

**THE OXIDATIVE STRESS STATUS OF RATS FED ON OIL BEAN SEED
MEAL**

BY

**EGENA PAULINE IHOTU
BC/2009/277**

**THE PROJECT RESEARCH IS SUBMITTED IN PARTIAL
FULFILLMENT OF THE AWARD OF BACHELOR OF SCIENCE (B.SC)
DEGREE IN BIOCHEMISTRY FACULTY OF NATURAL SCIENCES
CARITAS UNVERSITY AMORJI NIKE EMENE ENUGU STATE**

AUGUST, 2013

APPROVAL PAGE

This project as carried out by Egena Pauline Ihotu with registration number
BC/2009/277 of biochemistry department faculty of Natural Sciences Caritas
University Amorji Nike Emene Enugu State

Dr Yusuf Omeh

(Project supervisor)

Date -----

Mr Moses Ezenwali

(Head of Department)

Date -----

DEDICATION

I dedicate this project work to Almighty God for His unfailing love, mercy, grace and kindness upon me and to my late mother Mrs Deborah Omada Egena and all those that did everything possible to see me successful.

ACKOWNLEDEGEMENT

All praises, thanks and adoration are due solely to almighty God for making it possible for me once again to successfully pass this academic hurdle.

My sincere gratitude goes to my project supervisor, Dr. Yusuf Omeh without whose supervision, constructive criticism, suggestion and useful advice this work would not have been completed and H.O.D Mr. Moses Ezenwali and also to my departmental lecturers Mr. Peter Eze Steven, Dr. Ishiwu, Dr. Ikpe, Mr. ugwudike, Auntie Chineyere remain ever grateful to them also for the help and inspiration I derived from their scholarly experience.

My profound gratitude goes to my lovely brothers, Rev. Fr. Nelson, Pharm. Fred, Engr. Anyebe, Dr. Onuh, Enenche and Carol Onuh, Mrs. Jane Ochaje, Mr. Onah Audu for their support, prayers and finances and for their noble assistance right from my birth till now.

I equally want to appreciate my numerous friends and well wishers who in one way contributed and also those who gives good companionship as I grow .I am also grateful to thank my beloved friends Okeh Ogechukwu, Oguike Olachi, Nwaokocha Amaka, Agada Dominique, my colleagues in the department for their love, care and contribution of advice and encouragement they gave me. May the Almighty God reward you all in Jesus name Amen.

ABSTRACT

This study was on oxidative stress status of rat fed with *Pentaclethra macrophylla*, otherwise known as African oil bean seed oil in English or Ugba in Igbo. Sixteen male rats were distributed into four groups. 1 (control), then group 2, 3, and 4 as test groups. They were fed with their formulated meal (5%, 10%, 20% inclusions) for 28 days. Group 1 was the control and were fed with the normal feed, while group 2, 3 and 4 which were the test groups were fed with test feed formula.. The parameters determined were MDA concentrations and catalase activity. Serum MDA significantly increased ($p<0.05$) while the catalase activity significantly decreased ($p<0.05$). This finding may be clinically significant to individuals with predisposition to increased generation of reactive oxygen species (ROS) and other degenerative diseases.

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CHAPTER ONE

THE OXIDATIVE STRESS STATUS OF RATS FED ON OIL BEAN

SEED MEAL

1.0. INTRODUCTION

Ugba also called ukpaka is a popular food delicacy in Nigeria especially among Igbo ethnic group. It is rich in protein and is obtained by a solid state fermentation of the seed of African oil bean tree (*Pentaclethra macrophylla Benth*).

The natural fermentation of the seed which at present is still done at the house-hold level, renders the production nutritious, palatable and non-toxic (Enujiugha, 2002).

Its production, like many African fermented foods depends, entirely on mixed fermentation by microorganism from diverse source.

Pentaclethra macrophylla Benth is a large woody plant abundant in the rain forest areas of west and central Africa. It's origin in Nigeria is believed to be around 1937 (Ladipo, 1984); where it is found in the South Nigeria, (Mbajunwa *et al.*, 1998).

“Ugba” *Pentaclethra macrophylla Benth* belongs to the Family *Leguminosae* and sub-family *Microsoideae* (Keay, 1989 and NFTA, 1995).

Ugba seeds are irregular and oval; they are flat, black and hard pods. It is composed of oil, protein and small amounts of carbohydrate (Obeta, 1982).

1.1 AIM AND OBJECTIVES

- (i) To determine the concentration of Malondialdehyde (MDA, which indicates the peroxidation status) and
- (ii) The activity of Catalase (a marker of antioxidant status) in the serum of rats fed graded doses of African oil bean seed meal

CHAPTER TWO

2.0. LITERATURE REVIEW

The oil bean seeds are obtained from the African oil bean tree (*Pentaclethra macrophylla Benth*) a large perennial leguminous plant that grows to a height of 25m. The leaves are small and reddish when Young and but gradually turn to dark green (Enujiugha and Agbade, 2005).

The trees are planted along the sides of roads as shade trees and around communities as cash crops. The fruit is black, hard and woody pod measuring about 35-36cm long and 5-10cm broad. When mature it splits open explosively to release about eight diameters and weighing about 15-20grams (Keay *et al.*, 1964; Odunfan, 1986).

The compound leaves are usually about 20-45cm long and covered with rusty hairs giving a scurfy effect particularly along the upper surface but this eventually falls off. There are 10-12 pairs of stout pinnae, the middle pairs are 7-13cm long and also have rusty hairs along the central groove. There are usually 12 – 15 pairs of opposite stalk less pinnules (leaflets) each 12 – 15cm long and 5 – 10mm broad,

with the middle pairs longest. Leaflets often have a rounded tip but are sometimes notched, the base is unequal.

Flowers are creamy yellow or pinkish-white and sweet smelling, flowering commences at variable periods within West Africa. The main flowering season is between March to April with smaller flushes in June and November. Fruits are available at most periods of the year because the large woody pods are persistent. The pods are 40-50cm long and 5-10 wide. Fruits splits open explosively with the valves curling up. This is the form in which they appear on most trees, usually pods contain between 6-10 flat glossy brown seeds and are up to 7cm long. This is the edible product and sources of the oil, hence the name “the oil bean tree” (Aubre Ville, 1959).

