Causative Factors and Type of Anaemia Developed in Rabbits Following Chronic Consumption of Thermally Oxidized Palm Oil Diet

Elemi John Ani, Daniel Udofia Owu and Eme Efiom Osim

Several studies have documented negative effects following consumption of thermally oxidized palm oil on various systems of the body. This study seeks to investigate the effects of long term consumption of fresh and thermally oxidized palm oil diets on markers of anaemia. Eighteen male 5 months old New Zealand rabbits weighing 750-1000 g were used for this study. The animals were divided into 3 groups (n = 6), namely; control, fresh palm oil-fed group (FPO) and thermally oxidized palm oil-fed group (TPO). Fresh palm oil diet was prepared by mixing 15 g of fresh palm oil with 85 g of feed while thermoxidised palm oil diet was prepared by mixing 15 g of thermoxidised palm oil with 85 g of feed. At the end of 6 months of feeding, the animals were fasted for 12 h, after which blood samples were collected via cardiac puncture, under chloroform anaesthesia. Blood samples were then used for the different analysis. Red blood cell count, haemoglobin concentration and packed cell volume for TPO (4.58±0.30 millions mm$^{-3}$, 14.96±0.92 g dL$^{-1}$, 33.3±1.92%) group was significantly lower (p<0.05) compared with control (5.45±0.15 million mm$^{-3}$, 17.26±0.40 g dL$^{-1}$, 38.88±1.3 %). Erythropoietin concentration was significantly lower (p<0.01) in TPO group (7.45±0.83 pg mL$^{-1}$), compared with control (14.45±2.09 pg mL$^{-1}$) and FPO (11.63 ±0.68 pg mL$^{-1}$) groups, (p<0.05). Specific gravity of whole blood in the TPO fed group (1042±1.68) was significantly reduced (p<0.05), compared with control group (1052±1.67). On histology, the TPO fed group showed infiltration of the bone marrow with adipose tissue and reduction in the number of blood forming cells, while the liver in the TPO fed group showed extensive diffuse steatosis. This study has revealed the specific causative factors of anaemia following chronic consumption of thermally oxidized palm oil to be reduced serum erythropoietin, increased RBC hemolysis and damage to the bone marrow and kidneys. The class of anaemia has been identified in this study as normocytic and normochromic type.

Key words: Anaemia, bone marrow, erythropoietin, fresh palm oil, kidney, thermoxidized palm oil

Department of Physiology, Faculty of Basic Medical Sciences, College of Medical Sciences, University of Calabar, P.M.B. 1115, Calabar, Nigeria
INTRODUCTION

Oil palm is a tropical plant that originates from the gulf of Guinea in West Africa, hence its scientific name *Elaeis guineensis*. It grows in warm climates, at altitudes below 500 m above sea level. It is popularly called the African oil palm. Palm oil is obtained from the pulp of the fruits of this tropical tree (Ebong et al., 1999; Poku, 2002).

Several studies have documented a lot about the nutritional and health attributes of palm oil. However, some of these findings have been contradictory as, some say, it is beneficial to health, while others have documented various degrees of negative effects. Furthermore, the many reported beneficial effects appear to be skewed in favour of fresh palm oil over thermally oxidized palm oil.

Fresh palm oil, which some refer to as red palm oil, is the largest (Oyewole and Amosu, 2010), natural source of tocotrienol, which is the most potent form of vitamin E (Mukherjee and Mitra, 2009). The combination of tocotrienols, carotenes and other antioxidants make palm oil a super antioxidant that is relatively stable to oxidation (Fife, 2005). In addition to its antioxidant action, tocotrienols strengthen the immune system and protect skin cells from toxins and ultraviolet radiation (Jangdey et al., 2014). They have also demonstrated remarkable anticancer properties, far superior to most other antioxidants (Chinembiri et al., 2014). They do not only prevent cancer from taking hold, but actively block its growth and initiate apoptosis (Chinembiri et al., 2014).

