EVALUATION OF ANTIOXIDANT ACTIVITY OF *CARICA PAPAYA* LEAVES

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DECLARATION

I hereby declare that the project titled 'EVALUATION OF ANTIOXIDANT OF *CARICA PAPAYA* LEAVES' submitted to BIOCHEMISTRY PROGRAMME of the Department of BIOLOGICAL SCIENCES, COLLEGE OF BASIC AND APPLIED SCIENCES, MOUNTAIN TOP UNIVERSITY, Ogun State under the supervision of Mrs. O.O AYODELE is my research project report, which has been acknowledged in the text and the list of the references provided.

CERTIFICATION

This is to certify that research project report titled: EVALUATION OF ANTIOXIDANT ACTIVITY OF *CARICA PAPAYA* LEAVES was submitted by AJAYI OLUWATOYE with matriculation number; 15010102003 of Biochemistry in the Department of Biological Sciences, in partial fulfilment of the requirement for the award of Bachelor of Science (B.sc) degree in Biochemistry.

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DEDICATION

I would like to dedicate this project to the ALMIGHTY GOD for helping me reach a successful completion of this work.

ACKNOWLEDGEMENT

All thanks to God Almighty for his Grace and Mercy and for the completion of this project in good health.

My appreciations go to my Supervisor, Mrs O.O Ayodele, for her knowledge shared to me, thank you for your support, teachings and for your kind words, and always believing in me, to the Head of Department of biological sciences, Dr A.A. Adeiga for his emulating leadership personality; my father figured lecturer, Dr G.O Ajayi, for all his support and words of encouragement and fatherly love,.

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ABBREVIATIONS

C. papaya	*Carica papaya*
DNA	Deoxyribonucleic acid
H_2O_2	Hydrogen peroxide
mg	Millimeter
ml	Milliliter
ROS	Reactive Oxygen Species
SOD	Superoxide dismutase
STZ	Streptozotocin
TAC	Total antioxidant capacity
µg	Microgram

ABSTRACT

Oxidative stress is the underlying causative mechanisms for various diseases. It is an imbalance in the body between free radicals' generation and antioxidants. Oxygen-derived free radicals, which instigates damage to cellular constituents have been reported to play a decisive role in the pathogenesis of a broad spectrum of degenerative and chronic disorders such as diabetes mellitus and cancer. *C. papaya*, commonly called pawpaw is a flowering plant with succulent fruits and other components that are therapeutically and nutritionally beneficial. This study aims at evaluating the antioxidant activity of *C. papaya* leaf methanol extract in streptozotocin (STZ) induced diabetic rats.

The bioactive components from *C. papaya* leaves was extracted with 70% methanol, to get the crude extract. Qualitative phytochemical screening of the leaf extract was done, and the total antioxidant capacity (TAC) and Hydrogen peroxide radical scavenging activity of the extract were determined *in vitro* according to the methods by Prieto *et al.* (1998) and Ruch et al. (1989) respectively. Thirty (30) male wistar albino rats were randomly allocated into five (5) groups comprising of six (6) rats each; groups 1 and 2 were the control, groups 3 and 4 were the diabetic rats given 100mg/kg and 200mg/kg body weight of plant extract respectively, while group 5 were diabetic rats given the standard drug metformin. Diabetes was induced by single intraperitoneal injection of STZ (55mg/kg body weight) in citrate buffer (4.5) after fasting the animals overnight. Plasma glucose concentration in the experimental rats was determined according to glucose oxidase methods using Randox assay kit. The activities of superoxide dismutase and catalase in the plasma and liver of experimental animals were determined according to the method of Misra and Fridovich (1972) and Singha (1972) respectively.

The study showed that *C. papaya* significantly reduced the plasma glucose concentration in the diabetes treated groups. The total antioxidant capacity and hydrogen peroxide radical scavenging activity increases with reducing concentration of extract. The highest activities were recorded at the concentrations of 0.2mg/ml for TAC and 0.1mg/ml for H_2O_2 radical scavenging activity. There was no significant difference ($p > 0.05$) in SOD and catalase activities in the plasma and liver of experimental rats. Qualitative phytochemical screening of the plant extract showed the presence of flavonoids, phenol, polyphenols etc. with reported antioxidant activities.

This study shows that *C. papaya* leaves possess *in vitro* antioxidant and hypoglycaemic activities. Further research on *in vivo* antioxidant assays need be carried out to validate the results and to clearly understand the mechanism of antioxidant action of *C. papaya*.

Keywords: Oxidative stress, Ascorbic Acid, Hydrogen peroxide, SOD, Catalase, Antioxidant

CHAPTER 1

INTRODUCTION

1.1 Background to the study

Oxidative stress is an imbalance in the body between free radicals and antioxidants. Free radicals are molecules that have irregular electrons containing oxygen. The irregular amount makes it easy for them to interact with other molecules. Free radicals in the body can trigger great chemical chain responses because they interact with other molecules so readily. These responses are referred to as oxidation. They may be advantageous or dangerous (Martindale and Holbrook, 2002). Oxidation is a standard and essential method in the body. On the other side, oxidative stress is associated with an imbalance of free radical activity and antioxidants activity. Free radicals can assist combat pathogens when they function correctly. (Agarwal *et al.*, 2014).

Oxidative stress is the underlying causative mechanisms for various diseases. If there are more free radicals than antioxidants can keep them in balance, free radicals can cause fatty tissue, DNA, and protein damage in the body. A large part of the body consists of macromolecules, so that damage, can cause a wide number of diseases over time, which include: diabetes, cancer, atherosclerosis, heart disease, neurodegenerative diseases, such as Parkinson's and Alzheimer's. Oxidative stress also contributes to aging (Agarwal, 2014). Many traditional medicines played a major part in global health care. About 80% of Africans depend on herbal medicines (Okigbo and Mmeka, 2008).

C. papaya belongs in the Caricaceae family. It is a plant rich with antioxidants and phytochemicals, known for its fragile and unbranched soft stem, which yields copious latex and a cluster of lengthy, big stalked leaves. The leaves are traditionally used for the therapy of a large

spectrum of diseases such as malaria, jaundice and antiviral illnesses (Vijay *et al.,* 2014). Papaya can prevent oxidative stress and inhibit the activities of reactive oxygen species, and has been employed to treat distinct health problems such as diabetes mellitus, gastro intestinal tract disorders, etc in traditional medicine (Parle *et al.*, 2011).

1.2 Statement of research problem

Oxidative stress, being the underlying causative mechanisms for various diseases, is evaluated by testing the antioxidant activity of *C. papaya* methanolic extract on oxidative stress in diabetic male mammals.

1.3 Objectives of study

The main objective of this study is to investigate the *in vitro* and *in vivo* antioxidant activity of *C. papaya* leaf methanol extract in Streptozotocin (STZ) induced diabetic rats.

The specific objectives are to:

1. determine the phytochemical constituents of *C. papaya* leaf methanol extract.
2. determine the effect of *C. papaya* leaf methanol extract on blood glucose concentration of diabetic rats.
3. determine the Total antioxidant capacity and Hydrogen peroxide (H_2O_2) radical scavenging activity of *C. papaya* leaf methanol extract
4. determine the effect of *C. papaya* leaf extract on Superoxide dismutase (SOD) and Catalase activities in diabetic rats

1.4 Justification of study

In this study the effect of oxidative stress and the mechanism of action will be further evaluated in relation to antioxidants that scavenging reactive oxygen species linked to the cause of oxidant stress and the frequency of antioxidant scavenging and enzyme inhibition in *C. papaya*.

1.5 Significance of study

This study is conducted to evaluate the antioxidant activity of the *C. papaya* plant extract against streptozotocin induced oxidatively stressed diabetic male rats.