The fermented seed is called UGBA by the Igbo’s in the eastern part of Nigeria while the Efiks in the southern Nigeria call it UKANA. It is consumed by an estimate of about 15 million people in the eastern part of Nigeria majority of who are Igbo’s (Odunfa and Oyeyola, 1985)

2.1 CLASSIFICATION OF OIL BEAN

Kingdom: *Plantae*

Order: *Fabales*

Family: *Fabaceae*

Subfamily: *Mimosoideae*

Tribe: *Mimoseae*

Genus: *Pentaclethra*

Species *Pentaclethra macrophylla*

2.2 COMPOSITION OF SEED:

The oilbean seeds contain 4-17% carbohydrate, 44-47% oil which has been found to be rich in oleic acid (Nwokedi, 1975; Odoemelam, 2005) and linoleic acid (Onwuliri *et al.*, 2004). Onwuliri *et al.* (2004) also found out that the saturated fatty acid, lignoceric acid, occurred in high amounts constituting about 10% of the total fatty acid concentration. Some workers said that the oil content could be as low as 38% (Kar and Okechukwu, 1978). They also reported that the oil contains about 75% saturated fatty acids and 25% unsaturated fatty acids. (Achinewhu, 1983) showed the fatty acid content of the seeds. Both saturated and unsaturated fatty acids are found in the seeds. For the saturated fatty acids, lignoceric acid appears to be present in the largest amount constituting about 12% while palmitic acid is the least with 3.4%. Behemic acid is also present with 5.2%. The major unsaturated fatty acid in the seeds is linoleic acid constituting 42.8%. Oleic acid is

also present in appreciable amounts (29.0%). Linolenic and Gadoleic acids are present in very small amounts (3.2 and 0.28%, respectively).

2.3 FATTY ACID COMPOSITION OF AFRICAN OIL BEAN SEED

<u>Composition</u>	<u>Values</u>
Yield of oil (%)	46.3
Saturated fatty acid	
Palmitic acid	3.4
Behenic	5.2
lignoceric	12.0
Unsaturated fatty acid	
Oleic	29.0
Linoleic	42.8
Linolenic	3.2
Gadoleic	0.28

As percentage of total oil. Achinewhu (1983)

The presence of appreciable amounts of behenic and lignoceric acids is not desirable for edible oils (Odufan, 1986).

However, Odoemelam (2005) believes that the high degree of unsaturation makes it suitable for cooking purposes and for use as a drying oil for cosmetics, paints and varnishes.

Also they have been found to contain 36.2-43.89% **crude protein** which contains the 20 essential **amino acids**. However, the sulphur containing **amino acid** content is much lower than those found in other plant proteins (Mbadiwe, 1978; Mba *et al.*, 1974; Odoemelam, 2005). The high content of other essential **amino acids** makes the seeds a potential source of protein (Achinewhu, 1982). Glutamic acid appears to be the largest **amino acid** contained in the seeds. This may be responsible for its use as flavouring for soups in south eastern Nigeria. Aspartic acid, lysine and phenylalanine are also present in appreciable amounts in the seeds.

2.4 PHARMACOLOGICAL USES

Native to humid lowlands of West Africa, the African Oil bean plant is not only valued for its tasty large, glossy brown seeds and soil improvement properties; but for a variety of pharmacological uses. According to Nwanjo *et al.*, 2008, the oil bean's pharmacological uses from its seeds, roots, bark and leaves, benefit treatment for obesity, itching, heart problems, high blood pressure and wound management.

Pain and Itching Relief

A study by (Ugbogu *et al.*, 2009) details how an extract of Oil Bean leaves, seed, and bark justifies its use for treating itching and pain in animals and man and improving the anti-inflammatory response.

Healing Lotions

Oil expressed from the seeds has an anti-inflammatory quality that aids in wound management. Seeds are ground into a paste/lotion and render an antimicrobial effect promoting healing. An extract prepared from the bark is applied to leprosy sores. (Ehiagbonare *et al.*, 2008)

Anti-Diarrhoeal Agent

An extract prepared from the leaves of the Oil Bean plant can decrease propulsive bowel movements and the amount of fecal output. The extract also works as an anti-spasmodic and produces an antimicrobial effect. (Okorie *et al.*, 2006)

Decreases Anaemia

The rich mineral composition of fermented seed makes it a good, low-cost source of protein. Increased intake of the seed as food increases Hemoglobin values in test animals as reported by (Ugbogu *et al.*, 2009) Increased oxygenation of tissue enhances specific hormones that stimulate the production of red blood cells important in proper cardiac function.

Obesity

The Oil Bean plant can be used as a source of dietary estrogens (phytoestrogens). Dietary estrogens can be employed in nutritional supplements and pharmaceutical preparations and vitamin supplements to provide better nutrition and aid in controlling obesity. (Okorie *et al.*, 2006)

Abortion

The Oil Bean has abortion-inducing pharmacologic action. The seeds crushed and eaten with red ants can produce abortion and is used in undeveloped native African populations as a home abortion remedy. (Ehiagbonare *et al.*, 2008)

2.5 ANTI NUTRIENT IN HEALTH

Ugba a value added solid substrate fermentation product of African oil bean seed prepared by the Igbos in eastern Nigeria. Fermentation detoxifies the African oil bean seed with subsequent increase in nutrient availability and digestibility (Mbata and Orji, 2008). Unprocessed African oil bean seeds are bitter and possess anti nutritional factors amongst which are paucine, cyanide, oxalates, saponin, phytic acid, phytate and tannins (Achinewhu, 1983;, Enujinugba and Akanbi, 2005; Onwuliri *et al.*, 2004)

Processing drastically reduces the levels of the anti nutritional compounds in African oil bean seed with the concomitant increase in iron, calcium, potassium, thiamine and riboflavin levels, (Enujiugha and Ayodleoni 2003; Achinewhu and Riley 1986).The fermented products is rich in fats, protein and carbohydrate.

A study by Achinewhu (1983), showed the presence of saponins while Duke (1981) reported the presence of a poisonous alkaloid, paucine in the oilbean seeds. (Mbadiwe 1979) reported the presence of caffeoylputrescine, a growth depressant. However, hemagglutinnins were not found in the oil bean seeds (Toms and Western, 1971). The presence or absence of these toxic substances in the fermented beans has not been investigated. It is, however, believed that these substances are eliminated during the processing and fermentation of the seeds, especially during the soaking, where they can leach out into the water used for soaking. Other anti-

nutritional factors in the beans have been shown to reduce progressively during processing and fermentation (Onwuliri *et al.*, 2004). Ruiz-Teran and Owens (1999) have also shown that such substances are leached out during soaking. However, Akindahunsi (2004) observed that salting and soaking before cooking and fermentation did not have any effect on the level of tannins while the level of phytate increased. Thus these may be responsible for the poor performance of the bean seeds during feeding studies in rats by Isichei and Achinewhu (1988). However, there has not been any reported case of health problems resulting from the consumption of ugba over the years.