Generally, most dietary oils are consumed in thermally oxidized form. However, this thermoxidation is said to have deteriorative effects on the body. Some researchers have previously shown that uncontrolled thermal oxidation of fats and oils lead to formation of peroxides and other products, which are known to be very reactive, cytotoxic and destructive to tissues (Mesembe et al., 2004; Ani et al., 2015b). Long term consumption of oxidised oils and fats have been said to cause growth retardation, anaemia, thrombosis, fatty liver, essential fatty acid deficiency and nucleic acid deactivation of key metabolic enzymes (Mesembe et al., 2004). In addition, the very reactive free radical species that are generated have been implicated in the aetiology of diseases, such as cancer, atherosclerosis, diabetes, arthritis and cataract formation (Pryor, 1991).

Considering the widely documented negative effects of thermally oxidized palm oil on blood (Mesembe et al., 2004; Liu et al., 2014; Ng et al., 2014; Ani et al., 2015b), this study seeks to confirm the presence and possibly establish the specific causative factor and class of anaemia associated with chronic consumption of TPO diet. This is important since treatment of anaemia is usually type specific.

MATERIALS AND METHODS

Experimental animals and protocol: Eighteen male New Zealand rabbits (5 months old) weighing 750-1000 g were used for this study. The animals were purchased from the Department of Pharmacology, University of Calabar, Nigeria and were kept in the animal house facility of the Department of Physiology, University of Calabar, Nigeria. They were divided into 3 groups (n = 6), namely; control, fresh palm oil-fed group (FPO) and thermally oxidized palm oil-fed group (TPO). The control group received normal feed. The FPO group received animal feed mixed with fresh palm oil while TPO group received animal feed mixed with thermoxidised palm oil. All animals had access to food and water *ad libitum*. The feeding period lasted for 6 months after which the animals were used for the various experiments. They were kept in separate cages which were cleaned daily. Indeed, the animals were kept in accordance with the ethical standards laid down in the 1964 declaration of Helsinki.

Preparation of palm oil diets: Palm oil from the oil palm tree *Elaeis guineensis* was purchased from a local oil mill in Odukpani, Cross River State, Nigeria. The palm oil was divided into two equal parts. One part was used as Fresh Palm Oil (FPO) while the other part was thermally oxidised following the method used by Ani et al. (2014). Fresh palm oil and thermally oxidized palm oil were used to prepare the diets for FPO-fed group and TPO-fed group, respectively. Fresh palm oil diet was prepared by mixing 15 g of fresh palm oil with 85 g of feed while thermoxidised palm oil diet was prepared by mixing 15 g of thermoxidised palm oil with 85 g of feed as previously used (Ani et al., 2014, 2015a-c).

Collection of blood samples and measurement of haematological parameters: At the end of 6 months of feeding, the animals were fasted for 12 h, after which blood samples were collected using a syringe and needle through cardiac puncture, under chloroform anaesthesia. The blood samples were immediately emptied into EDTA bottles and mixed gently. A full automatic blood cell counter (Model PCE 210, Japan) was used to assess the blood samples. Values for Red Blood Cell (RBC) count, Packed Cell Volume (PCV), haemoglobin, Mean Corpuscular Volume (MCV), Mean Corpuscular Haemoglobin (MCH) and Mean Corpuscular
Haemoglobin Concentration (MCHC) were obtained. The results were displayed on the screen and were subsequently printed out. The machine was specially calibrated and dedicated for use on animals. Some of the results were confirmed by manual method of haemocytometry.

**Determination of red blood cell osmotic fragility:** Red blood cell osmotic fragility was assessed by osmometry, as described by Oyewale (1993). Ten small test tubes, each containing 2 mL of sodium chloride solution were prepared. The concentration of the sodium chloride in the ten tubes ranged from 1.0-0.1%, each tube differing by 0.1%. This was achieved by using a graduated pipette to measure from the stock 1% sodium chloride into each test tube, ensuring that each successive tube received 0.2 mL more of water and 0.2 mL less sodium chloride solution, to make a total of 2 mL in each test tube. A Pasteur pipette was then used to add a drop of blood to each tube. Each tube was covered with paraffin and mixed by inverting over the thumb, once. The tubes were then set aside for 1 h after which they were centrifuged. A spectrophotometer was then used to read the optical density of the supernatant at 540 nm.

**Determination of serum erythropoietin levels:** Quantitative sandwich enzyme immunoassay technique was used for determination of serum erythropoietin concentration. Blood samples from the animals were allowed to clot for 2 h at room temperature and then centrifuged for twenty minutes at 1000 rpm. The serum was then removed and assayed immediately, using a quantikine erythropoietin immunoassay kit (RnDSsystems, USA). All the reagents within the assay kit were kept at room temperature before use and the assay was carried out as outlined in the manufacturer’s manual.

