EFFECT OF CHRONIC EXPOSURE TO PETROLEUM HYDROCARBON POLLUTION ON SOME BIOCHEMICAL PARAMETERS OF INDIVIDUALS NATIVE TO EBOCHA, NIGER DELTA

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CERTIFICATION

This is to certify that this research work **"Effect of Chronic Exposure to Hydrocarbon Pollution on Some Biochemical Parameters of Individuals Native to Ebocha, Niger-Delta"** was carried out by **Elewor, Ihunanya Udodiri** with **(Reg No. 20144912578)**, in partial fulfillment for the award of the degree Master of Science (M.Sc.) in Biochemistry in the Department of Biochemistry, School of Biological Sciences, Federal University of Technology, Owerri.

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DEDICATION

This work is solely dedicated to the Almighty God for the wisdom, knowledge and understanding he gave me in the course of this work.

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ABSTRACT

The impact of pollution due to gas flaring on individuals native to Ebocha in Niger Delta Area was investigated. One hundred and five apparently healthy individuals between 21-70years who consented for the study were screened, seventy five individuals from Ebocha and thirty from Nsukka-a control location with no history of petroleum hydrocarbon pollution. Standard analytical procedures were used in the determination of the concentration of ascorbic acid (AA) of the individuals (males and females). The activities of antioxidant enzymes catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione reductase (GR) were analyzed. Results obtained for the ascorbic acid concentration of both male and female individuals from Ebocha were found to be significantly ($p<0.05$) lower than those from Nsukka. The results also revealed that the activities of antioxidant enzymes CAT and GPx for male individuals from Ebocha were found to be significantly ($p<0.05$) lower when compared to those from Nsukka. However, the results showed that increase in the duration of the exposure significantly ($p<0.05$) reduced the concentration of AA and activities of CAT and SOD in contrast to the significant increase in the activities of GPx and GR. Thus, these findings show that the concentration of AA and activities of all the antioxidant enzymes from the blood samples of individuals from Ebocha were altered, and it is possibly due to the chronic exposure to gas flaring in Ebocha community which might predispose them to various disease conditions.

Key Word: Chronic exposure, Biochemical, Gas Flaring, Petroleum, Niger Delta.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background of Study

Petroleum hydrocarbons are organic substances that are obtained from crude oil following oil exploration and exploitation activities. Since the first commercial production of crude oil at Oloibiri in 1956, in the present Bayelsa State, the oil sector has contributed immensely to the nation's economy to achieve an improved economic status. The sector is one of the major sources of revenue generation and industrialization in the country. The oil industries provided employment opportunities for an appreciable percentage of the labour force (Gbadebo, 2008).

Statistics have shown that there is a positive correlation between the operation of the oil sector and education in Nigeria. The number of educational institutions grew rapidly and this has been enhanced greatly by the role played by the oil companies as a result of their corporate social responsibility to community development through granting of scholarships at all levels, improvement of science laboratories and building of infrastructure in tertiary institutions (Gbadebo, 2008).

It is worthy of note that Niger Delta alone contributes to the national economy not only through petroleum and gas operations but also via agricultural production. The major food crops commonly found in the region are: water yam, cocoyam, maize, rice, melon, groundnuts, potato, plantain, banana and pepper. Also, economic trees like oil palm, coconut, rubber and cocoa as well as livestock abound (Ndiyo, 2008). The people of this region depend solely on their immediate environment for survival. Therefore, anything that alters their environment threatens their source of livelihood.

The word pollution, which was derived from a latin word “poluto”, meaning to “defile”, is defined as the introduction by man into the environment of some substances or energy liable to cause hazards to human health, harm to living things and ecological system, damage to structures and amenities, or interference with legitimate use of the environment (Holdgate,1979). There are different types of pollution which come from different sources, and most affected area is the Niger Delta Region, in Southern Nigeria, where oil exploration and mining activities have gone on for over five decades leading to emission of hazardous substances associated with oil and gas production. The operations of the petroleum industry, which include, exploration, production, refining, transportation and marketing of oil and gas products which is based mostly in the Niger delta has been the giant spider spinning the web of environmental degradation in the region.

All over the world, environmental pollution is an issue of great concern because of what the environment is to humans’ survival and indeed, the survival of the earth itself. The Ministry of Petroleum Resources in Nigeria revealed that between 1976 and 1990, there were 2,676 recorded oil spills. The ministry attributed 3.8% of the oil spills to equipment malfunction, 21% to corrosion of equipment and 18% to sabotage (Akpomuvie, 2011). However, sabotage of oil installations, which accounts for a significant number of spill incidences in the Niger Delta, is informed by protests, which is an expression of conflict between the host communities and the oil companies.

The World Bank (1995) observed that in 1991, whereas less than 5% of the world’s gas was flared, that of Nigeria was 76%. Nigeria’s percentage was more than 15times the world’s and more than

4times the Organization of the Petroleum Exporting Countries (OPEC) average. Flaring of associated gas mainly emits carbon dioxide (CO_2), carbon monoxide and a variety of air pollutants, such as VOCs, nitrogen oxides (NO_X), sulphur dioxide (SO_2), toxic heavy metals, methane and black carbon soot. Acute or chronic exposure to these, constitute a threat to the public health of both terrestrial and aquatic organisms.

As a result of gas flaring, which is one of the major operations of oil and gas industries, farmlands in the region now yield little or no harvest, because of soil infertility, it has also left the people of the region with a trail of devastation such as polluted air, acid rains that destroy roofing sheets, polluted water sources, destroying fishes and discouraging fish farming due to low income generation by fisher men (Audu and Arikawei, 2013).

The adverse effect of oil and gas operations is felt by the people of Niger Delta who are host to oil and gas industries, affecting their economic, political and social development. In order to investigate the degree of the effect on living organisms, both aquatic and terrestrial organisms, some biochemical parameters are usually investigated. These parameters include enzyme and non-enzyme antioxidants. Examples are ascorbic acid, glutathione, superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, xanthine oxidase among others.

1.2 Justification for the study

Air pollution has been identified as one of the most critical environmental challenges confronting the Niger Delta. Gas flaring releases air pollutants such as carbon dioxide (CO_2), methane (CH_4), nitrogen dioxide (NO_2), sulphur dioxide which contribute the largest volume of air quality degradation and they are also considered the most serious threat of all air pollutants to human health and welfare (Oghenejoboh *et al*., 2013). It has also been shown that the pollution caused by gas flaring in Ebocha has adverse effects on some biochemical parameters in the native fowl (*Gallus domesticus*) (Nwaogu *et al*., 2008) and common edible orange (*Citrus sinensis)* native to the environment (Nnorom *et al*., 2015). However, there is no information on whether the effect is the same in humans, native of that community, which prompted the study, to fill this research gap.