Although saponins have been reported to be toxic because they possess glucosides of both triterpenes and sterol (Basu and Rastogi 1967) and cause permeabilization and haemolysis on consumption (Price *et al.*, 1987; Cheeke 1996) but through mixed micelles bile formation (Okenful *et al.*, 1984) reduces lower plasma cholesterol. (Monago *et al.*, 2004) have shown that ugba fermented for up to four days decreased the level of plasma cholesterol in rats, the rate of decrease increasing with the time (days) of fermentation. Thus consumption of the well fermented product promotes health. Chidozie (2006) has shown that administration of the fermented seeds as a food supplement have greatly reduced the risk of cancer and some tobacco related diseases and cancer patients who had regular

fermented oil bean seeds as food supplement showed marked improvements in regaining quality health.

Phytic acid reduces nutritional value owing to limited availability of essential trace elements and dietary mineral (Brune *et al.*, 1992; Ryden and Selvendran 1993; Gus Tafsson and Sanberg, 1995) yet they possess hypoglycaemic, anti oxidant and anti carcinogenic activities.

Beside the anti nutritional factors of tannins of provoking astringent reaction in the mouth, they are known as important photochemical due to their free radical quenching activity more than other simple phenolics (Hagerman *et al.*, 1998)

2.6 OIL BEAN AND HUMANS

Oil bean is eaten after fermentation for 48 -72 hours. The vitamin content of the seeds is low while they are poor sources of calcium and phosphorus (Duke, 1981). Odoemelam (2005) has also shown that the seeds contain sodium (236.2 ppm) and potassium (181.3 ppm). The contents of niacin and riboflavin have been found to decrease during fermentation. Mineral content also decreased during fermentation while no phosphorus could be found in ugba (Duke, 1981).

However, since ugba is usually eaten with fish or added as a condiment to soup containing animal proteins, much of the needed calcium and protein may be obtained from these sources (Odunfa, 1986).

The major sugars found in the seeds are stachyose, galactose and fructose while saponins constitute about 2.1% of the seeds (Achinewhu, 1983). These saponins when hydrolysed would yield glucose, arabinose, rhaminose, Oleanolic acid and hederagenin. The content of these carbohydrate decreased significantly as fermentation time increased (Monago *et al.*, 2004).

However, Enujiugha (2003) has shown that fermentation for 72 hours slightly increased the crude protein and ash contents of ugba. The amino nitrogen increased steadily from 1.23 mg N g⁻¹ DM prior to fermentation to 13.68 mg N g⁻¹ DM after 72 hours of fermentation. He also found that the principal fatty acid linoleic acid increased from 60.68 to 67.57% of the total fatty acids while oleic acid decreased from 26.95 to 22.59%. Palmitic acid and other saturated fatty acids in the seed oil were also slightly affected by the fermentation. However, Onwuliri *et al*, (2004) found that fatty acid concentrations did not change appreciably with processing and fermentation. There was also accumulation of formic acid, acetic, lactic and butyric acids and got to 0.20, 0.18, 0.35 and 0.41 mg g⁻¹ respectively after 72 h of fermentation.

MINERAL COMPOSITION OF OIL BEAN SEED

<u>Componentts (mg/100g)</u>	<u>Unfermented ugba</u>	<u>Fermented ugba</u>
Minerals		
Phosphorous	172	--
Calcium	192	110
Iron	16	3.3
Vitamins		
Thiamine	0.07	0.07
Riboflavin	0.032	0.3
Niacin	0.9	0.3

(Duke 1981)

Isichei and Achinewhu (1988) studied the nutritive value of African oil bean seeds. The seeds were high in energy with a slight difference between the gross energy value of unfermented and fermented oil bean seeds. They also stated that the results obtained from the estimated protein energy ratio (p^e%) and net dietary protein calorie percent (NDpCal%) showed that the two processed forms of the seed have the potential to satisfy human protein and energy requirements.

2.6.1 OIL BEAN SEED AND ANIMALS

Feeding of rats with unfermented (UOB) and the fermented (FOB) seeds resulted in weight loss (-0.82 g and -0.11 g, respectively) However, the average daily intake by the rats was higher for the fermented (5.06 g) than the unfermented (4.72 g). The unfermented and fermented seeds produced a negative protein efficiency ratio (PER) in rats. The protein digestibility was also low. Although, the oil bean seeds are rich in protein (Achinewhu, 1982), they suffer source nutritional drawback as they could not promote nor maintain growth of rats. The poor performance has been attributed to the presence of toxic components in the seeds which impair protein utilization (Isichei and Achinewhu (1988). Mbadiwe (1978) attributed the poor nutritional quality to the presence of growth-depressing factors. Onwuliri *et al.*, (2004) have shown that the seeds contain some anti-nutritional factors which included cyanide, phytate, tannin and oxalate. The raw seeds were found to contain the highest concentrations of all the anti-nutritional factors except oxalate with the highest concentration (937.5 mg/100 g) in the boiled seeds. However, they observed a progressive reduction in the level of all the anti-nutritional factors at the different stages of processing and fermentation. The fully fermented ugba had a reduction of 73.49% for cyanide, 79.41% for tannin, 76.92% for oxalate and 45.98% for phytate. Akindahunsi (2004) studied the effect of salting, soaking before cooking and fermentation on the proximate, anti nutritional and mineral content of the bean seeds. They significantly decreased protein content by 10.5, 9.9

and 8.0%, respectively. However, the energy levels increased from 312.5 kcal mol⁻¹ in raw seeds to 450.9, 440.5 and 405.9 kcal mol⁻¹, respectively after treatments. The zinc levels increased while Mg, Na and K levels decreased.

2.7. OXIDATIVE STRESS

Oxidative stress is the term that refers to the imbalance between the generation of Reactive oxygen species and the activity of the antioxidant defences (Arouma, 1998).

Oxidative stress can also be defined as a condition manifested during the disturbance in the equilibrium status between pro-oxidants and antioxidants in a system in favour of pro-oxidants (Ozturk *et al.*, 2005).

Severe oxidative stress can cause cell damage and death. It is implicated in numerous human diseases. In mammalian cells, oxidative stress appears to lead to increases in the levels of free Ca²⁺ and iron within cells (Orrenius *et al.*, 1989 and Bast *et al.*, 1993).

If the equilibrium in the organism moves towards oxidative processes, then this is known as oxidative stress. Oxidative stress, for instance, is associated with the aging of body cells. Furthermore, a strong accumulation of reactive oxygen species

(ROS) along with drops in cellular concentrations of glutathione, (GSH), the major antioxidant produced by the body, is well known as a common cause of acute and chronic degenerative diseases, such as, arteriosclerosis, diabetes, stroke, Alzheimer's and Parkinson's diseases. (Conrad, 2008)

2.8.1. CATALASE

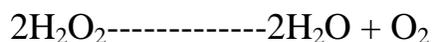
Catalase is a common enzyme found in nearly all living organisms. It was the first antioxidant enzyme to be characterized. Catalase is a tetramer of four polypeptide chains, each over 500 amino acids long. It contains four porphyrin heme (iron) groups that allow the enzyme to react with the hydrogen peroxide. The optimum PH for catalase is approximately 7.65, while the optimum temperature varies by species (Loew, 1990). Each of these four protein subunits also contain a molecule of NADPH each (Kirkman *et al.*, 1996)

Catalase is largely located within cells in the peroxisomes, which also contain most of the enzymes capable of generating hydrogen peroxide. The amount of catalase in cytoplasm and other sub-cellular components remains unclear, because

peroxisomes are easily ruptured during the manipulation of cells. The greatest activity is present in liver and erythrocytes but some catalase is found in all tissues.