CHAPTER 2

LITERATURE REVIEW

2.1 History of *C. papaya* (pawpaw)

C. papaya, also called papaw or pawpaw, is a succulent fruit of a large plant of the family Caricaceae. Though its origin is somewhat vague, the papaya may represent the fusion of two or more species of Carica native to Mexico and Central America. *C. papaya* has its origin in tropical America, ranging from Southern Mexico to the Andes of South America. It was spread to the south by Indians, and throughout the Caribbean with Spanish exploration. The Spanish also took it to Europe and the Pacific Islands. By the mid-17th century, papaya was pantropically distributed (Nurul, *et al.,* 20012).

Currently it is cultivated throughout the tropical world and into the warmest parts of the subtropics. The fruit is somewhat sweet, with an affable musky flavour, which is more distinct in some diversities and in some climates than in others. It is a prevalent breakfast fruit in numerous countries and is also used in juices, salads, pies, and confections. The immature fruit can be prepared like squash (Nurul, *et al.,* 2012).

2.1.1 General description of the plant

The fruits are usually spherical to cylindrical in shape, are between 75 to 500 mm (3 to 20 inches) or longer, and sometimes weighs as much as 9 to 11.5 kg (20 to 25.5 pounds). The flesh is extremely juicy and deep-yellow or salmon-coloured orange. Numerous, red and wrinkled black seeds are fixed along the walls of the wide main cavity (Petruzello, 2019).

Figure 1.1: Pictures of *C. papaya* plant and fruits. (Petruzello, 2019).

2.1.2 Botanical Description of the plant

Pawpaw is big, single-stemmed herbaceous perennial, to 30 ft, <20 ft in cultivation. Leaves are very large (up to 2 ½ ft wide), palmately lobed or intensely incised, with entire margins, and petioles of 1-3.5 ft in length. Stems appear as a stalk, are sunken, light green to tan brown, up to 8″ in diameter, and bear protuberant leaf marks.

Plants are dioecious, with cultivars producing only female or bisexual (hermaphroditic) flowers favoured in agriculture. Sometimes, *C. papaya* is alleged to be "trioecious" denoting that separate plants yield either male, female, or bisexual flowers. Female and bisexual flowers are ivory white, waxy, and carried on short peduncles laterally to the main stem in leaf axils. Flowers remain solitary or small cymes of three individuals. Position of ovary is superior. Preceding to opening, female flowers and bisexual flowers are shaped like a pear and are tubular

respectively. Since bisexual plants are self-pollinating and produce the most required fruit, they are favoured over male or female plants. Male papaya is illustrious by the smaller flowers carried on long stalks (left). Female flowers of papaya (right) are pear shaped when closed, and illustrious from bisexual flowers which are cylindrical (Rieger, 2012).

2.1.3 Cultivation and storage of the plant

Cultivation: Papayas are usually grown from seed. Their development is swift, with fruit being formed before the end of the first year. Under approving conditions, a plant could live five years or more.

Storage: Below 50°F, papayas experience alarming injury. Papayas are tremendously perishable; shelf life at room temperature arrays from 3 to 8 days, liable on storage atmosphere. (Rieger *et al*, 2012).

2.1.4 Classification of the plant

Scientific classification

Kingdom	:	Plantae
Subkingdom	:	Tracheobionta
Super division	:	Spermatophyta
Division	:	Magnoliophyta
Class	:	Magnoliopsida
Phylum	:	Streptophyta
Order	:	Brassicales
Family	:	Caricaceae
Genus	:	Carica
Species	:	Papaya
Scientific name	:	*C. papaya*

2.1.5 Chemical composition of the plant

Table 2.1: Chemical constituents identified from the fraction of *C. papaya* (Oche *et al.*, 2016)

Peak no.	Chemical name	Peak area (%)	Molecular weight	Molecular formula
1	Decylene	0.36	140	$C_{10}H_2O$
2	Trans-Geranylacetone	0.17	194	$C_{13}H_{22}O$
3	Methyl tridecanoate	0.34	228	$C_{14}H_{28}O_2$
4	Palmitic acid	0.91	256	$C_{16}H_{32}O_2$
5	Myristic acid, methyl ester	0.73	242	$C_{15}H_{30}O_2$
6	Myristic acid	2.06	228	$C_{14}H_{28}O_2$
7	Palmitic acid, methyl ester	7.62	270	$C_{17}H_{34}O_2$
8	Hexadecanoic acid	16.18	256	$C_{16}H_{32}O_2$
9	Linolelaidic acid, methyl ester	5.07	294	$C_{19}H_{34}O_2$
10	Methyl cis-6-octadecenoate	7.98	296	$C_{19}H_{36}O_2$
11	Stearic acid, methyl ester	3.54	298	$C_{19}H_{38}O_2$
12	Oleic acid	28.98	282	$C_{18}H_{34}O_2$
13	Stearic acid	7.29	284	$C_{18}H_{36}O_2$
14	15-Tetracosenoic acid	3.53	380	$C_{25}H_{48}O_2$
15	Methyl heptacosanoate	2.44	424	$C_{28}H_{56}O_2$
16	trans-13-Docosenoic acid	4.05	338	$C_{22}H_{42}O_2$
17	Methyl erucate	3.74	352	$C_{23}H_{44}O_2$
18	Methyl behenate	1.15	354	$C_{23}H_{46}O_2$
19	Heneicosanoic acid, methyl ester	2.08	340	$C_{22}H_{44}O_2$
20	Farnesyl cyanide	1.78	410	$C_{30}H_{50}$

The comestible portion of the fruit of *C. papaya* (pawpaw) contains both micro and macro minerals like: Na, K, Ca, Mg, Fe, Cu, Zn and Mn ((Bari et al., 2008). The plant is a source of carotenoids, vitamin C, thiamine, riboflavin, niacin, vitamin B6 and vitamin K; (Adetuyi *et al*., 2009). The leaves contain huge amounts of alkaloids, pseudocarpine and carpaine which creates positive effects on heart as well as on respiration. Leaves extract of *C. papaya* is well recognised as an anti-tumour agent (Vyas, 2014). The leaves also contain many active compounds that can reduce the level of lipid peroxidation and increase the power of total antioxidant in the blood such as chymopapain, cystatin, ascorbic acid, flavonoids, tocopherols glucosinolates and cyanogenic glucosides (Noriko Otsuki *et. al*., 2010)

2.1.6 Uses of the plant

The papaya plant carries a versatile fruit that is both mature and unripe. Young papaya leaves in some cuisines are consumed like a vegetable, and papaya leaf tea has an extensive range of presumed medicinal effects. *C. papaya* also has some traditional and medicinal use (Aravind, 2013).

Cancer

According to the research released in the ' Journal of Ethnopharmacology, ' Papaya leaf may demonstrate efficiency as a cancer prevention and therapy. The scientists indicated that their preliminary research findings show the immune-modulated characteristics of papaya leaf that may be of use in the therapy and avoidance of illnesses such as cancer, allergies and the components of certain vaccines (Krishna, 2008).

Ulcer

Papaya leaf tea protects against gastric ulcer in a study on laboratory animals published in the "West Indian Medical Journal Research reports have shown that papaya leaf tea shows potential for the treatment of gastric ulcer and oxidative stress on the stomach (Mark, 2012).

Gluten digestion

The enzyme papain in papaya leaves contributes in the protein digestion and is beneficial for treating gastrointestinal disorders. Papaya leaf tea can assuage the discomforts of heartburn and it stimulates appetite. Papaya leaf tea could also help digest gluten which is a wheat protein, which is problematic for some people to digest and causes an autoimmune disorder referred to as coeliac disease. This area still needs more research, so people with coeliac disease should not use papaya leaves to treat their condition.

2.2 Oxidative stress

Oxygen-derived free radicals, which instigates damage to cellular constituents have been accepted to play a decisive role in the pathogenesis of an extensive range of degenerative and chronic disorders (neurodegeneration cancer, aging, atherosclerosis, cataract), as well as in intense clinical conditions (Sadek, 2012).