**Determination of erythrocyte sedimentation rate (ESR):** Erythrocyte sedimentation rate was measured using Westergren’s apparatus as described by Ghai (2007). Blood was mixed with one fifth of its own volume of 3.8% sodium citrate. This was conveniently achieved by first drawing 0.4 mL of sodium citrate in a syringe and then drawing venous blood to the two milliliters mark. The mixture was expelled into a glass jar. Afterwards (within 3 h), a graduated glass tube (30 cm long and 25 cm in diameter) was used to draw the blood up to the 200 mm mark. The tube was then clamped in a special frame which held it vertically and occluded the lower opening. The length of the clear fluid at the top of the tube after 1 h was recorded as the ESR in mm h$^{-1}$.

**Determination of specific gravity of whole blood:** The specific gravity of whole blood was measured using the copper sulphate method as described by Ghai (2007). A total of 170 g of copper sulphate was dissolved in 1 L of distilled water to give a specific gravity of 1.100. This was used as the stock solution from which solutions of different specific gravities were now made. The different specific gravities were confirmed with a hydrometer. Copper sulphate solutions were prepared and put in a series of test tubes. These solutions had specific gravities of 1.040, 1.045, 1.050, 1.055, 1.060 and 1.065. A sample of heparinised venous blood was drawn into a pipette of narrow bore fitted with a teat. From a height of one centimeter, a drop of blood was introduced into each of the test tubes containing the copper sulphate solutions of different specific gravities and its behaviour observed within the next twenty seconds.

**Histological examination:** The liver, kidney and bone marrow were harvested and processed with Haematoxylin and Eosin (H and E) stains. The tissues were fixed in 10% neutral formalin after which they were dehydrated using alcohol and cleaned in xylene. They were then embedded in paraffin wax and thin sections cut at five microns. The sections were then stained with haematoxylin for 15 min, differentiated with 1% acid alcohol, counter stained in eosin for two minutes and mounted with DPX biological mounter. The sections were viewed under the microscope (magnification, X100) and photomicrographs taken.

**Statistical analysis:** All results are showed as Mean±SEM. The data was analyzed using one-way Analysis of Variance (ANOVA), followed by the post hoc multiple comparison test (least square difference procedure-LSD). Significant difference was placed at p<0.05. Computer software SPSS version 17.0 and excel analyzer were used for the data analysis.

**RESULTS**

**Comparison of mean monthly food and water intake in the different experimental groups:** The mean food intake at the beginning of the experiment was 95.5±0.1, 71.4±1.6 and 85.2±1.7 g, for control, FPO and TPO, respectively. This rose to 115.0±0.1, 114.9±0.0 and 114.9±0.0 g for control, FPO and TPO, respectively at the end of six months. There was no significant difference in the mean monthly food intake in the different experimental groups (Fig. 1). Mean monthly water intake for control, FPO and TPO group was 86.7±3.2, 72.3±2.2 and 60.7±0.1 mL, respectively. At the end of six months,
Comparison of mean monthly body weights in the different experimental groups: The mean body weight (g) at the onset for the control, FPO and TPO group was 971±13.3, 850±38.6 and 917±15.0 g, respectively. There was no significant difference in the initial body weights. This rose to 1813±34.2, 1720±40.5 and 1554±46.4 g, respectively, at the end of six months. Mean monthly body weight at the end of six months of feeding was significantly reduced in FPO (p<0.01) and TPO (p<0.001) fed groups, compared with control (Fig. 3).

Comparison of RBC count, PCV, Hb, MCV, MCH and MCHC in the different experimental groups: Table 1 shows that the RBC count in the control, FPO and TPO groups was 5.45±0.15, 5.16±0.24 and 4.58±0.30×10⁶ mm⁻³, respectively. Red blood cell count for TPO group was significantly less than that of control (p<0.05).

Table 1 also shows that the PCV for the control, FPO and TPO groups was 38.88±1.37, 35.32±1.89 and 33.33±1.92%, respectively. The PCV of the TPO group was significantly lower (p<0.05) than that of the control. However, there was no statistically significant difference between the TPO and FPO groups nor between FPO and control.