Its functions include catalyzing the decomposition of hydrogen peroxide to water and oxygen. Catalase has one of the highest turnover rates of all enzymes; one molecule of catalase can convert millions of molecules of hydrogen peroxide to water and oxygen (Boon *et al.*, 1978).

The action or reaction of catalase in the decomposition of hydrogen peroxide



2.7.2. MALONDIALDEHYDE

Malondialdehyde (MDA) is formed during lipid peroxidation of poly-unsaturated fatty acids (PUFAs) by the action of human platelet thromboxane synthetase on prostaglandins PGH₂, PGH₃ and PGG₂, and by the action of polyamine oxidase and amine oxidase on spermine.

MDA is a dialdehyde and is often thought of as a very reactive molecule. However, under physiological conditions (pH7.4), MDA exists as an enolate anion (-O—CH=CH—CHO), a form that is only fairly reactive, forming Schiff bases with molecules containing a free amine group.

Under more acidic conditions ($\text{pH} < 4$), however, β -hydroxyacrolein ($\text{HO}-\text{CH}=\text{CH}-\text{CHO}$) (β HHA) is the predominant form.

Proteins are much more reactive with MDA than free amino acids forming a variety of adducts and cross-links. MDA can also react with DNA bases producing a variety of mutagenic compounds. Furthermore, MDA has the potential to induce amino-imino-propen cross-links between complimentary strands of DNA and can also cause the formation of DNA-protein cross-links.

MDA is metabolized in the liver to malonic acid semialdehyde. This is unstable and spontaneously decomposes to acetaldehyde that is then converted to acetate by aldehyde dehydrogenase and finally to carbon dioxide and water. Some MDA eventually ends up as acetyl-CoA. Mammalian urine also contains enaminals derived from the hydrolysis of MDA modified proteins (Esterbauer *et al.*, (1991). Urinary output of MDA in humans is typically 0.2-0.8 $\mu\text{mol/L}$ (Tomita *et al.* (1990).

A common method used to measure MDA is through the formation of a chromophore which is produced when MDA is heated under acidic conditions with thiobarbituric acid – the TBAR (thiobarbituric acid reactive) test. The derivative can then be measured at spectrophotometrically (532nm (UV) or 553nm (fluorescence). Unfortunately, the TBAR test suffers from poor selectivity as other

aldehydes, bile acids, DNA bases, reducing carbohydrates and protein carbonyls can all take part in the TBA reaction. This can be overcome by using an HPLC separation to resolve the TBA-MDA adduct from other potential interferences. Another issue is that the stringent reaction conditions needed to form the fluorophore can lead to auto-oxidation of PUFAs leading to the artifactual production of MDA. This can be minimized by the use of antioxidants during the derivatization procedure.

There is an isocratic HPLC-fluorescence “TBARs” method that incorporates the use of the antioxidant, butylated-hydroxytoluene [BHT] (based on Lapage *et al.*, (1991). This method permits the measurement of real tissue MDA levels.

CHAPTER THREE

3.0 MATERIAL AND METHODS

3.1 MATERIALS

Equipment

Sample bottles,

Syringes and needles

Micropipette

Bench top centrifuge (800B)

New cline refrigerator (Model; DCR-IWI285-34 serial number 06/02781)

3.2 COLLECTION AND IDENTIFICATION OF PLANT MATERIALS.

The oil bean seeds were collected from Ogbe in Ahiazu Mbaise L.G.A of Imo State and were identified by Mr A. Ozioko of Bio-resources Development and conservation programme, (BDCP), Aku Road, Nsukka, Enugu state.

3.3 ANIMALS

Mature male Wister albino rats were obtained from the laboratory animal units of the faculty of Veterinary Medicine, University of Nigeria, Nsukka were used for the experiment. The animals were kept in a well ventilated stainless steel cages at room temperature of about 28⁰C. Normal growers feed (Vital feed, Nigeria) and clean drinking water was provided to the animals until the time of the experiment.

The animals were allowed 1 week for acclimatization before the experiment and ethical rules guiding the use of laboratory animals according to Zimmerman (1983) was strictly followed.

3.4 PREPARATION OF OILBEAN SEED MEAL FOR ANIMAL FEEDING

The oil bean seeds dehulled and the outer coat were discarded while the inner seed were oven dried at the temperature of 60⁰C for 180 minutes. With the aid of mechanical grinder it was crushed to a powder form for the meal preparation.

3.4.1. OIL BEAN SEED MEAL INCLUSION DIET PREPARATIONS

The dried grinded oil bean seeds were mixed at different percentages (5, 10 and 20%) with the normal feed for the different group of test rats while the control groups had normal feed only.

3.4.2. OIL EXTRACTION

The solvent extraction method was used in the extraction and the solvent used was n-hexane.

Principle: A fat soluble solvent such as petroleum ether and n- hexane extracts fats from samples.

A large quantity of the sample was soaked in n-hexane, with different bottles and cooked lightly to avoid the escape of the hexane since it is highly volatile. The sample was allowed to stand for 24hours (overnight) at room temperature. The next day, the soaked samples were thoroughly shaken for 10mins using mechanical shaker. This was followed by filtering the mixture through a filter paper. The

filtrate obtained, was transferred into the oil extractor flask and connected to soxhlet extraction apparatus. This was aimed at recovering the solvent and also separating the solvent from the oil. The solvent was then recovered after heating while the oil remained in the extraction flask. The oil in the extraction flask was brought out and dried further at a temperature of 60 °C for some time until the smell of the solvent is no longer perceived. The oil was cooled with dedicator and turned into a laboratory sample bottle and labelled (Laxminatrain and Hildebert, 2007).

3.5 FORMULATION OF OIL BEAN SEED MEAL DIET

Wister albino rats were housed in metabolic cages at temperature condition of 27 – 28°C. They were allowed to acclimatize for 7 days by feeding them on grower’s marsh normal feed after which they were fed with the oil bean seed meal. They were divided into four groups. Groups 1 (control group) consisted of 4 males who received the normal growers marsh diet, Group 2 (Test group 1) also comprised of 4 male rats, received 5% oil bean seed meal, Group 3 (Test group 2) which also comprised of 4 male rats, received 10% oil bean seed meal, and the Group 4 (Test group 4) also had 4 male rats but received 20% oil bean seed meal.