Oxidative stress is the precursor to oxidative damage. Oxidative stress happens when there is a disproportion between free radicals' production and the body's ability to counteract their destructive effects through nullification with antioxidants. Oxidative damage is the impairment sustained by cells and tissues that are not able to carry on with the production of free radical (Kresser, 2018).

2.2.1 Oxidative damage

A free radical is an uncharged ion, molecule, or atom with an unpaired electron in its outermost valence shell. In an expedition to fill their partially empty valence shells, free radicals move about the body larceny electrons from other atoms in the cells and tissues. This electron-larceny activity, oxidatively harm proteins, cells, and DNA and is known as an underlying factor in many chronic diseases, including autoimmune disease, cardiovascular disease, and type 2 diabetes. (Kresser, 2014).

According to the scientific works, three principal factors initiate and spread oxidative damage: deficient antioxidant status, consumption of rancid fats and oxidative stress.

Insufficient antioxidants

Insufficiency of antioxidants leads to oxidative damage. Antioxidants Bergh circulating lipids, cells, tissues and cell membranes from oxidative damage. Obtaining of antioxidants from a nutrient-filled diet rather than supplements is preferable. In fact, studies investigating the effects of antioxidant supplements specify that they have no advantage and may well cause harm. There are several elucidations for this astounding phenomenon:

- Antioxidants in nutriments are packed with enzymes and cofactors that augment their action and can be better absorbed than artificial antioxidants.
- Additional compounds in antioxidant-rich foods might play dynamic roles in the antioxidant effects of nutrient-filled foods, creating effects that cannot be imitated with a synthetic, remote antioxidant (Kresser, 2014).

Other brilliant sources of antioxidants include, glutathione, vitamin E, and the antioxidant enzyme superoxide dismutase.

Rancid vegetable oils

The ingesting of rancid dietary fats compromises cell membrane health and encourages oxidative damage. The principal sources of rancid fats in the Average Human Diet are industrial vegetable oils. Omega-6 polyunsaturated fatty acids (PUFAs) are high in industry vegetable fats, including canola, soybeans, seed and safflower oils. The omega-6 PUFAs remain sensitive and very prone to heat and light damage. Unfortunately, omega-6 PUFAs expose themselves to thermal, metal and other chemical substances in the very method by which industrial vegetable oils are produced, causing oxidative damage to the fatty acids and producing "rancid" fats. But the damage does not stop there; when heated during the cooking process, vegetable oils are further oxidized (Kresser, 2014).

Unsaturated fat oxidation in industrial oils leads to advanced lipid oxidation end products (ALEs) that pose a major danger for human health (Kanner, 2007).

The ALEs also co-oxidize the body's antioxidant store, which depletes the vitamins A, C and E. The combination of swelling and depletion of antioxidant induced by the intake of industrial vegetable oils spreads an oxidative harm chain reaction in the body (Kresser, 2014).

2.2.2 Other oxidative damage causes

Chronic Psychological Stress

Being anxious than ever before about finances, politics, health, safety, and relationships. (Ballal *et. al.*, 2010) The chronic psychological stress fight not only reduces living conditions but also encourages oxidative damage by continuous initiation of the HPA axis.

Environmental Toxins

A patient's living environment can be a significant source of oxidative stress (Allen *et al.,* 2009). Exposure to particulate air pollution in urban areas and mould and biotoxins in water-damaged buildings promotes oxidative stress by depleting antioxidant reserves. (Doi and Uetsuka, 2011).

Physical Inactivity

Sedentary living increases the stress of oxidation. Instead, regular physical activity affects the body hermetic.; It causes short-term generation of free radicals, but improves long-term output of antioxidants. (Kresser, 2014).

Iron Overload

An overaccumulation of iron in the body, a condition referred to as iron overload, is associated with the development of several chronic diseases, including diabetes and cardiovascular disease. One of the mechanisms by which iron overload promotes chronic disease is through the generation of hydroxyl free radicals, which promote oxidative stress. (Simcox and McClain, 2013).

Infections

Oral infections with microbes such as *P. gingivalis* increase oxidative damage; this may explain why periodontitis is linked to several chronic diseases, including cardiovascular and neurodegenerative disease (Abbayya *et al.*, 2015). H. pylori, hepatitis C, and Chlamydia pneumoniae infections are associated with higher levels of oxidized LDL, indicating that oxidative stress with an infectious origin plays a role in atherosclerosis. (Huang *et al.*, 2011).

2.2.3 Prevention of oxidative damage

Oxidative damage can be prevented by the avoidance of rancid vegetable oils, steering clear of packaged, processed foods, eating an antioxidant-rich, whole-foods diet. This type of diet supplies the body with the antioxidants and cofactors it needs to combat oxidative stress.

The total abstinence from cigarettes. Engaging in daily stress-reduction practices. Meditation, yoga, spending time in nature, and taking “technology breaks” alleviate chronic stress, which causes oxidative stress when allowed to continue unabated (Aschbacher, 2013)

Environmental toxin exposure can also be avoided. Stopping the use of pesticides on the lawns and gardens. Buying of organic food as often as possible, avoidance of storage of food in plastic containers and handling receipts, safely eliminating sources of heavy metal exposure such as dental amalgams, and filter drinking and bathing water.

Treating infections can also prevent oxidative stress. Chronic infections are a significant cause of oxidative stress and must be addressed to halt the free radical cascade.

Address iron overload. curcumin and green tea are two options for attenuating iron overload-induced oxidative damage. (Elsevier, 2019).

2.3 Reactive oxygen species (ROS)

Reactive Oxygen Species (ROS) comprises the reduced form of oxygen and its reaction products with other molecules. Some but not all ROS are free radicals. ROS frequently have high reactivity due to the extra-unpaired electron at their outer shells and their half-lives are in the order of milliseconds. Hydroxyl radical and Superoxide anion are examples of radical ROS, whereas hypochlorite ion and hydrogen peroxide are non-radical ROS. The hydroxyl radical is the greatest damaging radical for the reason that it cannot be scavenged simply in our bodies. (Silva and Lima, 2014).

ROS	
Free radicals	Non-radicals
Hydroxyl radical (•OH)	Hydrogen peroxide (H2O2)
Superoxide anion (•O2−)	Singlet oxygen (1O2)
Lipid peroxyl (•LOO−)	Ozone (O3)
Thiyl (•RS)	Lipid peroxide (LOOH) Peroxynitrite ($ONOO^-$)

Table 2.2: Radical and non-radical ROS (Silva and Lima, 2014).

Types of Reactive Oxygen Species

Numerous ROS are created as by-products through mitochondrial electron transport. In addition, ROS are formed as required intermediates of metal catalysed oxidation reactions. Atomic oxygen has two unpaired electrons in distinct orbits in its outer electron shell. This electron structure makes oxygen vulnerable to radical formation. The sequential reduction of oxygen through the addition of electrons leads to the development of a number of ROS including: superoxide; hydrogen peroxide; hydroxyl radical; hydroxyl ion; and nitric oxide.

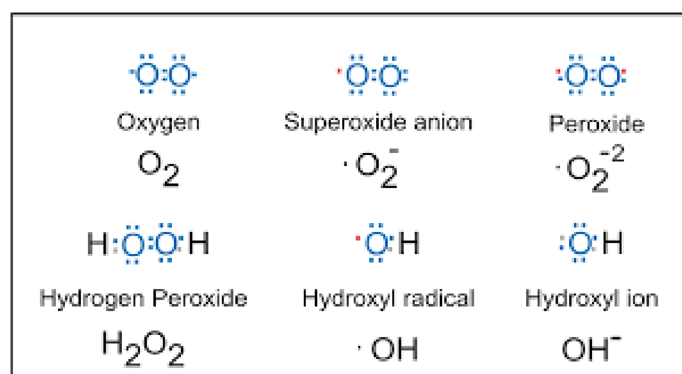

Figure 2.1: Types of ROS (Frey, *et al.,* 2009).