1.3 Aim of the Study

The aim of study was to investigate the effect of chronic exposure to petroleum hydrocarbon (PHC) pollution on some biochemical parameters (mainly antioxidants) in individuals native to Ebocha, Niger Delta.

1.4 Specific Objectives of the Study

The specific objectives of the study included:

(a) Determination of the serum concentration of ascorbic acid

(b) Assay of the activities of the following enzymes;

i. Catalase (CAT)

ii. Superoxide dismutase (SOD)

iii. Glutathione peroxidase (GPx)

iv. Glutathione reductase (GR).

CHAPTER TWO

2.0 LITERATURE REVIEW

Niger Delta Area is the centre for oil and gas production in Nigeria and the richest part of Nigeria in terms of natural resources. The region has the largest natural gas reserve and it is the second largest oil reserve in Africa. It is the continent's primary oil producer (Kadaya, 2012). The region has about 606 oil fields with 355 situated onshore, 251 situated offshore; 5,284 drilled oil wells and 7,000km of oil and gas pipelines (Onuoha, 2008). Furthermore, it has more than 123 flaring sites, thereby making Nigeria one of the highest emitter of greenhouse gases in Africa and the world at large. Increase in the number of gas flaring sites in Niger Delta; put the region in high risk of exposure to hazardous chemicals, emissions and pollutants associated with gas flaring, especially for those living close to the facilities (Uyigue and Agho, 2007).

2.1 Historical Development of Petroleum Hydrocarbon Pollution

Nigeria's energy resources especially oil and gas are exploited by Shell, Chevron, Exxon Mobil, with impunity and disregard for the ecosystem for more than 40 years. These multinational oil companies as they are fondly called get profits from the energy resources, while the innocent local communities in the Niger-Delta live with the daily pollution coming from non-stop gas flaring and oil spillage, along with their health consequences (Friends of the Earth, 2008).

Gas flaring is a dangerous activity and human rights violation that is not tolerated in advanced, developed nations because the gas pollutants are harmful, poisonous and unfriendly to the natural ecosystem. With reference to the Niger-Delta, gas flaring manifests in the forms of gas leakages directly into the atmosphere causing fire incidents and heating up the atmospheric air. Nigerian Liquefied Natural Gas (NLNG) pipelines have been reported to have leaked and caught fire in several communities, which burnt uncontrollably for days destroying plants and animals in the affected areas (Zabbey, 2004).

Official reports indicate that between 1976 and 1997, there were about 5,334 reported cases of crude oil spillages causing serious environmental degradation. Oil spillage results to release of approximately 2.8 million barrels of oil into the land, swamp, estuaries and coastal waters of Niger-Delta communities (Dublin-Green *et al*., 1998). The environment of Ebocha-Egbema in the Niger Delta has been shown to be polluted as a result of gas flaring, which have gone on there for over five decades (Nwaogu *et al*., 2008), It was also observed that the pollution in Ebocha had adverse effects on some biochemical parameters of the native fowls (*Gallus domestical*), native to the environment (Nwaogu *et al*., 2008), while in 2010, the concentration of glutathione and citric acid in *Citrus sinensis* were seen to be markly reduced due to petroleum hydrocarbon pollution in the community which induced some changes in the metabolic activities of the plant (Nnorom *et al*., 2015).

2.2 POLLUTION

Pollution is widely defined as the introduction by man into the environment of some substances or energy liable to cause hazards to human health, harm to living things and ecological system, damage to structures and amenities, or interference with legitimate use of the environment (Holdgate,1979). Pollution was also defined by Ademoroti (1996) as "an undesirable change in the physical, biological, and the chemical characteristics of air, land and water, which can affect the healthy activities and survival of man and other living organisms".

Pollutants are substances released into the environment (air, land or water) that could have adverse effects on the ecosystem. A pollutant can be of natural origin or man-made and can be classified as primary or secondary pollutants. Primary pollutants are usually produced from a process, such as ash from a volcanic eruption. Other examples include carbon monoxide from motor vehicle exhaust, or the sulfur dioxide released from factories. Secondary pollutants on the other hand, are not emitted directly, rather, they are formed when primary pollutants react or interact with other substances. Ground level ozone is a prominent example of a secondary pollutant. Some pollutants may be both primary and secondary i.e they can be emitted both directly and/or formed from other primary pollutants.

2.2.1 Types Of Pollution

There are different ways by which the environment can be polluted, and this can be classified into three major types: air, water, and soil (land), noise and nuclear pollution.

2.2.2 Air Pollution

Air pollution is the introduction of particulates, biological molecules, or other harmful materials into the atmosphere, causing diseases, death to humans, damage to other living organisms such as animals and food crops, or the natural environment. Air pollutants may come from anthropogenic or natural sources. The atmosphere is a complex natural gaseous system that is essential to support life on planet earth. According to the WHO report, air pollution in 2012 caused the deaths of around 7 million people worldwide (WHO, 2014). Air pollutants are substances released into the air that could have adverse effects on humans and the ecosystem. The substance can be solid particles, liquid droplets, or gases.

(i) Gaseous Pollutants: These substances are gases at normal temperature and pressure as well as vapors evaporated from substances that are liquid or solid. Among pollutants of greatest concern are carbon monoxide (CO), hydrocarbons, hydrogen sulfide *(*H_2S*)* nitrogen oxides *(*N_xO_y*)*, ozone *(*O_3*)* and other oxidants, sulfur oxides *(*S_xO_y*)*, and CO_2. Pollutant concentrations are usually expressed as micrograms per cubic meter *(*$\mu g/m^3$*)* or for gaseous pollutants as parts per million (ppm) by volume in which 1 ppm = 1 part pollutant per million parts (10^6) of air.

(ii) Particulate Pollutants: Fine solids or liquid droplets can be suspended in air. Some of the different types of particulates are defined as follows:

- **a) Dust:** Relatively large particles about 100 μm in diameter that come directly from substances being used (e.g., coal dust, ash, sawdust, cement dust, grain dust).
- **b) Fumes**: Suspended solids less than 1 μm in diameter usually released from metallurgical or chemical processes, (e.g., zinc and lead oxides).
- **c) Mist:** Liquid droplets suspended in air with a diameter less than 2.0 μm, (e.g. sulfuric acid mist).
- **d) Smoke:** Solid particles *(*0.05–1.0 μm*)* resulting from incomplete combustion of fossil fuels.
- **e) Aerosol***:* Liquid or solid particles (<1.0 μm*)* suspended in air or in another gas.

