN results in the decrease or increase in activities of reactive oxygen species. This indicates that Rutin and DMN can regenerate excessive oxidant in a system which alters the redox system in the liver and the liver can be damaged (Subhan, 2012).

In addition, catalase (CAT) is involved in the detoxification of hydrogen peroxide either generated by a prior step involving SOD from superoxide radical or generated from other means. In this study, we observed that when the rats were treated with the toxicants, the amount of H202 consumed increased due to the ability of Rutin and DMN to generate reactive oxygen species such as H202.

SOD being the first line of antioxidant defense system, converts superoxide radicals to form hydrogen peroxide and consequently preventing the deleterious effects of the oxygen radicals.

SOD activity was increased in the kidney of rats exposed to Rutin and DMN . The activities of SOD were increased in the co-administered groups. This study shows that SOD activity was increased in the kidney and toxicants are reduced in the kidney.

The most valuable peroxidase, GPx responsible for the detoxification of hydroperoxides. It catalyzes GSH dependent reduction in hydroperoxides and hydrogen peroxide. In the present study the concentrations of GPx and GST are significantly increased in the kidney. Both GPx and GST are reduced in the toxicant when compared with the normal control group which goes in line with the

report given by Orisakwe in 2014.

Glutathione , a known tripeptide observerd to act as a non enzymatic antioxidant by direct interaction or is thiol group with reactive oxygen species and higly essential in maintaining the cellular integrity of the red blood cells (RBC). In healthy cells and tissues, more than 90% of the total glutathione pool is in the reduced form (GSH) and less than 10%% exists in the disulfide form (GSSG). GSH is capable of reducing any disulfide bond formed within cytoplasmic proteins to cysteine by serving as electron donor. From this study, GSH and TSH levels were decreased in the

kidney and reduced in the toxicants when compared to the control group. GSH decreasing and GST increasing at the same time may be connected to an increased use of GSH to defend them from oxidative stress, by suppression of both redox cycling and free radical reproduction as reported by Hanigan, M. H. in 1998 during his research on glutathionase expression and function in carcinogenesis.

Lipid peroxidation (LPO) is assessed by measuring the malondialdehyde (MDA) level. Initiation of LPO is caused by attack of any specie that has sufficient reactivity to abstract a hydrogrn atom from a methylene group upon a poly unsaturated fatty acid (Sodergen, 2002).RONS are byproducts of the normal metabolism of oxygen and they function in cell signaling and homeostasis. They are beneficial to cells supporting basic cellular processes and viability RONS and LPO serve as markers for oxidative stress. From this study, the activities of RONS and LPO are increased in the kidney of rats treated with Rutin and dimethylnitrosamine. They show significant increase in the toxicants compared to the control groups.

Xanthine Oxidase (XO) generates reactive oxygen species. The enzyme catalyzes the oxidation of hypoxanthine to xanthine and can further catalyze the oxidation of xanthine to uric acid which is excreted by kidneys. This study shows that XO activities are increased in the liver and activity increases in the co-treated groups compared to the control.

Urea and Creatine levels are used to evaluate the function of the kidney as they serve as indicators of kindneyinjury. Urea test measures the amount of urea nitrogen in your blood. This study evaluated the kidneys of male Wistar rats treated with Rutin and DMN and the result showed as increase in the levels of urea and creatine across the cohorts except the control group alone which shows a decrease.

Lipid peroxidation (LPO) is assessed by measuring the malondialdehyde (MDA) level. Initiation of LPO is caused by attack of any specie that has sufficient reactivity to abstract a hydrogen atom upon a polysaturated fatty acid (Sodergen, 2002).

Nitric Oxide (NO) can act as a cardiovascular signaling molecule while Myeloperoxidase (MPO) is a key element of the immune system which provides defence against invading pathogens.

They both suggest the presence of inflammation. MPO and NO activities in the liver of animals

treated with Rutin and DMN are increased in co-treated groups when compared to control but high

dose has a more pronounced effect according to this study.

4.1.2 CONCLUSION

Finally, we have conclude that we have established this fact demonstrating the implications of Rutin and DMN in renal totoxicity and in accordance

with the results of this study, the co-exposure of Rutin and DMN increases renal damage. Hence, controlled exposure of dimethylnitrosamine should be monitored as its combination with Rutin may affect renal functions in male Wistar Albino rats which could result in infertility.