2.3.1 Physiological roles of ROS

During normal cell metabolism, ROS are created. They contribute in many physiological procedures, including immune system activation. For instance, activated phagocytes release hydrogen peroxide as a portion of the immune system to kill pathogens. Also recognised to play a part in fighting infections is superoxide anion (Frey *et al.,* 2009). ROSs released through fibroblast, nonphagocytic cells, including endothelial and cardiac myocytes, have been used to control numerous intercellular signalling pathways. These cells have diverse isoforms of NADPH oxidase. In addition, in hyperactivation, sperm capsulation, acrosome response and sperm-oocyte fusion, physiological ROS is also needed (Droge, 2002).

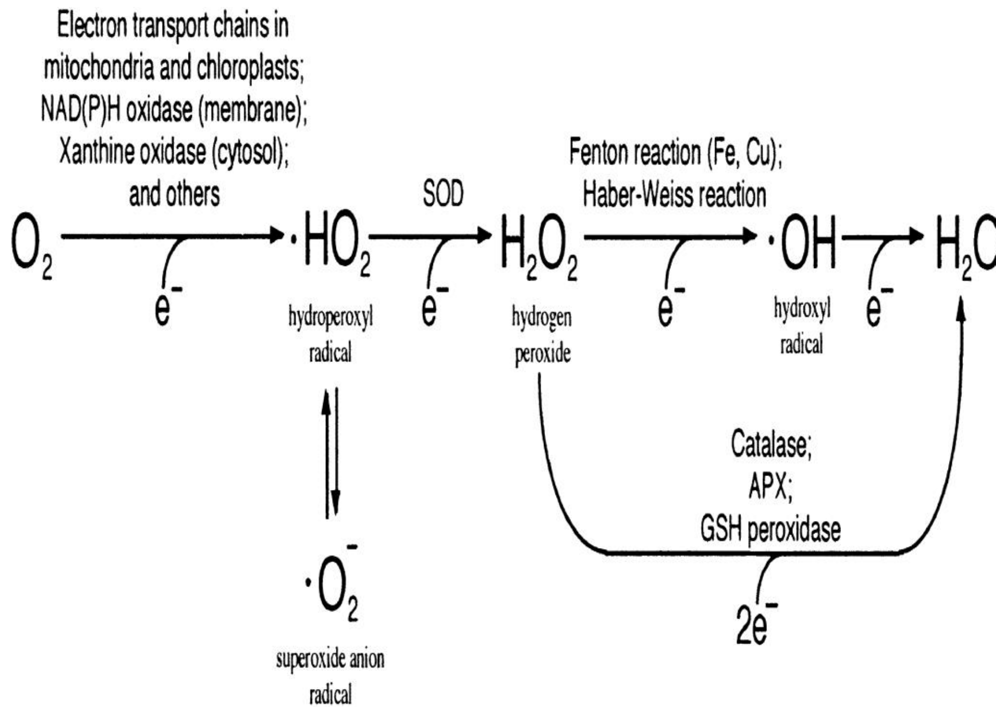

Figure 2.2: Electron transport of ROS (Droge, 2002)

2.4 Streptozotocin (STZ)

Streptozotocin is a chemotherapeutic alkylating agent and has also been used as antimicrobial agent. STZ is a glucosamine-nitrosourea compound that is toxic and causes damage to the cells of the DNA. DNA damages encourages the initiation of Poly Adenine diphosphate Ribose

Polymerase (PARP) which is likely more substantial for diabetes induction than the DNA damage itself.

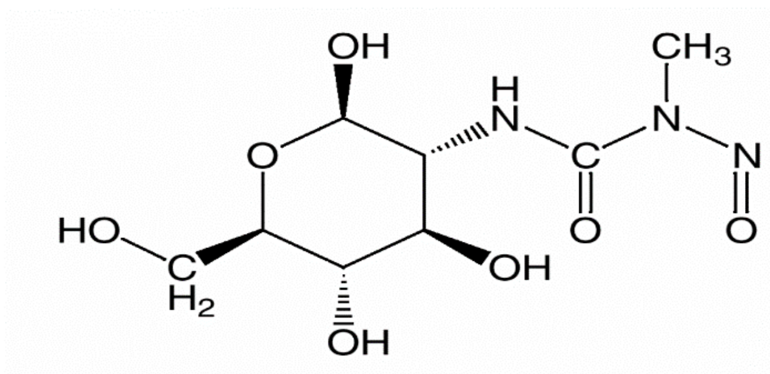

Figure 2.3: chemical structure of STZ

2.5 Treatment of diabetes

Pharmaceutical drugs like biguanides and sulfonylureas are used for the treatment of diabetes but these have objectionable side effects or contraindications or are either too affluent (Debidas, *et al.,* 2005). Metformin is a common anti diabetic drug used as the standard drug for this study.

2.5.1 Metformin

Metformin is a biguanide that has a strong base with a pKa of 12.4, and consequently exists principally as a protonated cation at physiological pH. Despite its hydrophilicity, metformin can be transported across cell membranes via organic cation transporters (OCT) (Mustafa, *et al.,* 2015).

The molecular action of this drug has not been clearly recognized. However, the mainstay of action of this drug can be accredited to the hepatic effects. Hepatic sensitivity to insulin is augmented thereby reducing gluconeogenesis and glycogenolysis which contributes to the post prandial plasma glucose-lowering effects (Shaw, *et al.,* 2005).

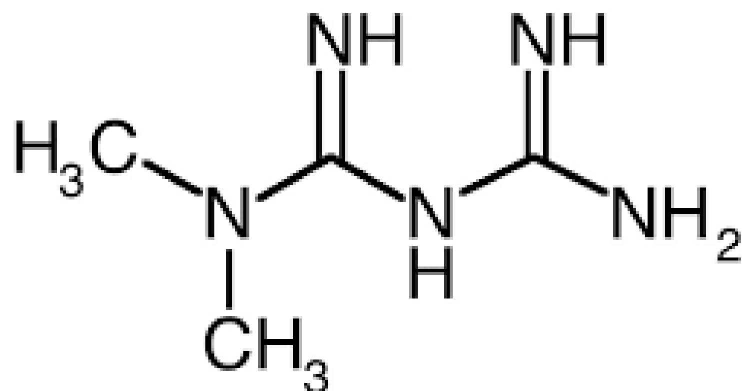

Figure 2.4: chemical structure of metformin

CHAPTER THREE

METHODOLOGY

3.1 Materials and Reagents

Test tubes, Test tube racks, Beaker, Volumetric flask, weighing balance, Filter Paper, Funnel, Dropper, Fume Cupboard, Rotary Evaporator, Spatula, Water Bath, Nose mask, Eye googles, Hand gloves, Distilled Water, H_2SO_4, 1%HCL, 3.5%FeCl, Glacial Acetic Acid, Chloroform, Potassium Iodide, Mercuric chloride, Ammonium, Acetic Anhydride, 1% Potassium Ferricyanide, Potassium Hydroxide, 10%NaOH, Benedicts' reagent, and α-Naphthol.

3.2 Collection and preparation of plant materials

The Male species of fresh pawpaw leaves (*C. papaya)* was plucked directly from trees at a local farm at Amofe Adetoye Street, Ayobo, Lagos, in January 2019, and was authenticated in the Department of botany, Faculty of biological sciences, University of Lagos. Fresh and clean water was used to wash the leaves, so as to remove soil content, dirt and dust, after which they were dried in a hot air oven at 40^oC.

After drying to a crispy state, a laboratory blender was used to pulverize the leaves. The powder was then kept in a translucent glass jar and refrigerated at 4^oC until procedure.

Fine powder of the papaya leaves was soaked in 320 ml of 70% methanol, by dissolving 70g of the fine powder of papaya in a jar with the methanol which was then tightly closed and intermittently shaken over a 3-day space to permit a better interaction between the solvent and the plant. This procedure is termed maceration.