The haemoglobin content of the control, FPO and TPO groups was 17.26±0.40, 16.22±0.78 and 14.96±0.92 g dL⁻¹, respectively. Haemoglobin concentration in the TPO group was significantly lower (p<0.05), compared to the control (Table 1).
The mean corpuscular volume in the control, FPO and TPO group was 72.5±1.95, 68.35±1.15 and 73.45±4.16 fl, respectively. There was no statistically significant difference in MCV among the groups in this study (Table 1).

The mean value for MCH for control, FPO and TPO group was 32.17±0.60, 31.38±0.40 and 32.92±1.94 pg, respectively. There was no statistically significant difference in MCH among the groups studied (Table 1). The mean MCHC concentration in the control, FPO and TPO group was 44.43±0.74, 45.93±0.38 and 44.92±1.38%, respectively. There was no significant difference in MCHC among the three groups.

Comparison of red blood cell fragility in the different experimental groups: Figure 4 compares the red blood cell fragility in the control, FPO and TPO groups. It shows the degree of haemolysis (%) in the red blood cells of each of the three groups in different concentrations of sodium chloride solution. The most severe haemolysis was in the TPO group which had 7.3% haemolysis at sodium chloride concentration of 0.9% (minimum resistance). The Median Corpuscular Fragility (MCF) was also highest in the TPO group (0.88% sodium chloride) compared to FPO (0.67% sodium chloride) and control (0.52% sodium chloride). At 0.8% sodium chloride solution, 72% haemolysis was recorded for the TPO group compared to 14% for FPO and 0% for control. The point of maximum resistance for all the three groups was at 0.1% sodium chloride solution.

Comparison of erythropoietin concentration in the different experimental groups: Erythropoietin concentration (pg mL⁻¹) in the control, FPO and TPO group was 14.45±2.09, 11.63±0.68 and 7.45±0.83 pg mL⁻¹, respectively. Erythropoietin concentration was significantly lower (p<0.01) in TPO group, compared with control and FPO (p<0.05). There was no statistically significant difference in erythropoietin concentration among the oil fed groups (Fig. 5).

Comparison of erythrocyte sedimentation rate in the different experimental groups: Figure 6 shows that the erythrocyte sedimentation rate in the control, FPO and TPO group was 0.20±0.19, 0.23±0.03 and 0.27±0.03 mm h⁻¹, respectively. There was no statistically significant difference among the three groups (p>0.05).

Comparison of specific gravity of whole blood in the different experimental groups: Figure 7 shows that the specific gravity of whole blood in the control, FPO and TPO group was 1052±1.67, 1047±1.66 and 1042±1.68,
respectively. Specific gravity of whole blood in the TPO fed group was significantly reduced (p<0.05), compared with the control group. Although, whole blood specific gravity was reduced in FPO fed group, it was not significantly different from the control or TPO group (p>0.05).

**Photomicrograph of the bone marrow in the different experimental groups:** Figure 8 shows photomicrographs of the bone marrow in (a) control, (b) FPO and (c) TPO group. Bone marrow in the control group shows normal Blood Forming Cells (BFC) and Adipose Tissue (AT). The Myeloid-Erythroid (M.E) ratio is normal (1:1). The FPO fed group also showed normal BFC and AT, with normal cytoarchitecture and myeloid-erythroid ratio of 1:1. The TPO fed group on the other hand shows infiltration of the bone marrow with adipose tissue and reduction in the number of blood forming cells, such that the ratio of adipose tissue to blood forming cells is about 4:1.

**Comparison of photomicrograph of the liver in the different experimental groups:** Figure 9 shows photomicrographs of the liver in (a) control (b) FPO and (c) TPO groups. Photomicrograph of the liver in the control group shows normal liver architecture, displaying the Central Vein (CV) and Limiting Plate (LP). The hepatic lobules and portal tracts are normal. The FPO fed group shows areas of Diffuse Steatosis (DS). There is no necroinflammation and the limiting plates are intact. Photomicrograph of the liver in the TPO fed group on the other hand shows extensive diffuse steatosis. There is progressive inflammation with Hepatocellular Necrosis (HN). The limiting plates are damaged in many places.