2.2.2.1 Sources of Air Pollutants

(a) Natural Air Pollutants: Many pollutants are formed and emitted through natural processes. An erupting volcano emits particulate matter as well as gases such as sulfur dioxide, hydrogen sulfide, and methane; such clouds may remain airborne for long periods of time. Forest and prairie fires produce large quantities of pollutants in the form of smoke, unburned hydrocarbons, carbon monoxide, nitrogen oxides, and ash. Dust storms are a common source of particulate matter in many parts of the world, and oceans produce aerosols in the form of salt particles. Plants and trees are a major source of hydrocarbons on the planet, and the blue haze that is so familiar over forested mountain areas is mainly from atmospheric reactions with volatile organics produced by the trees. Plants also produce pollen and spores, which cause respiratory problems and allergic reactions.

(b) Anthropogenic Air Pollutants*:* These substances come primarily from three sources: (1) combustion sources: burning of fossil fuel for heating and power, or exhaust emissions from transportation vehicles that use gasoline or diesel fuels; (2) industrial processes; and (3) mining and drilling. The principal pollutants from combustion are fly ash, smoke, sulfur, and nitrogen oxides, as well as carbon monoxide and carbon dioxide. Combustion of coal and oil, both of which contain significant amounts of sulfur, yields large quantities of sulfur oxides. One effect of the production of sulfur oxides is the formation of acidic deposition, including acid rain. Nitrogen oxides are formed by thermal oxidation of atmospheric nitrogen at high temperatures; thus almost any combustion process will produce nitrogen oxides (Hodgson *et al*., 2004). Carbon monoxide is a product of incomplete combustion. Transportation sources, particularly automobiles, are a major source of air pollution and include smoke, lead particles from tetraethyl lead additives, carbon monoxide, nitrogen oxides, and hydrocarbons. Since the mid-1960s there has been significant progress in reducing exhaust emissions, particularly with the use of low-lead or no-lead gasoline as well as the use of oxygenated fuels—for example, fuels containing ethanol or methyl *t*-butyl ether, MTBE. Industries emit various pollutants relating to their manufacturing processes such as acids (sulfuric, acetic, nitric, and phosphoric), solvents and resins, gases (chlorine and ammonia), and metals (copper, lead, and zinc).

2.2.2.2 Examples of Air Pollutants

(i) Carbon Monoxide (CO): Carbon monoxide combines readily with hemoglobin (Hb) to form carboxy-hemoglobin (COHb), thus preventing the transfer of oxygen to tissues. The affinity of hemoglobin for carbon monoxide is approximately 210 times its affinity for oxygen. Concentrations of 100 ppm can cause headaches, dizziness, nausea, and breathing difficulties (Hodgson *et al*., 2004). An acute concentration of 1000 ppm is invariably fatal. Carbon monoxide levels during acute traffic congestion have been known to be as high as 400 ppm; in addition, people who smoke elevate their total body burden of carbon monoxide as compared with nonsmokers. The effects of low concentrations of carbon monoxide over a long period are not known, but it is possible that heart and respiratory disorders are exacerbated.

(ii) Sulfur Oxides (S_xO_y): Sulfur dioxide is a common component of polluted air that results primarily from the industrial combustion of coal, with soft coal containing the highest levels of sulfur. Oxidation of sulfur dioxide (SO_2), usually in the presence of a catalyst such as NO_2, forms H_2SO_4, and thus leads to acid rain (WHO, 2014). The sulfur oxides tend to adhere to air particles and enter the inner respiratory tract, where they are not effectively removed. In the respiratory tract, SO_2 combines readily with water to form sulfurous acid, resulting in irritation of mucous membranes and bronchial constriction. This irritation in turn increases the sensitivity of the airway to other airborne toxicants (WHO, 2014)

(iii) Nitrogen Oxides: Nitrogen dioxide (NO_2), a gas found in photochemical smog, is also a pulmonary irritant and is known to lead to pulmonary edema and hemorrhage. The main issue of concern is its contribution to the formation of photochemical smog and ozone, although nitrogen oxides also contribute to acid deposition.

(iv) Ozone (O_3): A highly irritating and oxidizing gas is formed by photochemical action of ultraviolet (UV) light on nitrogen dioxide in smog (Hodgson *et al*., 2004) as shown in the equation below. The resulting ozone can produce pulmonary congestion, edema, and hemorrhage.

NO_2 + UV light ⟶ NO + Ož

Ož + O_2 ⟶ O_3

At this point it is worth distinguishing between “good” and “bad” ozone. Tropospheric ozone occurs from 0 to 10 miles above the earth’s surface, and it is harmful. Stratospheric ozone is located at about 30 miles above the earth’s surface, is responsible for filtering out incoming UV radiation and thus is beneficial. It is the decrease in the stratospheric ozone layer that has been of much concern recently. It is estimated that a 1% decrease in stratospheric ozone will increase the amount of UV radiation reaching the earth’s surface by 2% and cause a 10% increase in skin cancer (Hodgson *et al*., 2004). Major contributors to damage to stratospheric ozone are thought to be the chlorofluorocarbons (CFCs). Chlorine is removed from the CFC compounds in the upper atmosphere by reaction with UV light and is then able to destroy the stratospheric ozone through self-perpetuating free radical reactions.

$$Cl + O_3 \longrightarrow ClO + O_2$$

$$2ClO + O_2 \longrightarrow 2Cl^- + 2O_2$$

Before being inactivated by nitrogen dioxide or methane, each chlorine atom can destroy up to 10,000 molecules of ozone.

(v) Hydrocarbons (HCs) or Volatile Organic Compounds (VOCs): These are derived primarily from two sources: approximately 50% are derived from trees as a result of the respiration process (biogenic); the other 45% to 50% comes from the combustion of fuel and from vapor from gasoline (Hodgson *et al*., 2004). Many gasoline pumps now have VOC recovery devices to reduce pollution.

(vi) Lead: One of the most familiar of the particulates in air pollutants is lead, with young children and fetuses being the most susceptible. Lead can impair renal function, interfere with the development of red blood cells, and impair the nervous system, leading to mental retardation and even blindness. The two most common routes of exposure to lead are inhalation and ingestion. Neurological effects of lead in children have been documented at exposure levels once thought to cause no harmful effects (less than10μg/dl) (Canfield *et al*., 2003). Lead –exposed adults may also experience many of the neurological symptoms experienced by children, although the thresholds for adults tend to be higher (ATSDR, 2005).

(vii) Solid Particles: Dust and fibers from coal, clay, glass, asbestos, and minerals can lead to scarring or fibrosis of the lung lining. Pneumoconiosis is a condition common among coal miners that breathe coal dust, while silicosis is caused by breathing silica-containing dusts, and asbestosis from asbestos fibers. These are all well-known industrial pollution diseases.