3.3 Filtration

The mixture of the methanol and plant was sieved using a sterile cotton over an empty beaker. The filtrate was then gathered into a beaker and the residues as well into another beaker. After filtration the filtrate was allowed to settle, mixed and then filtered again, this was done twice to ensure fine filtration.

3.4 Concentration

A rotary evaporator machine was used to concentrate the filtrate, which had a water bath attached. The tube or flask containing the filtrate was suspended in the water bath which was set to a temperature of $45^{o}C$ which is the boiling point of the solvent and that is methanol. At the conclusion of the concentration, the rotary evaporator had successfully separated the solvent from the crude extract, the crude extract was then collected in a beaker and dried to completeness in the oven at $40^{o}C$, after which it was weighed to get the percentage yield.

3.5 Phytochemical screening

Analysis of the leaf quantitatively to identify these chemicals:

1. Carbohydrates
2. Alkaloids
3. Saponin
4. Terpenoids
5. Protein
6. Phenols
7. Polyphenols
8. Fat & Oil
9. Phytosterol
10. Tannin
11. Flavonoids
12. Glycosides

3.6 Qualitative phytochemical analysis

3.6.1 Test for Carbohydrates

5ml of distilled water was used to dissolve 0.5g of the extract, it was then Filtered to the filtrates obtained. Benedict and Molisch's tests were carried out

3.6.1.1 Benedict's Test

2ml of filtrate was placed in a clean test tube, 1-2 ml of the Benedict's reagent was added. It was then heated slightly.

3.6.1.2 Molisch's Test

2ml of filtrates were placed in a test tube 2-4 drops of α-Naphthol were added. Mixture was well shaken. Few drops of concentrated H_2SO_4 were added carefully along the side of the test tube.

3.6.2 Alkaloid (Mayer's Test)

0.5g of methanolic extract was dissolved in HCL It was Filtrated. Mayer's reagent (potassium mercuric iodide) was added to the Filtrate.

3.6.3 Saponin (Froth Test)

1g of extract was dissolved in 20 ml of distilled water, solution was shaken continuously for 15 minutes

3.6.*4* Terpenoids (Salkowski Test)

2ml of chloroform was added to the extract It was then filtrated, 3 ml of H_2SO_4 was carefully added to the filtrate. Mixture was well shaken and allowed to stand.

3.6.4 Protein (Ninhydrin Test)

2ml of 0.2% Ninhydrin reagent was added to the extract, it was then heated and allowed to boil for some minutes in the water bath.

3.6.5 Phenol (Ferric Chloride Test)

1-2g of extract was dissolved in distilled water, 3-4 drops of FeCl3 solution were added to the solution.

3.6.6 Polyphenol

0.5g of methanolic extract was dissolved in 5ml distilled water, it was then heated in a water bath for 30 minutes. To the heated mixture, 1ml of $FeCl_3$ was added and 1ml of potassium Ferricyanide was added.

3.6.8 Fat & Oil (Spot Test)

A small quantity of extract was placed in-between 2 filter papers and pressed.

Oil stains on the paper indicates the presence of fat and oil.

3.6.9 Flavonoids (Alkaline reagent Test)

Add few drops of 10% NaOH solution to the extract.

3.7 Experimental animals

Male wistar albino rats were weighed and placed in a clear and dry animal cage, every morning upon entering the animal house light is switched on and by evening light is turned off hence the rats are exposed to light for 12 hours and darkness for another 12 hours daily.

A total number of 30 rats were used and divided into 5 groups with each group containing 6 rats.

Group 1 – Normal control

Group 2 – Diabetic control

Group 3 – Diabetic rats given 100mg of leaf methanol extract of *C. papaya*.

Group 4 – Diabetic rats given 200mg/kg of leaf methanol extract of *C. papaya*.

Group 5 – Diabetic rats given 100mg/kg metformin (standard drug).

Administration was done orally for a period of 14 days (2 weeks).

3.7.1 Induction of diabetes

STZ was used to induce diabetes experimentally through intraperitoneal injection (55mg/kg body weight). STZ was dissolved in citrate buffer (0.01M, pH 4.5) to the rat, after overnight fasting (12hours after last feeding). Injected rats were returned to their cages and provided with 5% glucose solution for 12hours to overcome STZ-induced hypoglycaemia. After 72hours of STZ administration, fasting blood glucose (FBG) level was measured using a glucometer. Animals with FBG level higher than 200 mg/dL (11.1 mmol/L) were considered diabetic and were used for the study.

3.7.2 Drug administration

The administration of metformin and methanolic extract of *C. papaya* started after 72 hours of STZ administration. The extract and metformin were suspended in distilled water and administered orally. The volume of administrated extract was 1ml for each animal. The normal group and diabetic control group were administered only with vehicle.

After fourteen (14) days of administration of plant extract and metformin, blood samples and organs were collected for assays.

3.7.3. Collection and preparation of Blood plasma

After fourteen (14) days of administration of plant extract and metformin, the animals were sacrificed under chloroform anaesthesia. The blood sample was collected from eyes, venous pool and the heart. The liver and kidney were excised and washed in 0.9% NaCl for assay. Blood samples were transferred to Lithium heparin (EDTA) bottle and centrifuged at 2500g for 15mins within 1hour of collection to obtain plasma.

3.7.4 Harvesting of organs

The rats were then dissected from the abdominal to the thoracic region using dissecting scissors and forceps. Blood was collected in heparinized tubes and essential organs (kidney and liver) were harvested from the rats, rinsed in normal saline solution and kept in ice-cold cooler box.

3.7.5 Homogenization of organs

The harvested organs were cut into a weight of 0.2 g and were homogenised in 1 ml of Phosphate buffer (pH 7.4). This was then centrifuged at 4000 rpm for 10 minutes, after which the supernatants were collected into Eppendorf tubes and refrigerated at $4^{o}C$.

3.8 Assay Methods

3.8.1 Determination of blood glucose level

The glucose level was investigated, using Randox kit. Test tubes were labelled according to group and identity given to the experimental animals, along with one test tube for blank and one test tube for standard.

200µl of working reagent and standard reagent (20µl) was pipetted into a test tube This was repeated into corresponding test tubes. All the test tubes were incubated at 37°C for 10minutes and was read at a wavelength of 500nm against the blank that contained only working reagent. Concentration of Plasma glucose was obtained by calculating:

$$\frac{\text{Absorbance of sample X concentration of standard (103mg/dL}}{\text{Absorbance of standard}}$$

3.8.2 *In vitro* antioxidant assay methods.

3.8.2.1 Determination of Total Antioxidant Capacity

Total antioxidant capacity of the sample was carried out conferring to the method of Prieto *et al.,* (1998) and is grounded on the decrease of molybdenum (VI) to molybdenum (V) by the extract and the consequent formation of a green phosphate/molybdenum (V) complex at an acidic pH.

PROCEDURE

1ml of the reagent solution which consist of 0.6M sulphuric acid, 4mM ammonium molybdate and 28mM Sodium phosphate was added to 0.1ml of the sample extracts or standard solutions of (Vitamin C) ascorbic acid (20, 40, 60, 80, 100µg/ml). The reacting mixture was incubated in the water bath at 95^oC for 90mins, chilled to room temperature and using a spectrophotometer, the absorbance was read at 695nm against a blank, which consists of the reacting mixture comprising distilled water in place of the extract. The antioxidant activities of the extracts were explicated as an ascorbic acid equivalent.

3.8.2.2 Determination of Hydrogen Peroxide (H_2O_2) radical scavenging activity

The extracts ability to scavenge hydrogen peroxide was determined conferring to the method of Ruch et al. (1989).

PROCEDURE

Hydrogen peroxide solution (2 mM) was prepared in 50 mM phosphate buffer (pH 7.4).