**Photomicrographs of the kidney in the different experimental groups:** Figure 10 shows photomicrographs of the kidney in the (a) Control, (b) FPO and (c) TPO groups. The control group shows normal Glomeruli (GL) and Tubules (TU). The kidney architecture is essentially normal. The FPO fed group also shows normal kidney architecture. The TPO fed group on the other hand shows infiltration by Inflammatory Cells (IC)-(glomerulonephritis). There are also Atrophic Tubules (AT) and tubule-interstitial nephritis, with progressive tubular destruction. There is narrowing of the Bowman’s space and vasculitis in some areas.

---

Fig. 7: Comparison of specific gravity of whole blood in the different experimental groups. Values are Mean±SEM, n = 6, *p<0.05 vs control

Fig. 8(a-c): Photomicrograph of a cross section of the bone marrow in the (a) Control, (b) FPO and (c) TPO group (Magnification×100), AT: Adipose tissue, BFC: Blood forming cells
Fig. 9(a-c): Photomicrograph of a cross section of the liver in the (a) Control (b) FPO and (c) TPO Group (magnification×100), CV: Central vein, LP: Limiting plate, DS: Diffused steatosis, HN: Hepatocellular necrosis

Fig. 10(a-c): Photomicrograph of a cross section of the kidney in the (a) Control, (b) FPO and (c) TPO groups stained with H and E. Magnification×100, GL: Glomerulus, TU: Tubule, AT: Atrophic tubule, IC: Inflammatory cells

**DISCUSSION**

Studies have discussed various detrimental effects following thermally oxidized palm oil ingestion. In our study, serum erythropoietin concentration was significantly decreased in the thermoxidised palm oil diet fed group, compared to the control and fresh palm oil diet fed groups. Erythropoietin is the principal factor that stimulates red blood cell production (Guyton and Hall, 2000). The decrease in erythropoietin in circulation must have contributed to the anaemia in the thermoxidised palm oil diet fed group, which manifested as a decrease in erythrocyte count, packed cell volume and haemoglobin concentration.
Considering that most of the erythropoietin in the body is produced specifically by the interstitial peritubular cells of the kidney (Davidson et al., 1999), it is safe to say that the destruction of the kidney tubules by thermoxidised palm oil diet seen in this study, is responsible for the decrease in erythropoietin in this group, which further resulted in anaemia. The importance of lack of erythropoietin to development of anaemia is supported by previous findings that irradiation of the kidneys causes anaemia (Green and Ezeilo, 1977). Secondly, animals with their kidneys removed, show no response to lack of oxygen (Green and Ezeilo, 1977). Normally, lack of oxygen should lead to an increase in red blood cell count (polycythaemia), as a compensatory mechanism to ensure adequate oxygen carrying capacity of the red blood cells and guarantee tissue oxygenation. In this situation where the kidney is damaged, the liver, which produces 10-15% of the body’s erythropoietin requirement (Guyton and Hall, 2000), would have maximized production to try and ameliorate the situation. However, the liver in the thermoxidised palm oil diet fed group in this study was also found to be damaged. Specifically, there was diffuse steatosis (fatty liver) with progressive inflammation and hepatocellular necrosis seen in the photomicrograph of the liver. Therefore, compensation from this angle was closed.

Small amounts of erythropoietin is also reported to be produced by some other tissues and cells, including: neural tissues (astrocytes and neurons), testis (sertoli cells), uterus, placenta and erythroid progenitors (Lacombe, 1997; Magnanti et al., 2001). The quantity of erythropoietin produced by these tissues is negligible. However, even if it were to be much, erythropoietin on its own cannot produce red blood cells. Rather, it would act through stimulation of the bone marrow to produce red blood cells. In this study however, the bone marrow of the thermoxidised palm oil diet fed group was also found to be damaged. Specifically, there was fat infiltration of the marrow, with reduction in the progenitor and mature cells. Therefore, red blood cell production in the bone marrow was also hampered.