3.5.1 OIL BEAN SEED BASED TREATMENT DIET (g/100g DIET)

The meal was prepared based on grams

	Normal feed (g)	Oil bean seed (g)
Control group	100	0
For 5% group	95	5
For 10% group	90	10
For 20% group	80	20

3.5.2 CHEMICALS/BIOCHEMICALS

All the chemicals used in this research were of the purest grade commercially available and were obtained from Merck, Germany, BDH chemicals Ltd., Poole, England; May and Baker Ltd., England; Riedel-De Haen Ag Seilze -Hannover, Germany; and Hopkin and Williams, Essex, England.

3.6. PHYTOCHEMICAL SCREENING

3.6.1. TEST FOR TANNINS

Ferric chloride test as described by Harborne (1973) was employed.

Procedure: An aqueous extract of the sample was obtained by dispensing 2.00g of the sample in 10ml of distilled water. The mixture was shaken thoroughly and filtered through a whatman filter paper No 4.0 to get the filtrate which served as an aqueous extract. 2 ml of the filtrate was measured into a test tube; 3ml of distilled water was added to the tube and shaken gently to mix well. 2 drops of dilute ferric chloride (FeCl_2) solution was added to the mixture. The formation of a very dark precipitate was indicative of the presence of tannins.

The test was repeated two more times and conducted against a blank control consisting of distilled water and ferric chloride without the extract.

$$\% \text{ Tannin} = \frac{100}{W} \times \frac{A_u}{A_s} \times \frac{C}{100} \times \frac{V_f}{V_a} \times D$$

Where,

W = Weight of sample analysed

Au = Absorbance of the test sample

As = Absorbance of standard tannin solution

- C = Concentration of standard in mg/ml
Vf = Volume of filtrate analysed
D = Dilute factor where applicable

3.6.2. TEST FOR ALKALOIDS

The presence of alkaloids in the test sample was investigated using the test described by Harborne (1973).

Procedure:

An alcohol extract was used and obtained by dispensing 2.00 of the sample in 10 ml of ethanol. The mixture was thoroughly shaken before filtering with a whatman filter paper No 4.0. The resulting filtrate was used for the tests:

2ml of the filtrate was measured into a test tube and 3 drops picric acid added and shaken to mix well. In a separate test tube containing 2ml of ethanol, 3 drops of picric acid was mixed with it. The content of the tube were examined visually. The formation of light green coloration indicated the presence of alkaloids. In another separate confirmatory test, 2 ml of the filtrate from the sample was measured into a test tube, while 2 ml of ethanol was put in a separate test tube for the blank. To both test tubes, 3drop of iodine was added and shaken to mix well. The presence of dark-green coloration in the test sample indicated the presence of alkaloids.

The experiment was repeated two more times for affirmation

3.6.3. TEST FOR SAPONIN

The presence of in the test sample was determined by the froth test as well as the emulsion test described by Harborne (1973).

Procedure: An aqueous extract of the test sample was obtained by dispensing 2.00g of the sample in 10ml of distilled water. The mixture was thoroughly shaken and filtered through a whatman filter paper No 4.0 and 2ml of the aqueous extract was mixed with 6 ml of distilled water in a test tube and shaken well. The formation of stable form confirms the presence of saponin.

For emulsion test, 3drops of groundnut oil is added to test tube containing the sample and observed for the presence of stable emulsion. The presence or formation of stable emulsion confirms the presence of saponins. The froth test was 3repeated two more times for confirmation.

The experiment was repeated two more times to obtain the average

$$\% \text{ Saponins} = \frac{W_2 - W_1}{W_3} \times \frac{100}{1}$$

Where: W_1 = Weight of dish and sample

W_2 = Weight of evaporating dish

W_3 = Weight of sample

3.6.4. TEST FOR FLAVONOIDS

The presence of flavonoids in the test sample was determined by the acid alkaline test described by Harborne (1973).

Procedure: The aqueous extract of the sample was obtained by dispensing 2.00g of sample in 10 ml of distilled water. The mixture was shaken thoroughly and filtered through a whatman filter paper 4.0. 2 ml of the aqueous extract was put into a test tube and a few drops of bench concentrated ammonia (NH_3) were added. The formation of a yellow coloration was recorded as positive test.

In a confirmatory test, a few drops of concentration hydrochloric acid (HCl) were added to the yellow solution which turned colourless. This confirmed the presence of flavonoids.

The experiment was repeated two more times with blank control without the extract.

3.6.5. DETERMINATION OF PHENOLS

Phenol content of the sample was determined by Follins method described by Pearson (1976).

Procedure:

0.2g of the sample was weighed out and added 10ml of methanol in a test tube and then shaken thoroughly. The mixture was left to stand for 5 minutes before filtering with Whatman filter paper No. 4.0. The extract was placed into a test tube and 1ml of Follins reagent was added to 5ml of distilled water. The colour was allowed to develop for about 3 to 4 hours. Then, it was subjected to electronic spectrophotometer and the absorbance of the colour was read at 760nm.

The experiment was repeated two more times to obtain an average. The phenol content is expressed as percentage of the sample and is obtained as shown below:

$$\% \text{ Phenols} = \frac{100}{W} \times \frac{A_u}{A_s} \times \frac{C}{100} \times \frac{V_f}{V_a} \times D$$

Where: W = Weight of Sample analyzed, As = Absorbance of standard solution,

Vf = Total filtrate volume, Au = Absorbance of test sample

D = Dilution factor

C = Concentration of standard in mg/ml

3.7. DETERMINATION OF FATTY ACID COMPOSITION

Principle

After saponification, the methyl esters of constituent fatty acid are prepared using methanol and boron fluoride catalyst and are then separated and identified by gas chromatography.

Procedure

The potassium hydroxide prepared in 2N of methanol was added to one gramme of the oil sample to saponify the oil. The oil was emulsified by addition of prepared Conc. HCl with methanol (1.4) in a soap solution. This follows by addition of normal heptanes to select the oil. Brine was added to salt out the outer composition 0.5µl micro syringe was used to measure out and transfer to the machine. After 10mins, the fatty acids were identified in a graph, having peaks, their area and carbon number.