Different concentrations (100, 300 and 500 ug/ml) of extracts were transferred into the test tubes and their volumes made up to 0.4 ml with 50 Mm phosphate buffer (pH 7.4). After addition of 0.6 ml (2 mM) hydrogen peroxide solution, tubes were vortexed and after 10 min, absorbance of

the hydrogen peroxide was read at 230 nm against a blank solution containing phosphate buffer without H_2O_2 (Ilhami *et al.,* 2005; Kumar and Kumar, 2009). The percentage H_2O_2-scavenging of extract and standard was calculated using the formula":

$$\% \text{ Scavenged}[H_2O_2] = [(A_0 - A_1)/A_0] \times 100$$

Where A_0 was the absorbance of the control, and A1 was the absorbance in the presence of the sample and standards".

3.8.3 *In vivo* antioxidant assay methods

3.8.3.1 Determination of superoxide dismutase (SOD)

In accordance with Misra and Fridovich (1972), the level for superoxide dismutase (SOD) activity was determined.

PRINCIPLE

SOD is an enzyme capable of suppressing adrenaline (epinephrine) autoxidation at pH 10, this reaction thus constitutes the basis of a simple assay. Radical superoxide (O^{-2}) is produced as a consequence of the reaction of xanthine oxidase. Its presence results in epinephrine oxidation to adrenochrome such that the level of adrenochrome produced for each O^{-2} introduced increases with increasing pH (Valerino and McCormack, 1971) as well as increasing concentration of epinephrine. The result was that SOD could inhibit a free, radical chain response involving superoxide (O^{-2}) such as epinephrine autoxidation.

PROCEDURE

9 ml of distilled water was used to dilute 1 ml of sample to make a 1 in 10 dilution. 0.2 ml of the diluted sample was added to 2.5 ml of 0.05M carbonate buffer (pH 10.2) to equilibrate in the spectrophotometer and the reaction began by the addition of 0.3 ml of freshly prepared 0.3 mM

adrenaline to the mixture which was rapidly mixed by inversion. The blank, comprises of 2.5 ml buffer, 0.3 ml of substrate (adrenaline) and 0.2 ml of water. The absorbance increase was measured at 480 nm at every 30 seconds for 150 seconds.

3.8.3.2 Determination of Catalase activity

Catalase activity was estimated following the method defined by Singha (1972).

Principle

When heated in the presence of H_2O_2, dichromate in acetic acid is concentrated to chromic acetate with the creation of perchromic acid as an intermediate that is unstable. The chromic acetate then formed is measured calorimetrically at 570 - 610 nm. Dichromate has no optical density in this region, hence, its presence in the assay mixture will not interpose with the colorimetric determination of chromic acetate. The catalase preparation further cleaves H_2O_2 at various time intervals. At a particular time, the reaction is stopped by adding a mixture of dichromate/acetic acid and the remaining H_2O_2 is quantified after heating the reaction mixture by measuring chromic acetate calorimetrically.

PROCEDURE

Colorimetric determination of H_2O_2

Briefly, in a clean test tube, 2 ml of dichromate/acetic acid was added to different amounts of H_2O_2 (10 to 100 μmoles) each. Out rightly, an unstable blue precipitate of perchromic acid was produced. It was then heated for 10 minutes in a boiling water bath, and colour change of the solution was observed to a stable green, due to the formation of chromic acetate. It was then cooled at room temperature and made up to the volume of the reaction mixture to 3 ml.

Absorbance was read at 570 nm. The concentrations of the standard were plotted against absorbance.

Test tube 1 2 3 4 5 6 7

H_2O_2 (ml) 0.00 0.10 0.20 0.30 0.40 0.50 0.60

Dichromate/acetic acid (ml) ` 2.00 2.00 2.00 2.00 2.00 2.00 2.00

Distilled water (ml) 1.00 0.90 0.80 0.70 0.60 0.50 0.40

Determination of catalase activity of samples

1 ml of sample was mixed with 19 ml distilled H_2O to give 1 in 20 dilution of the sample. The assay mixture comprised of 4 ml of H_2O_2 solution (800 µmoles) and 5 ml of phosphate buffer in a 10 ml flat bottom flask. 1ml of diluted enzyme preparation was rapidly mixed with the reaction mixture and vortexed gently at room temperature. 1 ml of the reaction mixture was pipetted and blown into 2 ml mixture of dichromate/acetic acid reagent at 60 seconds intervals. The hydrogen peroxide contents of the withdrawn sample were determined by the method described above.

3.9 Waste disposal

Tubes containing residual blood and tissue samples were incinerated, while the carcasses of experimental rats were buried in designated location.

3.10 Statistical analysis

The statistical analysis was done using Graph pad prism 6.0. The results were reported as mean ± SEM (standard error of mean). The collected data were subjected to Analysis of Variance

(ANOVA) to test for variations of the different parameters observed in the study. Test of significance was at 0.05% probability ($p<0.05$).

CHAPTER FOUR

RESULTS

4.1 Result for phytochemical screening

The qualitative phytochemical screening of *C. papaya* leaf methanolic extract presented positive results for the presence of various bioactive compounds.

Table 4.1: Qualitative phytochemical constituents of *C. papaya* leaf methanol extract

	Phytochemicals	Results
1.	Alkaloid	Positive
2.	Fat and oil	Positive
3.	Terpenoids	Positive
4.	Phenol	Positive
5.	Flavonoids	Positive
6.	Tannins	Positive
7.	Glycosides	Positive
8.	Phytosterol	Positive
9.	Polyphenol	Positive
10.	Saponin	Positive
11.	Anthraquinone	Positive

4.2 Plasma glucose determination in experimental rats

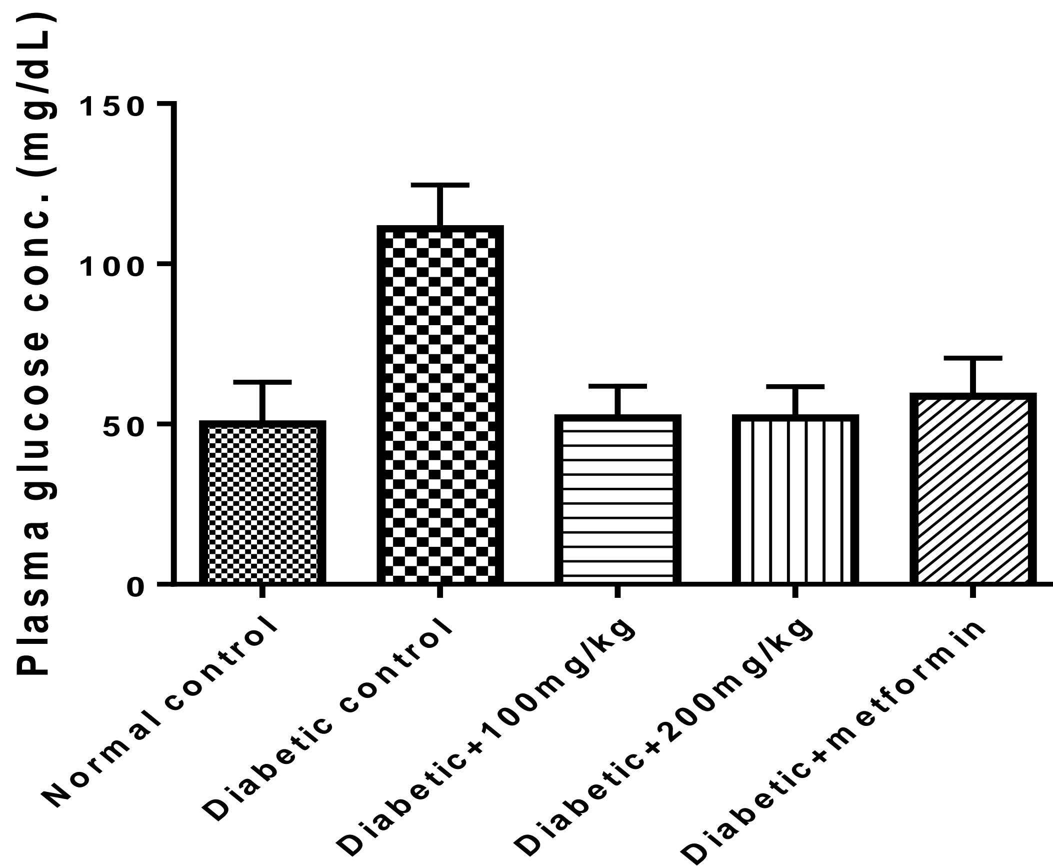

Figure 4.2: Results Plasma glucose concentration of Normal control and diabetic rats.