The osmotic fragility of the red blood cells was also tested in this study. It revealed that the red blood cells from the animals in the thermoxidised palm oil diet fed group were the most fragile, compared to the control and fresh palm oil diet fed groups. As a consequence, haemolysis was more severe in the thermoxidised palm oil diet fed group and also appeared at a higher concentration (0.9%) of sodium chloride solution. Ideally, there should be no haemolysis in this physiological solution but 7.3% haemolysis was recorded here. The concentration at which 50% of the cells are haemolysed (median corpuscular fragility), was also highest in the thermoxidised palm oil diet fed group, at a value of 0.88% sodium chloride solution. This was also too high and normally, haemolysis should not even have started at this concentration. At 0.8% sodium chloride solution, 72% of the red blood cells in the thermoxidised palm oil diet fed group had haemolysed, whereas normal red blood cell fragility should ideally begin at 0.48% saline and be complete at about 0.36% saline (Ghai, 2007). This normal range may not follow strictly for all species of animals, but it serves as a guide especially, where there is no control. However, the haemolysis in the thermoxidised palm oil diet fed group was high even, when compared to control and this is represented by a shift of the curve of the graph to the right.

Therefore, the anaemia in the thermoxidised palm oil diet-fed group was caused by a number of factors, namely; reduction in erythropoietin concentration, increased osmotic fragility of the red blood cell membrane and destruction of the bone marrow. It was further compounded by the damaged kidneys and liver. It is interesting to know how the animals in TPO diet fed group remained alive despite all these factors. A possible explanation is hyperplasia of the bone marrow. It is reported that destruction of major portions of the bone marrow by any means, especially x-ray therapy, causes hyperplasia of the remaining parts of the bone marrow in an attempt to supply the demand for red blood cells in the body (Guyton and Hall, 2000). This may have been responsible for the few red blood cells with which the animals were surviving.

The absolute corpuscular values of Mean Corpuscular Volume (MCV), Mean Corpuscular Haemoglobin (MCH) and Mean Corpuscular Haemoglobin Concentration (MCHC) were also studied. These values give more information about the condition of an average red blood cell. This information is vital for diagnosis of the type of anaemia present in a given blood sample (Guyton and Hall, 2000). In this study, there was no significant change in the MCV, MCH and MCHC in the control, fresh and thermoxidised palm oil diet fed groups. Therefore, the type of anaemia present in the thermoxidised palm oil diet fed group is a normocytic, normochromic anaemia. Normocytic, normochromic anaemia is also seen in acute blood loss, anaemia of chronic disease, aplastic anaemia (bone marrow failure), drug therapy and haemolytic anaemia, among others. Of these, the two most common causes are chronic disease and drug therapy (Opasich et al., 2005; Angelousi and Larger, 2014).

Among the causes listed above, bone marrow failure and haemolytic anaemia directly apply to this study and we have already implicated them in the aetiology of the
anaemia found. Testing for the absolute corpuscular values therefore helped in diagnosing the type of anaemia. Diagnosis is important since management of anaemia is now targeted at specific causes and types. For instance, Iron deficiency anaemia, which is microcytic, hypochromic in nature, is usually treated with Iron supplements and not necessarily blood transfusion, while vitamin $B_12$ and folate have been used in treatment of some macrocytic anaemias. In anaemic heart failure in children, packed red blood cells are transfused rather than whole blood, because the intent is to improve the anaemia without overloading the heart. All these point to the fact that treatment of anaemia is type and cause-specific and the first step begins with diagnosis of the type.

The specific gravity of whole blood was demonstrated in this study to be significantly reduced in the thermoxidised palm oil diet fed group, compared to the control and fresh palm oil diet fed group. This test is a quick and accurate method for estimation of serum and plasma protein concentration. The value is probably decreased in the group fed on thermoxidised palm oil diet because the liver was damaged in this group. Even though plasma proteins were not assayed directly in this study, it is logical to believe that they were decreased, since the liver is responsible for production of plasma proteins except gamma globulins. Specific gravity is also decreased in anaemia and kidney disease (Guyton and Hall, 2000). These two factors may also have contributed to the reduced specific gravity in the thermoxidised palm oil diet fed group.

The erythrocyte sedimentation rate was also measured and it showed no significant change among the three groups studied. It is usually raised in most anaemias. Although, ESR of the thermoxidised palm oil diet-fed group was highest in this study, it was not significantly different from that of the other groups despite the anaemia in this group.

CONCLUSION

This study has revealed the specific causative factors of anaemia following chronic consumption of thermally oxidized palm oil to be reduced serum erythropoietin, increased RBC hemolysis and damage to the bone marrow and kidneys. The type of anaemia has been identified in this study as normocytic and normochromic type.

ACKNOWLEDGMENT

Authors hereby acknowledge the efforts of all laboratory staff of Department of Physiology, University of Calabar, Nigeria.

REFERENCES