3.7.1. DETERMINATION OF ANTHOCYANIN

Anthocyanin content of the samples was determined by gravimetric method described by Harborne (1973)

Procedure:

2g of sample was hydrolysed by boiling in 50ml of 0.1N HCl solution for about 30 minutes and filtered through a what-man filter paper No 4.0. The filtrate was transferred to a separating funnel and equal volume of ethyl acetate was added to the solution and mixed well. The resulting mixture was allowed to layer and the ethyl acetate layer (extract) was recovered and the aqueous layer was discarded. The extract was dried at 100% for 5 minutes over a steam bath and concentrated amyl alcohol was added to extract the anthocyanins. The turbid solution was

properly filtered using a pre-weighed whatman filter paper No 4.0. The precipitation was dried in an oven at 100% for 5minutes and cooled in a desiccator and reweighed. The experiment was repeated two more times to get an average.

$$\% \text{ Anthocyanin} = \frac{W_2 - W_1}{W_3} \times 100$$

Where: W_1 = Weight of filter and sample

W_2 = Weight of filter Paper and precipitate

W_3 = Weight of Sample

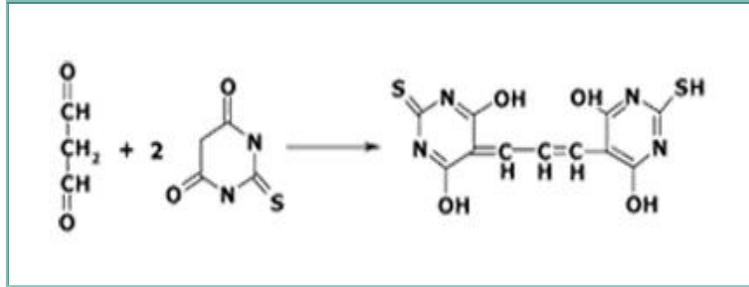
3.8. COLLECTION OF BLOOD SAMPLE

The rats were sacrificed by dazing and the blood collected through cardiac puncture. The pooled blood from each group was centrifuged at 300rpm for 10 minutes¹ after which the serum was collected and kept in the refrigerator for analysis and the sediment discarded.

3.9. TEST FOR MALONALDEHYDE

The NWLSS NWK-MDA01 assay is based on the reaction of malondialdehyde (MDA) with thiobarbituric acid (TBA); forming a MDA-TBA₂ adduct that absorbs strongly at 532 nm.

This reaction is the most popular method for estimating MDA in biological samples. However, interference can be a significant problem in some biological samples if not dealt with appropriately.



Method

The NWLSS method minimizes *ex vivo* lipid peroxidation and maximizes recovery of MDA by carefully optimizing the reaction conditions.

- BHT and EDTA are added to the sample and reaction mixture to minimize artefact oxidation.
- Reaction temperature has also been reduced to minimize the decomposition of lipid hydro peroxides.
- Reaction pH has been optimized to facilitate hydrolysis of MDA-protein adducts for better recovery of MDA.
- Cleaner output through optimized data reduction using single wavelength ABS_{532} or 3rd derivative $SCAN_{400-700}$ analysis.

3.9.1 CATALASE ASSAY

There are numerous assays for catalase. Gregory and Fridovich (1974) report on a sensitive activity stain for catalase applicable to a polyacrylamide gel electrophoretogram, Haining and Legan (1972) describe a polarographic assay utilizable in tissue homogenates, and Kroll *et al.*, (1989) discuss a rapid method for estimating the bacterial content of foods. The subject has been reviewed by Maehly

and Chance (1954) and Chance and Maehly (1955). The assay used at Worthington follows:

Method: Essentially that described by Beers and Sizer (1952) in which the disappearance of peroxide is followed spectrophotometrically at 240 nm. One Unit decomposes one micromole of H₂O₂ per minute at 25°C and pH 7.0 under the specified conditions.

Reagents

- 0.05 M Potassium phosphate, pH 7.0
- 0.059 M Hydrogen peroxide (30%) in 0.05 M potassium phosphate, pH 7.0

Enzyme

$$\text{Mg / protein / ml} = A_{260} \quad X \quad 0.0667$$

Immediately prior to use dilute the enzyme in 0.05 M phosphate buffer, pH 7.0 to obtain a rate of 0.03-0.07 ΔA/min.

Procedure

Adjust the spectrophotometer to 240 nm and 25°C.

Pipette into each curvette as follows:

Reagent grade water	1.9 ml
0.059 M Hydrogen peroxide	1.0 ml

Incubate in spectrophotometer for 4-5 minutes to achieve temperature equilibration and to establish blank rate if any. Add 0.1 ml of diluted enzyme and record decrease in absorbance at 240 nm for 2-3 minutes. Calculate $\Delta A_{240}/\text{min}$ from the initial (45 second) linear portion of the curve.

Calculation

$$\frac{A_{240}/\text{min}}{43.6 \text{ xmg enzyme/ml reaction mixture}} \times 1000$$

CHAPTER FOUR

4.0 RESULT AND DISCUSSION

The figures below are the results obtained.

Figure I show the types and concentration of the fatty acids present in oil bean seed oil after extracted. It shows that it has more of unsaturated fatty acids.

Figure II shows the plasma level of malondialdehyde. An increased level of malondialdehyde can serve as a good indicator of disturbance in oxidative – antioxidant balance, hence increased oxidative stress activity.

Figure III shows the plasma level of catalase in the serum of Wister albino rats fed with oil bean seed. It shows that catalase is significantly reduced in two groups but increase significantly in a group.

4.1 FIGURE 1 Shows fatty acid composition of extracted oil of *Pentracllethra macrophylla*. It shows that the oil has more of poly unsaturated fatty acid in which Linoleic acid has the highest composition followed by oleic acid.