Data are mean ± SEM; n=4

The effect of the methanolic extract of *C. papaya* on plasma glucose concentration in figure 4.2 shows that animals that has been induced diabetically with no treatment (diabetic control) show a significant increase in plasma glucose concentration when compared with the control. However,

significantly (P<0.05) lowered plasma glucose concentrations were recorded in the C. papaya treated diabetic rats compared to the diabetic control rats.

4.4 Results of *in vitro* antioxidant assays

The results of Total antioxidant capacity and Hydrogen peroxide radical scavenging activity of *C. papaya* leaf methanol extract is shown in Table 4.2.

Table 4.2: Total antioxidant capacity and Hydrogen peroxide radical scavenging activity of *C. papaya* leaf methanol extract

Total Antioxidant Capacity		**H_2O_2 Radical Scavenging Activity**	
Concentration (mg/ml)	% Inhibition	Concentration (mg/ml)	% Scavenged
0.02	89	0.10	64.3
0.04	88	0.30	47
0.06	82	0.50	39.4
0.08	81		
0.10	75		

The total antioxidant capacity results show the percentage inhibition in concentration of methanolic extract of *C. papaya.* And there is an increase in inhibition with a decrease in the concentration. The highest percentage inhibition of 89 and 88 were recorded with concentration of 0.02mg/ml and 0.04mg/ml respectively, and a higher concentration of 0.10mg/ml gave a

lower percentage inhibition.

The Hydrogen peroxide radical scavenging activity results show the percentage scavenged in concentration of methanolic extract of *C. papaya.* And there is a decrease in scavenging activity with an increase in the concentration. The highest percentage scavenged of 64.3 was recorded with concentration of 0.01mg/ml, and a higher concentration of 0.50mg/ml gave a lower percentage scavenging activity.

4.5 Results of *in vivo* antioxidant assays

The results of SOD activities in the plasma and liver and Catalase activities in the plasma are shown in figure 4.3 to figure 4-5.

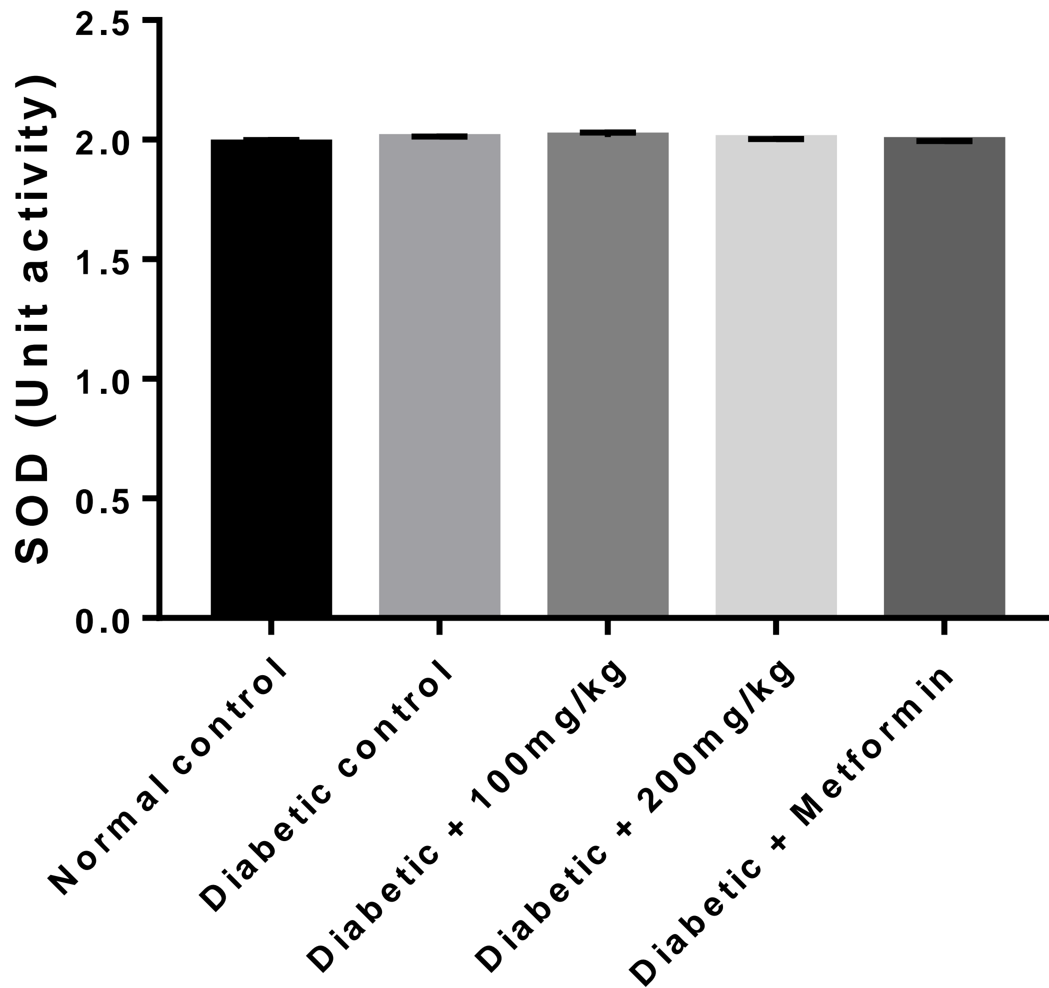

Figure 4.3: Plasma SOD activities of Normal control and Diabetic rats treated with *C. papaya* leaf methanol extract

Data are mean ± SEM; n=4

There were no significant differences (P>0.05) in SOD activities in the plasma of experimental groups.

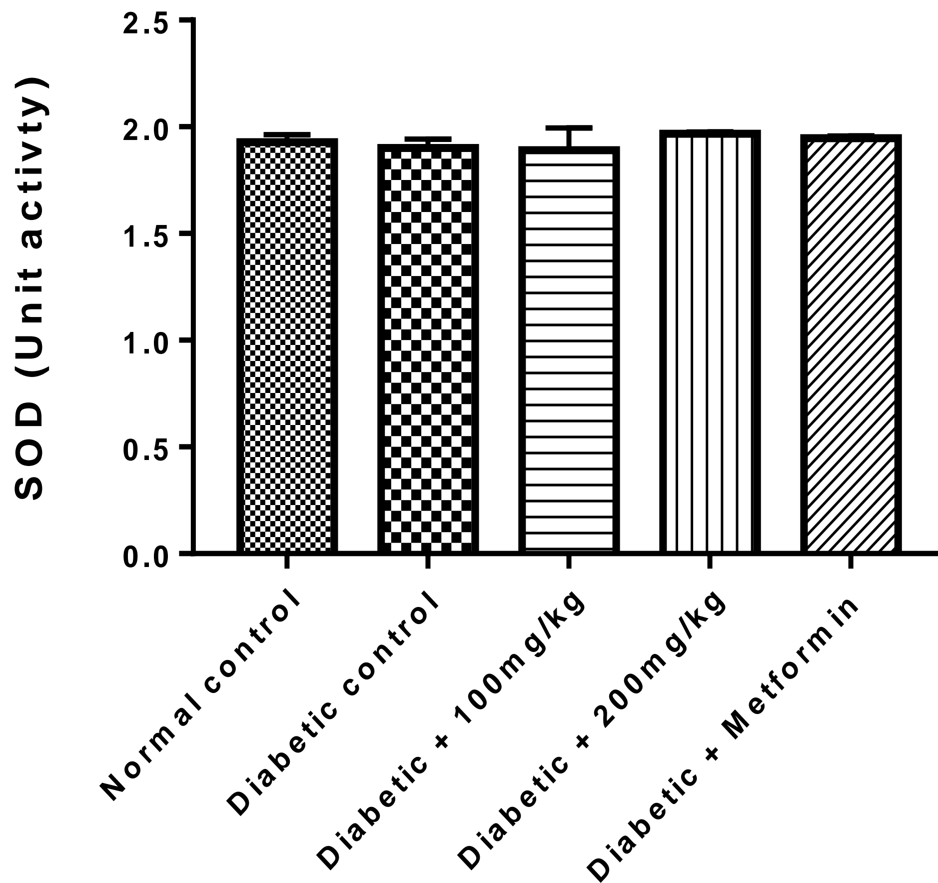

Figure 4.4: Liver SOD activities of Normal control and Diabetic rats treated with *C. papaya* leaf methanol extract

Data are mean ± SEM; n=4

There were no significant differences (P>0.05) in SOD activities in the liver of experimental groups.