2.2.2.3 Environmental Effects of Air Pollutants

a) **Vegetation*:*** Pollutants may visibly injure vegetation by bleaching, other color changes, and necrosis, or by more subtle changes such as alterations in growth or reproduction. Air pollution damage to vegetation produces a variety of gross symptoms on leaves which may have similar features as to color changes (Morris and Violet, 2012). Air pollution can also result in measurable effects on forest ecosystems, such as reduction in forest growth, change in forest species, and increased susceptibility to forest pests. High-dose exposure to pollutants, which is associated with point source emissions such as smelters, frequently results in complete destruction of trees and shrubs in the surrounding area.

b) **Domestic Animals*:*** Although domestic animals can be affected directly by air pollutants, the main concern is chronic poisoning as a result of ingestion of forage that has been contaminated by airborne pollutants. Pollutants important in this connection are arsenic, lead, and molybdenum. Fluoride emissions from industries producing phosphate fertilizers and derivatives have damaged cattle throughout the world. The raw material, phosphate rock, can contain up to 4% fluoride, some of which is released into the air and water. Farm animals, particularly cattle, sheep, and swine, are susceptible to fluoride toxicity (fluorosis), which is characterized by mottled and soft teeth, and osterofluoritic bone lesions, which lead to lameness and, eventually, death.

c) **Materials and Structures:** Building materials have become soiled and blackened by smoke, and damage by chemical attack from acid gases in the air has led to the deterioration of many marble statues in Western Europe. Metals are also affected by air pollution; for example, sulfur dioxide causes many metals to corrode at a faster rate. Ozone is known to oxidize rubber products, and one of the effects of Los Angeles smog is cracking of rubber tires. Fabrics, leather, and paper are also affected by SO_2 and sulfuric acid, causing them to crack, become brittle, and tear more easily.

d) **Atmospheric Effect:** The presence of fine particles or nitrogen dioxide in the atmosphere can result in atmospheric haze or reduced visibility due to light scattering by the particles. The major effect of atmospheric haze has been degradation in visual air quality and is of particular concern in areas of scenic beauty, including most of the major national parks such as Great Smoky Mountain, Grand Canyon, Yosemite, and Zion Parks. There is also concern over the increase in CO_2 in the atmosphere because CO_2 absorbs heat energy strongly and retards the cooling of the earth. This is often referred to as the greenhouse effect; theoretically an increase in CO_2 levels would result in a global increase in air temperatures. In addition to CO_2, other gases contributing to the greenhouse effect include methane, CFCs, nitrous oxide, and ozone.

e) Acidic Deposition: Acidic deposition is the total of wet and dry acidic deposition, with wet acidic deposition being commonly referred to as acid rain. Uncontaminated rain has a pH of about 5.6, but acid rain usually has a pH of less than 4.0. In the eastern United States, the acids in acid rain are approximately 65% sulfuric, 30% nitric, and 5% other, whereas in the western states, 80% of the acidity is due to nitric acid. Many lakes in North America and Scandinavia have become so acidic that fish are no longer able to live in them. The low pH not only directly affects fish but also contributes to the release of potentially toxic metals, such as aluminum, from the soil. The maximum effect occurs when there is little buffering of the acid by soils or rock components. Another area of concern is that of reduced tree growth in forests. The leaching of nutrients from the soil by acid deposition causes a reduction in growth rates of trees able to survive in the altered environment. In addition to the change in soil composition, there are direct effects on the trees from sulfur and nitrogen oxides as well as ozone.

2.2.3 Water Pollution

Water is the most abundant substance in living systems, making up 70% or more of the weight of most organisms. The amount of water in river systems at any time is but a tiny fraction of the Earth's total water; 97 percent of all water is contained in the oceans and about three-quarters of fresh water is stored as land ice; nearly all the remainder occurs as groundwater. Lakes hold less than 0.5 percent of all fresh water, soil moisture accounts for about 0.05 percent, and water in river channels for roughly half as much, 0.025 percent, which represents one four-thousandth of the Earth's total fresh water (Adefemi and Awokunmi, 2010; Belingham, 2012). Rivers have been of fundamental importance throughout the human history. Water from the rivers is a basic natural resource, essential for various human activities. Therefore, the river banks have attracted settlers from ancient times. These settlements have now become big cities, using rivers for irrigation, navigation, hydro-power generations of special significance for country like India, where agriculture is the major source of livelihood of the majority of its population (Lawson, 2011). Water is a dynamic system, containing living as well as nonliving, inorganic, soluble as well as insoluble substances. So its quality is likely to change day by day and from source to source. Any change in the natural quality of water may disturb the equilibrium system and would become unfit for designated uses.

Water pollution is the contamination of water bodies such as lakes, rivers, oceans, aquifers, groundwater (Goel, 2006). It occurs when pollutants are discharged directly or indirectly into water bodies without adequate treatment to remove harmful compounds. Like air pollution, pollution of water bodies affects humans, aquatic plants and animals and other forms of organisms living in water. In almost all cases, the effect is damaging not only to individual species and populations, but also to the natural biological communities (Edward, 2007).

2.2.4 Soil (Land) Pollution

Soil pollution is the build-up of toxic chemical compounds e.g salts, pathogens or radioactive materials in the soil that can affect living organisms (Ramesh, 2017). Most air pollutants resulting from gas flares often find their way into the soil after rainfall, thereby polluting the land. Improper disposition of untreated sewage, oil spills, industrial wastes are also known to be contributors of soil pollution. Fertilizers and pesticides from agricultural use which reach the soil as run off are growing cause of soil pollution with negative effects (Efe, 2003).

2.2.4.1 Water and Soil Pollutants

With three-quarters of the earth's surface covered by water and much of the remainder covered by soil, it is not surprising that water and soil serve as the ultimate sinks for most anthropogenic chemicals. Until recently, the primary concern with water pollution was that of health effects due to pathogens, and in fact this is still the case in most developing countries. In the United States and other developed countries, however, treatment methods have largely eliminated bacterial disease organisms from the water supply, and attention has been turned to chemical contaminants. Contamination of soil and water results when by-product chemicals are not properly disposed of or conserved.

2.2.4.2 Sources of Water and Soil Pollutants

Surface water can be polluted by point or nonpoint sources. An effluent pipe from an industrial plant or a sewage-treatment plant is an example of a point source while a field from which pesticides and fertilizers are carried by rainwater into a river is an example of a nonpoint source (Hodgson *et al*., 2004). Industrial wastes probably constitute the greatest single pollution problem in soil and water. These pollutants include organic wastes such as solvents, inorganic wastes, such as chromium and many unknown chemicals.