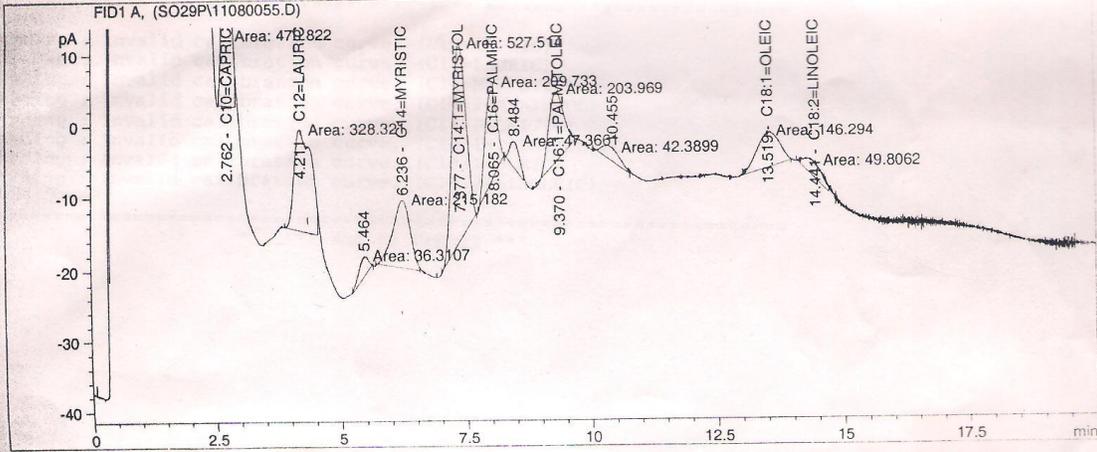
Signal 1: FID1 A,

Peak #	RetTime [min]	Type	Width [min]	Area [pA*s]	Area %	Name
1	2.762	MM	0.1757	470.82208	20.67086	C10=CAPRIC
2	4.211	MM	0.3914	328.32083	14.41452	C12=LAURIC
3	5.464	MM	0.2142	36.31073	1.59418	?
4	6.236	MM	0.3921	215.18240	9.44732	C14=MYRISTIC
5	7.377	MM	0.3195	527.51410	23.15985	C14:1=MYRISTOL
6	8.065	MM	0.2649	209.73300	9.20807	C16=PALMITIC
7	8.484	MM	0.2135	47.36605	2.07955	?
8	9.370	MM	0.3493	203.96922	8.95502	C16:1=PALMITOLEIC
9	10.455	MM	0.3701	42.38994	1.86108	?
10	13.519	MM	0.4836	146.29445	6.42288	C18:1=OLEIC
11	14.441	MM	0.3529	49.80620	2.18668	C18:2=LINOLEIC

Totals : 2277.70901

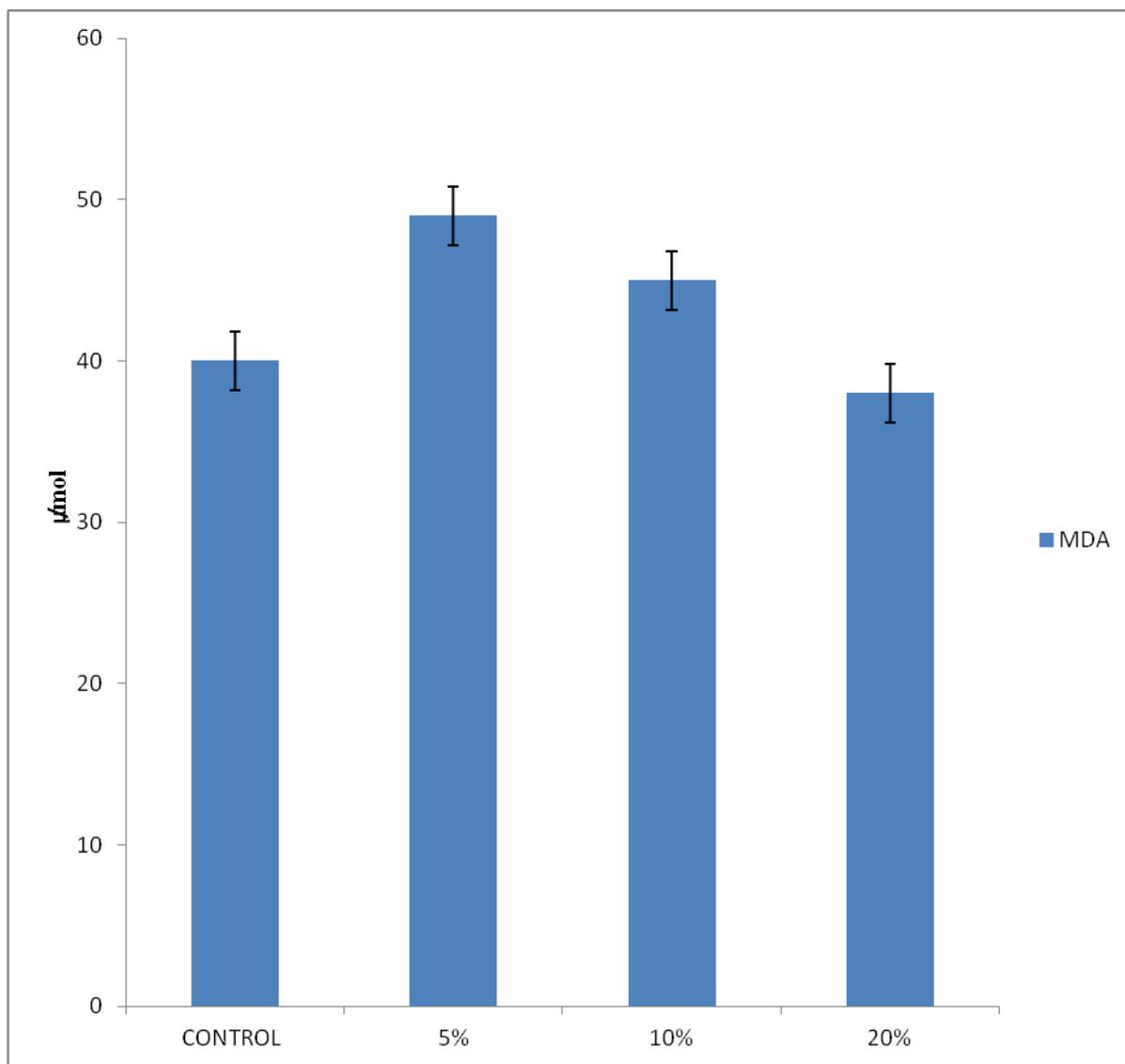
Results obtained with enhanced integrator!
9 Warnings or Errors :