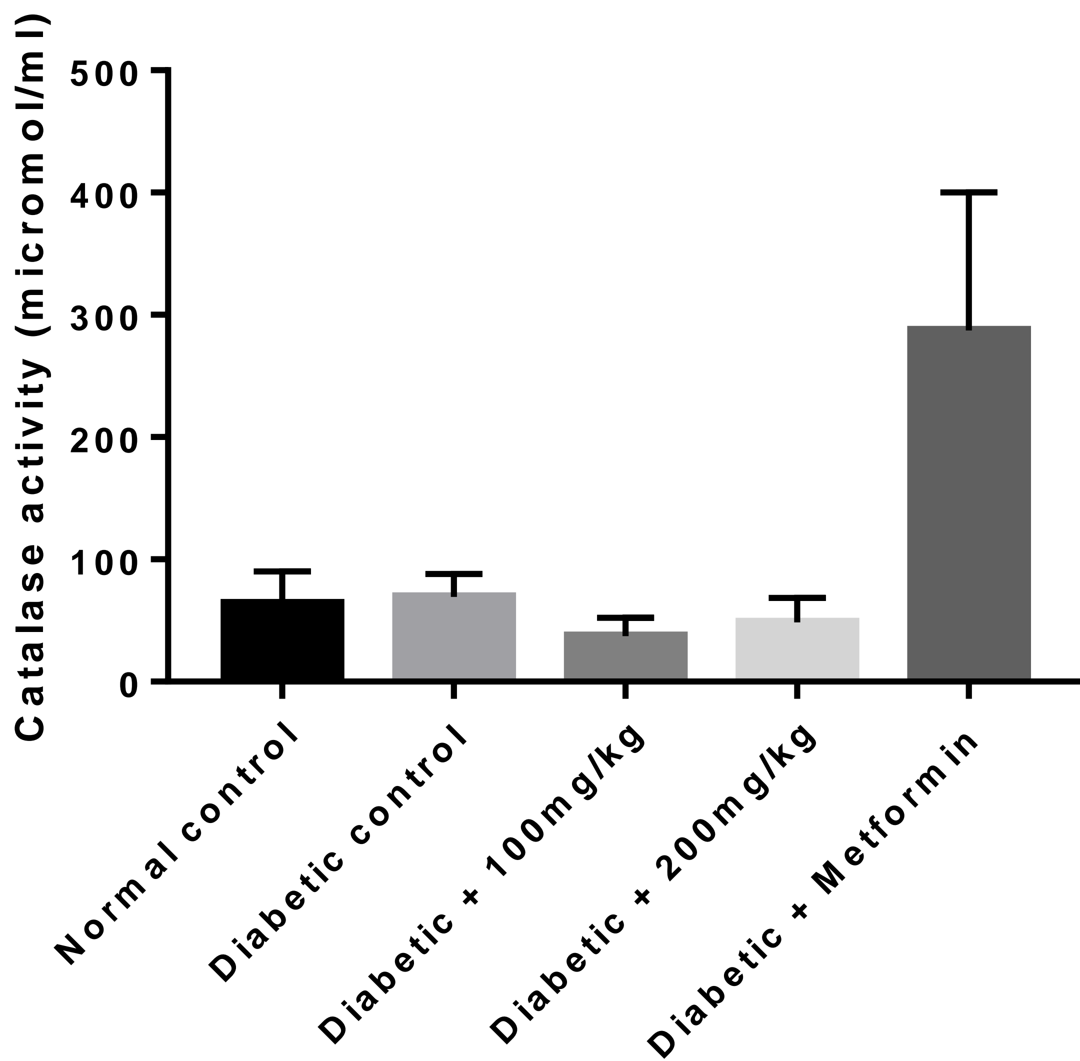

Figure 4.5: Plasma catalase activity of Normal and diabetic rats treated with *C. papaya* leaf extract.

Data are mean ± SEM; n=4

The effect of methanolic extract of *C. papaya* on plasma Catalase activity in figure 4.5 shows that animals treated with metformin alone show a significant increase in the activity of catalase when compared with the control groups. There was no insignificant difference ($P>0.05$) between the Diabetic treated groups and the control groups.

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATION

Oxidative stress is a disproportion in the body between antioxidants and free radicals. It is the underline causative mechanisms for various diseases, and the leaves o*f C. papaya* contain many bioactive compounds that counters oxidative stress.

This study evaluates the antioxidant activity of *C. papaya* by screening the phytochemicals present, checking the plasma glucose concentration of the animals and by using both *in vitro* and *in vivo* assay methods, which were total antioxidants capacity and H_2O_2 radical scavenging activity for *in vitro* and superoxide dismutase and catalase activities for *in vivo.*

From the results of plasma glucose concentration, it was detected that the effect of the methanolic extract of *C. papaya* on plasma glucose concentration showed that the diabetic control had a significant rise in plasma glucose concentration when compared with the control and a lowered plasma glucose concentration were recorded in the C. papaya treated diabetic rats when contrasted to the diabetic control rats.

From the results, it was detected that the Total antioxidation capacity, showed an increase in inhibition with a decrease in the concentration of the *C. papaya* extract. From the results, it was observed that the Hydrogen peroxide radical scavenging activity, showed a decrease in scavenging activity with an increase in the concentration of the *C. papaya* extract.

From the results, it was detected that there was no significant alteration ($p>0.05$) recorded in the action of the enzymatic antioxidant SOD in the plasma and liver of the group of animals administered *C. papaya* extract and metformin at different doses when compared with the control

group. However, there was a significant increase ($p>0.05$) in the activity of the enzymatic antioxidant Catalase in the plasma when comparing the control group and group administered metformin only.

C. papaya has been described to counter lipid peroxidation by maintaining the activities of antioxidant enzymes like superoxide dismutase and catalase (Reddy and Lokesh, 1992). Metformin administration also elevates activity of some antioxidant enzymes such as catalase and SOD. Since hydrogen peroxide can inactivate SOD, whereas, superoxide anion can inactivate catalase, an overproduction of superoxide anion in diabetic state could represent the initiator of the observed effect on activities of both enzymes (Ribiere *et al.,* 1985).

5.1 Conclusion

This study shows the *in vitro* antioxidative effect of *C. papaya*, and hypoglycaemic effect in streptozotocin induced diabetic rats. The results indicate that *C. papaya* could be considered as a potent nutraceutical plant which contains a range of bioactive compounds that can be applied in the prevention of oxidative stress-based diseases such as diabetes mellitus.

5.2 Recommendation

The constituents of the of *C. papaya* leaf extract contains compounds, micronutrients and phytochemicals which may be responsible for its observed antioxidant activity. This research study recommends that the plant possesses antioxidant activities which can counteract the oxidative damage.

Further research on *in vivo* antioxidant assays must be carried out to validate the results and to clearly understand the *in vivo* antioxidant capacity of *C. papaya*.

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