Domestic and municipal wastes, both from sewage and from disposal of chemicals, are another major source of chemical pollutants. Pollution of soil and water also results from the use of pesticides and fertilizers. Persistent pesticides applied directly to the soil have the potential to move from the soil into the water and thus enter the food chain from both soil and water. In a similar way, fertilizers leach out of the soil or runoff during rain events and flow into the natural water systems.

Pollution from petroleum compounds has been a major concern since the mid-1960s (Hodgson *et al*., 2004). In 1967, the first major accident involving an oil tanker occurred. Other sources, such as gas flaring, improper disposal of used oil by private car owners and small garages, further contribute to oil pollution.

2.2.5 Toxicity

Toxicity which is the adverse effect of pollutants on living organism can be grouped into two based on duration of exposure and dosage. Acute toxicity, as a result of sudden exposure to substantial quantities of toxicant, either from a single or multiple sources in a short period of time. Chronic toxicity, which result from a long term exposure to a low dose of pollutant.

2.2.5.1 Factors that Influences Toxicity

Toxicity of a particular pollutant varies from one organ to another, and also from one organism of the same specie to another. Some factors are responsible for this variation. These include age, exposure route (the toxicity of a particular compound may vary with the portal of entry into the body, whether through the alimentary canal, the lungs, or the skin.) as seen in figure 1, and duration of exposure, dose of the pollutant, gender, diet, physiological condition, or the health status of the organism (Hodgson *et al*., 2004). As opposed to experimental animals, which are highly inbred, genetic variation is the most important factor in human toxicity since the human population is highly outbred and shows extensive genetic variation. Even the simplest measure of toxicity, the LD_{50} (the dose required to kill 50% of a population under stated conditions) is highly dependent on the extent to which the above variables are controlled. LD_{50} values, as a result, vary markedly from one laboratory to another (Hodgson *et al.*, 2004). Exposure of humans and other organisms to toxicants may result from many activities: intentional ingestion, occupational exposure, environmental exposure, as well as accidental and intentional (suicidal or homicidal) poisoning. The measurement of toxicity is complex.

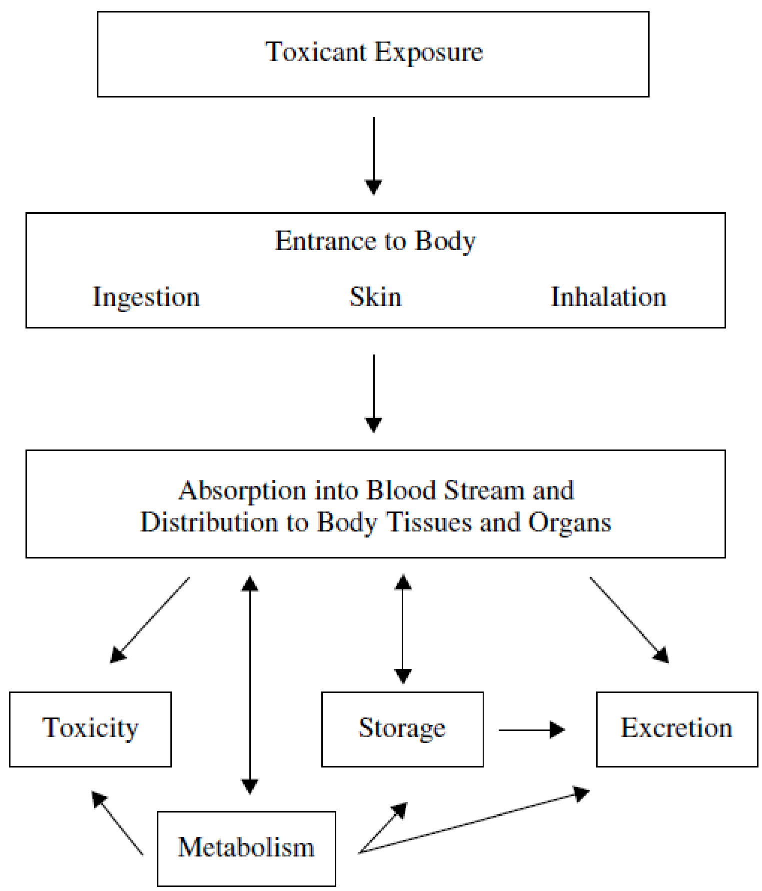


Fig. 1: Chart of How Pollutants Enter the Body and Their Distribution
Source: (Hodgson *et al.*, 2004).

2.3 PETROLEUM HYDROCARBON POLLUTION

Natural gas is a mixture of hydrocarbons which consist of about 95% methane, 2.5% ethane, 0.2% propane, 0.06% butane and 0.02% some higher alkanes (C_5H_{12} + $C_{10}H_{22}$), 1.6% nitrogen (N_2), 0.7% carbon dioxide (CO_2) and trace amounts of hydrogen sulfide (H_2S), water (H_2O) as well as other non-combustible components. According to Brown *et al* (2010), composition of natural gas associated with crude petroleum mainly consists of methane and other gaseous components which vary with the individual production oil field. Nigerian natural gas can be roughly described as 90% methane, with 1.5 – 2.0% carbon dioxide, 3.9 – 5.3% ethane, 1.2–3.4% propane, 1.4–2.4% heavier hydrocarbons and trace amount of sulfur (Ashton-Jones *et al*., 1998). Although it is classified as 'sweet' due to its low sulfur contents, the results of a study on flaring operation in the Niger Delta region showed that sulfur dioxide (SO_2) is one of the gases released during the operation (Obioh *et al*., 1994). Apart from other anthropogenic sources, gas flaring and venting process associated with petroleum exploitation and production operations in the Niger Delta discharge significant amount of air pollutants into the environment over the past five decades.

2.3.1 Gas Flaring

Gas flaring is a common practice of burning off unwanted, flammable gases via combustion in an open atmosphere, non-premixed flame. Gas Flaring is the most significant source of air pollution from offshore oil and gas installations (Nwankwo and Ogagarue, 2011). During most of the activities in the oil and gas industry, wastes, either in solid, liquid or gaseous form are generated and discharged into the environment (Gobo *et al*., 2009).

According to World Bank estimate in 2011, the annual volume of natural gas being flared and vented stood at about 138 to 140 billion cubic meter (bcm) (GGFR, 2012), this is enough to provide for the annual gas consumption of Central and South America or that of Germany and Italy(Gerner *et al*., 2004). Russia still tops the world's flaring countries, followed by Nigeria, Iran and Iraq. Inconsistent data and under-reporting of gas flaring by governments and companies has complicated the global effort to track progress on flaring reduction (GGFR, 2012).