4. Method number SO29P Packed GC



Area Percent Report

4.2 FIG II: Malondialdehyde concentration of test and control animal

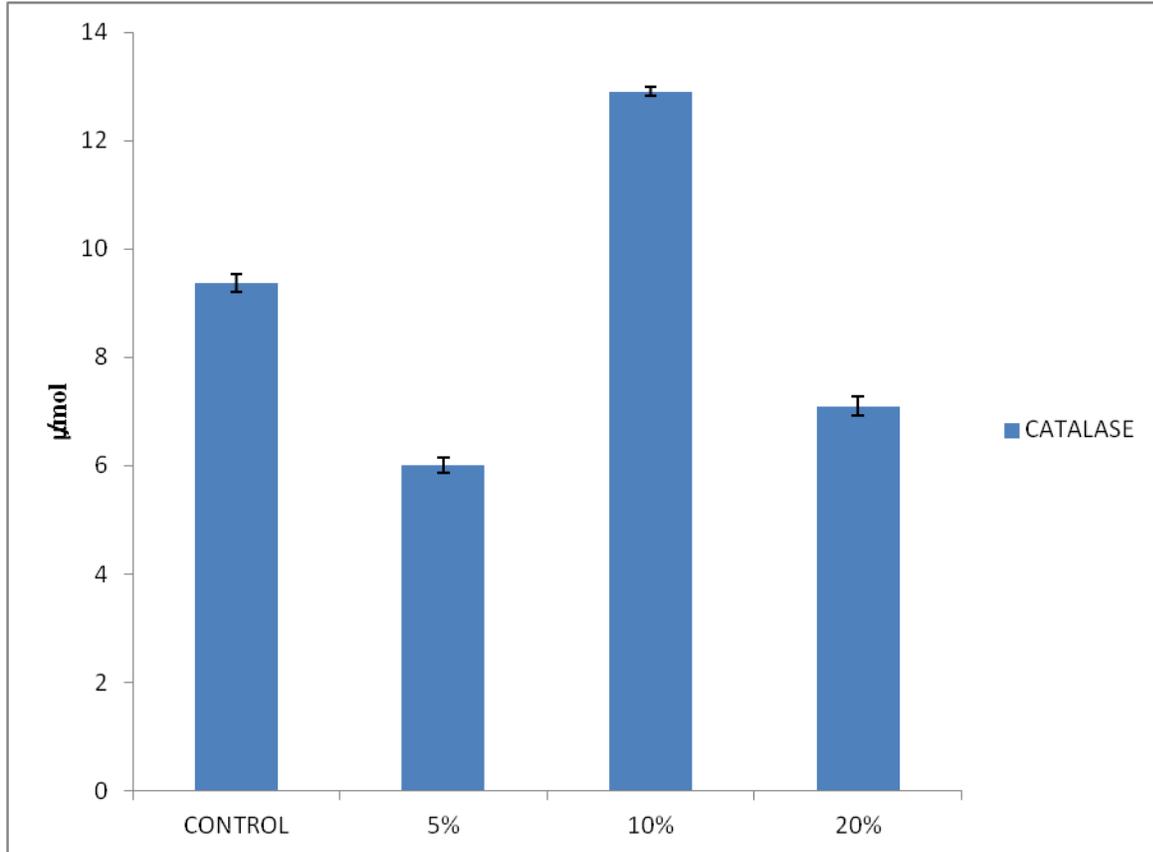


In **Figure II** above, Malondialdehyde (MDA) increased significantly and decreased significantly as the concentration of the oil bean seed increased, hence there was decreased lipid peroxidation with higher intake of the oil bean seed.

The result showed there was a significant increase ($p < 0.05$) at 5% meal inclusion (49.00 ± 1.83) compared to the control (40.00 ± 1.83). There was an observed significant increase ($p < 0.05$) in the 10% when compared with the control

(45.00±1.83), but in 20% (38.00±1.83) there was a significant decrease ($p>0.05$) when compared to the control.

4.3 FIG III: Catalase concentration of test and control animals.



In **Figure III**, there was a significant decrease ($p>0.05$) in catalase activity when comparing 5% and control, (6.00±0.14) and (9.37±0.20) respectively, an observed significant increase ($p<0.05$) in 10% (12.90±0.08). In 20%, (7.10±0.20) there was a significant decrease ($p<0.05$) (7.10±0.20).

CHAPTER FIVE

5.0. DISCUSSION

Oxidative stress has been associated with increased production of reactive oxygen species (ROS) or a significant decrease in the effectiveness of antioxidant defences (Schafer and Buettner, 2001). It has been reported to play a major role in the complications of several diseases and aging process. Lipid peroxidation is an indicator of oxidative stress and a cause of cellular injury in animals and tissues (Faix *et al.*, 2005).

The poly-unsaturated fatty acids of cell membranes are the principal target of reactive oxygen species (ROS). Attack results in the formation of lipid peroxide which undergo decomposition to sub-product such as malondialdehyde.

Lipid peroxidation can be measured with the reaction of malondialdehyde and thiobarbituric acid, despite it is not specific, yet it represents a small percentage of all lipid peroxidation decomposition products and hence it is far from being a reliable marker of oxidative stress (Meagher and FitzGerald, 2000).

Exposure of MDA leads to accumulation of intra cellular reactive oxygen species and dysfunction of the mitochondria (De Grot *et al.*, 1998).

In the result above (Fig II) MDA increased, which showed that lipid peroxidation increased, hence there was continuous peroxidation of poly unsaturated fatty acid producing malondialdehyde. This agrees with the work of Hartley *et al.*, (1999) who reported an increase in α , β -unsaturated aldehydes such as S-hydroxynonenal (4-HNE) and malondialdehyde resulting from lipid peroxidation. These aldehydic secondary products of lipid peroxidation are generally accepted markers of oxidative stress.

Reactive oxygen species and lipid peroxidation products impair the respiratory chain in hepatocytes, either directly or indirectly through oxidative damage to the

mitochondria genome, these feature in turn lead to the generation of more reactive oxygen species, and a vicious cycle ensues, (Faix *et al.*, 2005) hence that accounts for the increase in MDA in the result above. The catalase activity in Fig II decreased. Catalase is a prominent anti oxidant enzyme in the cell; it decomposes H_2O_2 to H_2O and O_2 .

Oxidative stress has been associated with increased production of ROS, leading to increase in concentration of MDA, and a decrease in the effectiveness of anti oxidant defence such as catalase (Schafer and Buetter, 2001)

The decrease in the catalase activity in the result could also be as a result of the flavonoid and tannin content of the oil bean seed which scavenges free radicals. Oil bean seed has flavonoid content of 0.48mg/kg and tannin 0.76mg/kg. They are polyphenolic compound. Polyphenols are reactive species toward oxidation. (Schafer, 2001) It and its flavan-3-ol and its derivative are in high concentration in the erythrocytes. The sudden increase in catalase concentration in 10% could be traced to animal handling which may have increased the stress levels of the animals in the process of sample collection.

High catalase activity in erythrocyte seems to provide anti oxidant defence for tissues with low catalase activity particularly pancreatic β cells; hence it is important in anti oxidant defence against hydrogen peroxide (Robertson *et al* 2003) Generally, decrease in catalase in the blood or catalase deficiency may increase the chance of developing type 2 diabetes (Lazlo Goth, 2008) and also play a role in the greying of hair, because H_2O_2 is naturally produced by the body, and catalase breaks it down. If catalase level decline H_2O_2 cannot be broken down as well. This gives H_2O_2 the ability to bleach the hair from the inside out (Hiti M. 2009).

5.1. RECOMMENDATIONS

It could be worthy of note to say since *pentracllethra macrophylla* has high fat content with some antioxidants such as flavonoids, despite it increasing the level of lipid peroxidation which brings about the formation of MDA, an oxidative stress marker. Awareness should be created to encourage its consumption, because these antioxidants help reduce oxidative stress.

An alternative technology method should be employed to enhance the mass production of *Pentracllethra macrophylla* oil for cooking and other uses such as pharmacological uses in the production of drugs as supplement, and in the manufacturing of hair cream for those that want early grey hair.

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