Nigeria flared 14.6 bcm of natural gas in 2011 and most of this occurs in the Niger Delta region. During gas flaring, incomplete combustion also emits various hazardous air pollutants such as volatile organic compounds (VOCs), polycyclic aromatic hydrocarbons (PAHs), soot and also benzene, naphthalene, styrene, acetylene, fluoranthene, anthracene, pyrene, xylene and ethylene (Strosher,2000). Benzene and toluene in particular are hazardous due to their inherent toxicity in mammals, while their wide use in industry and high volume of production lead to substantial environmental releases. These volatile hydrocarbons, can be absorbed into the blood via the respiratory tract, as well as transfer to man through the food chain have various potential health effects (Eyong, 2000; USEPA, 2004).

Gas flaring is one anthropogenic activity that results to unpredictable weather changes and major natural disasters because it emits a cocktail of benzene and other toxic substances that are harmful to humans, animals, plants and the entire physical environment (Worldbank, 1995). The main components of these flared gases include carbon (iv) oxide (CO_2), methane (CH_4), nitrogen (iv) oxide (NO_2), water vapour, sulphur (iv) oxide. The emitted gases return to the soil as acid rain (Ikoro, 2003). The amount of gas flared between the year 2000-2011 are shown in table 1.

Table 1: Amount of gas flared in Nigeria from the year 2000 – 2011.

YEAR	GAS FLARED (Mm^3)
2000	25592.0
2001	27890.3
2002	75772.7
2003	22796.0
2004	24592.0
2005	23429.0
2006	18376.9
2007	22748.1
2008	17844.8
2009	13457.2
2010	13499.3
2011	16200.5

Source: Data and information sourced from the Nigerian National Petroleum Corporation (NNPC), Department of Petroleum Resources (DPR) and Central Bank of Nigeria (CBN) Estimates (Ite and Udo, 2013).

2.3.2 Oil Spillage

Oil is a general term used to denote liquid petroleum products which mainly consist of hydrocarbons. The release of oil into the natural environment is termed oil spill (Onyinloye and Olamiju, 2013) and the phenomenon is known as oil spillage. In addition to gas flaring, accidental spills during tanker accidents, the extraction, refining, transportation and storage of crude oil further contributes to the pollution of the environment. Oil spills also occur due to deliberate acts such as sabotage, oil bunkering, lack of maintenance of engineering equipments. Nigerian crude oil is known to contain heavy metals such as Al, Zn, Fe, Pb, Co, Cu, Cr, and Mn, (Idodo-Umeh and Ogbeibu, 2010). The frequent spills of crude oil on land and water are the major anthropogenic sources of heavy metal enrichment in terrestrial and aquatic habitats in oil producing areas of Niger Delta of Nigeria.

2.3.3 Socio-Economic And Environmental Effect Of Petroleum Hydrocarbon Pollution In Niger Delta

Niger Delta region, encompassing over 20,000 km in Southern Nigeria, is the center of oil and gas production in Nigeria (World Bank, 1995). Despite being the richest part of Nigeria, the region's potentials for sustainable development remains unfulfilled and its future is being threatened by diverse environmental problems, of which pollution is the most paramount. Exposure to petroleum hydrocarbon pollutants could be from gasoline fumes at the pumps, gas flaring, spilled oil, leakage from underground storage tanks into the ground waters, while some of fractions are released into the soil thereby contaminating plants grown in such soil.

2.3.4 Effect of Petroleum Hydrocarbon Pollution on water body

Water bodies such as streams, rivers, lakes, oceans etc. house many aquatic organisms ranging from small crabs, periwinkles, to big fishes and other aquatic plants and animals. Therefore the release of pollutants into these water bodies, either directly or indirectly, poses a great threat to these living organisms, due to their ability to alter the physicochemical parameters of the water. Water pollution is a major environmental challenge facing the Niger Delta communities. A study carried out by Nwaogu and Onyeze (2010) revealed that flared gases caused an increase in the acidity of water body, which could be as a result of gases released during this practice, leading to acid rain which eventually find its way into water bodies, thereby cause a shift in the metabolism of living things in the water. Table 2 revealed that water sample from polluted site had its total hardness at 192± 1.20, a value that is far above the WHO/FEPA standard. (FEPA, 1991)

Table 2: Mean ± Standard Deviation Values of physicochemical Parameters of water from Ebocha (polluted site) and Mbaise (unpolluted area), alongside the WHO/FEPA Standards.

Physicochemical parameters(mg/l)	**Water samples**		WHO/FEPA Standards
	Ebocha	Mbaise	
pH	5.42	6.58	6.5 -8.5
Total Hardness	192 ± 1.20	40.0 ± 1.40	0 – 0.5
Calcium Hardness	160 ± 1.30	32.0 ± 1.25	No limit
Total Solid	800 ± 10.0	70.0 ± 2.22	No limit
Chlorine	56.74 ± 2.50	1.28 ± 0.03	250
Total Dissolved Solid	600 ± 5.20	66.0 ± 1.30	100 – 1000
Total Suspended solid	200 ± 2.50	4.0 ± 0.25	No limit
Alkalinity	73.75 ± 3.25	2.25 ± 0.22	No limit
Sulphate	12.0 ± 1.22	2.50 ± 0.25	250 – 500
Phosphate	0.126 ± 0.02	0.008 ± 0.002	< 5.0
Nitrate	8.00 ± 0.02	2.00 ± 0.01	10

Source: Nwaogu and Onyeze (2010)

Also surface and underground waters in petroleum hydrocarbon polluted environments, via oil spill tends to have increased concentration of heavy metals such as lead, barium, cadmium, selenium and copper more than water bodies in non-polluted areas (Nwankwo and Ogagarue, 2011). Odoemelam *et al.* (2013), observed that the levels of heavy metals in sediment, fish and water from Cross River varied in their study, iron was found to be the most abundant heavy metal in sediment (99.78 mg/kg), fish (11.45 mg/kg) and water (4.85 mg/L). And the variation in concentration is according to: sediment > Fish > water. The effects of these spills depend on the oil dosage, the oil type, metrological conditions and physical geography of the area. Statistics have shown that during 1976-1980, the majority of oil spill incidents occurred in the purely mangrove swamp zones and the offshore areas of the Niger-Delta, which constitute the most productive biological areas. Within six months, mangrove vegetation started dying in the polluted waters. Crabs, molluscs and periwinkles died, while associated fire hazard spread to about 25 hectars of land. Worse still, pollution of the top soil from below was noted about two years after the incident while water table was affected across 15.1 hectars.

Petroleum hydrocarbon pollution on the water surface could prevent natural aeration, leading to the death of trapped marine organisms. In some cases, fish may ingest the spilled oil or other food materials impregnated with oil and as such become inedible and unpalatable (Oshwofasa *et al*., 2012), also making the water unfit for domestic and industrial use and if used can lead to water borne diseases such as amoebaisis, cholera, ascariasis.

2.3.5 Effect of Gas Flaring on climate

The issue of global warming has become more highly profiled in the world, the practice of gas flaring by oil companies is attracting more attention. This is not only because gas flaring is environmentally unfriendly but also because it is literally destroying valuable natural resources. Gas flaring produces enormous amounts of greenhouse gases (GHGs) including carbon dioxide (CO_2), methane (CH_4), and propane (Kaldany, 2001). World Bank estimated that about 10% of global CO_2 emission comes from flaring of gas. Nigeria's gas flaring alone releases about 35 million tons of CO_2 and 12 million tons of methane, which has a higher warming potential than carbon monoxide (Watts, 2001). Other pollutants such as volatile organic compounds (VOCs) and chlorofluorocarbons are also involved in ozone layer depletion. VOCs are well-known outdoor air pollutants which are categorized as either methane bond or not. Methane is an extremely efficient greenhouse gas which contribute to enhanced global warming. The chlorofluorocarbons can also come in contact with other gases and damage the ozone layer. This allows harmful ultraviolet rays to reach the earth's surface, which can lead to skin cancer, eye diseases and can also cause damage to plants.

2.3.6 Effects of Petroleum Hydrocarbon Pollution on buildings

The primary cause of acid rain is the emission of sulphur dioxide (SO_2) and nitrogen oxides (NO) which combine with atmospheric moisture to form sulphuric acid and nitric acid respectively. Acid rain and building degeneration have been linked to the activity of gas flaring. In addition, acid rain accelerates the decay of building materials and paints. Roofs of buildings in the Niger Delta regions have accelerated corrosion by the composition of the acid rain that falls as a result of gas flaring. Investigation carried out in Umutu-Ebedei, Delta State, physical observation revealed that most buildings especially those with iron sheet roof experienced massive damage resulting in frequent leakages (Oseji, 2011).

Nkwocha and Pat-Mbano (2010) examined the impact of gas flaring on the buildings at Obrikom and Omoku communities in River State, and the results revealed a strong association between emissions from the flare point and some impacts observed on the buildings in the area. And they concluded that the most probable air pollutants that caused these observed impacts were SO_2, NO_2 and particulate matter (PM) that is less than 10microns, as they matched the criteria for causative agents in this case. Acid rain acidifies lakes and streams, and damages vegetation. Prior to falling to the earth, sulphur dioxide and nitrogen oxide gases and particulate matter derivatives, sulphate and nitrate, contribute to visibility degradation and harm to public health.

2.3.7 Effect of Petroleum Hydrocarbon Pollution on Soil Nutrients and Agriculture

Acid rain through one means or the other finds its way into the soil, thereby affecting soil nutrients {organic carbon content, sulphate (SO_4) and nitrate (NO_3)}, although the mean values for both SO_4 and NO_3 are below the WHO/FEPA soil standards, the high soil acidity creates chemical and biological conditions which is harmful to both plants and soil microorganisms and its capacity for productive agriculture (Nwaogu and Onyeze, 2010). One of such conditions is the reduction in the capacity of plants to absorb cations (Wild, *et al*., 2000).

The environment of Ebocha-Egbema in the Niger Delta has been shown to be polluted as a result of oil exploration and exploitation, which have gone on there for over five decades, and native fowls (*Gallus domesticus*) native to the environment are not excluded from the negative effects of this pollution, as investigation revealed that petroleum hydrocarbon (PHC) pollution has adverse effects on some biochemical parameters of the native fowls in such polluted areas (Nwaogu *et al*., 2008).

Oil spill on the land could also lead to retardation of vegetation growth for a period of time and in extreme cases destruction of vegetation occurs, it also renders the soil unfit for cultivation (Oshwofasa *et al*., 2012). Soils in gas flare sites are known to have high temperature and low moisture content, which can be attributed to the detrimental effect from high intensity heat generated by the process of gas flaring. The high temperature affects the survival of microbes thereby reducing soil fertility and germination of plants (Ubani and Onyejekwe, 2013), while the low moisture content, as experienced near the flared points, leads to reduction in the rate of translocation of soil nutrients within the plant system and affect microbial activities (Okeke and Okpala, 2014).

In Ogbia local government Area of Bayelsa state farming is the predominant occupation among the households representing about 58% of their population. When investigated, over 70% of the respondents showed strong perception that gas flaring has a negative impact on their well-being over time and poses serious threat to their livelihood (Beulah and Obot, 2013). This was supported by the report filed by Olaniyi and his colleagues (Olaniyi *et al.,* 2008). In summary, petroleum hydrocarbon pollution causes depletion of soil nutrients by causing low cation (Ca^{2+}, k^{+}, Mg^{2+} etc.) exchange capacity, increased soil acidity, reduced organic matter, reduced water holding capacity of the soil, reduced aeration, reduced soil microorganisms and this caused loss of soil fertility which resulted to:

(i) Reduction in nutritional values of crops.

(ii) Low yield of agricultural produce due to decrease in solubility and nutrient uptake of plant crops.

(iii) Stunted growth and withered crops etc.

2.3.8 Effects of Gas Flaring on Air Quality

Flared gases contain hazardous substances which when released into the atmosphere affects air quality. A study on air quality by Nwaogu and Onyeze (2010) revealed alterations in air quality parameters (CO, NO_2, SO_2, CH_4, and particulates) of the surroundings, close to a gas flaring site. The values obtained were above the WHO/FEPA standard permissible limit for normal environment. It was also observed that air quality indices decreased as the distance from flaring site increased, an indication that gas diffusion is proportional to distance (Craft *et al*., 1986). Concentration of particulates in Igwuruta / Umuechem Community in Rivers State was found excessive, exceeding the allowable regulatory limits across all the stations investigated (Gobo *et al*., 2009). All these findings are indications that gas flaring has a negative impact on the Niger Delta atmosphere.

2.3.9 Effect of Petroleum Hydrocarbon Pollution on Humans

In the last few years, attempts have been made regarding the evaluation of health impact of petrol fumes in human and laboratory animals in Nigeria (Pranjic *et al*., 2002). Several studies have reported toxicological implications of inhalational exposure to petrol fumes in human and animal models (Azeez *et al*., 2012). Data from study carried out suggested that oxidative stress is associated with occupational exposure to petrol fume in individuals investigated. Petroleum attendants therefore should take necessary precautionary measures and have regular medical check-up to ascertain their health condition (Odewabi *et al*., 2014).

The following include some of the effects of petroleum hydrocarbon on human health, they include:

(i) Release of carcinogenic substances: The Warri Women Protesters in 2001 who besieged the headquarters of SPDC and Chevron clearly highlighted the deleterious impact of oil exploration on public health. The preliminary survey by Akoroda (2000) indicated that an emergent trend of carcinogenic diseases in the Niger Delta is traceable to exposure of people to the substances emitted from gas flaring e.g PAHs (Polycyclic aromatic hydrocarbons), benzene, pyrene and some of the heavy metals in the gas being flared. Toxicity of benzene most likely results from oxidative metabolism of benzene to reactive products. Benzene can be enzymatically bioactivated to reactive intermediates that can lead to increased formation of reactive oxygen species (ROS). The metabolism of benzene metabolites, phenol, and hydroquinone, result in arachidonic acid peroxidation and oxygen activation to superoxide radicals, respectively. Ashraf and Hoda (2008) reported a decrease in superoxide dismutase (SOD) activity in the plasma of petrol pump workers who are exposed to benzene. Silva *et al*.(2003) found that hydroquinone can be metabolized to benzoquinones that are potent haematotoxins, genotoxins, and carcinogens that can also induce the formation of radical species, i.e. they can also ultimately predispose cells to oxidative damage. These people suffer respiratory diseases, which are consequences of long exposure to gas flaring (Jike, 2004). There is an apparent upsurge of carcinogenic diseases e.g. skin and lung cancer in the Niger Delta. Skin rashes and eye irritation are also in widespread in this region. The leaders of the Warri Women Protest of 2001 quite rightly traced the prevalence of bronchial diseases and eye abnormality to unrestrained gas flaring by oil companies in the Niger Delta (Oshwofasa *et al*., 2012).

(ii) Renal Dysfunction: Prolonged exposure to low dose emissions of flared gases caused an increase in serum concentrations of biomarkers such as urea, creatinine, potassium, inorganic phosphate and uric acid, which are indicators of renal dysfunction. This suggests that residents of gas flared environments are prone to developing kidney diseases than the un-exposed (Dennison *et al*., 2013).

(iii) Affects blood indices: Benzene is a known systemic toxicant in humans at any concentration which when inhaled for a prolonged period is haematotoxic and causes bone marrow depression. Studies have shown that there is decrease in the packed cell volume (PCV), hemoglobin (Hb) and red blood cell (RBC) counts among petrol station attendants (Okoro *et al*., 2006), which could be as a result of inhalation of petrol fume at the station. Naphthalene on the other hand, which is also one of the products of the incomplete combustion of associated gas in crude oil, when inhaled or ingested in large amount, is known to destroy the membrane of red blood cells leading to its breakdown (Owu *et al*., 2005).

(iv) Causes reduction in respiratory flow rate: The volatile nature of petroleum hydrocarbon makes it readily available in the atmosphere any time it is dispensed, especially at petrol filling stations, depots and gas flaring sites. PHC contains mixture of volatile hydrocarbons; this property makes inhalation the most common form of exposure to these chemicals (Cecil *et al*., 1997). The effect of flared gases on the air quality causes irritation to the eyes, respiratory tract and skin. Study has shown that petroleum hydrocarbon pollution has negative impact on the integrity of lungs of children and adults (males and females) resident in Niger Delta communities by reducing their mean peak expiratory flow rates. The severity of the impact on peak expiratory flow rate worsens with longer exposure to gas flaring and hence reduction in peak expiratory flow rate (Aloamaka *et al*., 2012).

Analysis of medical records showed a greater frequency of disease types such as asthma, cough, breathing difficulty, eye/skin irritation in Igwuruta/Umuechem community that has a long history of gas flaring compared to Ayama with no flaring history (Gobo *et al*., 2009). This subset of diseases accounted for 22.4% and 5.9% (ratio 4 to 1) of all cases reported at the respective health centers. The high level of particulates in the dry season constitutes a greater short-term exposure risk to residents and workers, with the particular risk of respiratory irritation, itching/eye irritation and cough being endemic in the area surrounding the flare site (Gobo *et al*., 2009).

2.3.10 Effect of Gas Flaring on Nigeria's Economy

Nigeria is rated the second largest gas flaring country in the world after Russia. Nigerian National Petroleum Corporation (NNPC) disclosed in its annual statistical bulletin (ASB) that Nigeria lost up to $868.8 million, equivalent to #173.76 billion to gas flaring in 2014 (NNPC, 2015). Available data showed that oil and gas companies operating in Nigeria burn over $3.5 to $5 billion yearly from the over 257 flow stations in Niger Delta. Specifically, the country flared about 17.15% of the 95,471 metric tonnes of gas produced in June 2015 alone, according to the data from Nigerian National Petroleum Corporation (NNPC). In addition, loss due to low yield of agricultural produce and damage to infrastructures all contribute to low revenue generation by the Federal Government of Nigeria.

In the bid to end the negative impact of flaring gas, the "Zero Routine Flaring 2030 Initiative" was launched in April, 2015. The initiative was launched by the United Nation Secretary General, Ban Ki Moon, and the World Bank Group President, Jim Yong Kim. And fortunately, about 45 governments, oil companies and development institutions have endorsed the initiative, Niger Delta Petroleum Resources of Nigeria inclusive (World Bank, 2015).

Pollutants from gas flaring and oil spills affect living things including man, animals, plants and their environment. They are carcinogenic (Ruiz and Rizo, 2007), immune-toxic (Olsgard *et al.*, 2008), causes chronic respiratory diseases such as asthma, and neurotoxic. It also causes increase in temperature (thermal gradient), acid rain, and low agricultural productivity. Therefore, the study of the impact of petroleum hydrocarbon pollution cannot be overemphasized.

2.4 OXIDATIVE STRESS

Oxidative stress reflects an imbalance between the systemic manifestation of reactive oxygen/nitrogen species and a biological system's ability to readily detoxify the reactive intermediates or to repair the resulting damage. In other words, oxidative stress result from an imbalance between reactive oxygen/nitrogen species (ROS/RNS) production (such as superoxide O^{2-}, hydroxyl OH^-, peroxy ROO^-, hydrogen peroxide, H_2O_2) and antioxidant's capacity to counter it. This can occur as a result of either increased ROS/RNS generation, impaired antioxidant system, or a combination of both. In the presence of oxidative stress, the ROS attack will modify and denature functional and structural molecules, leading to tissue injury and dysfunction as seen in figure 2.

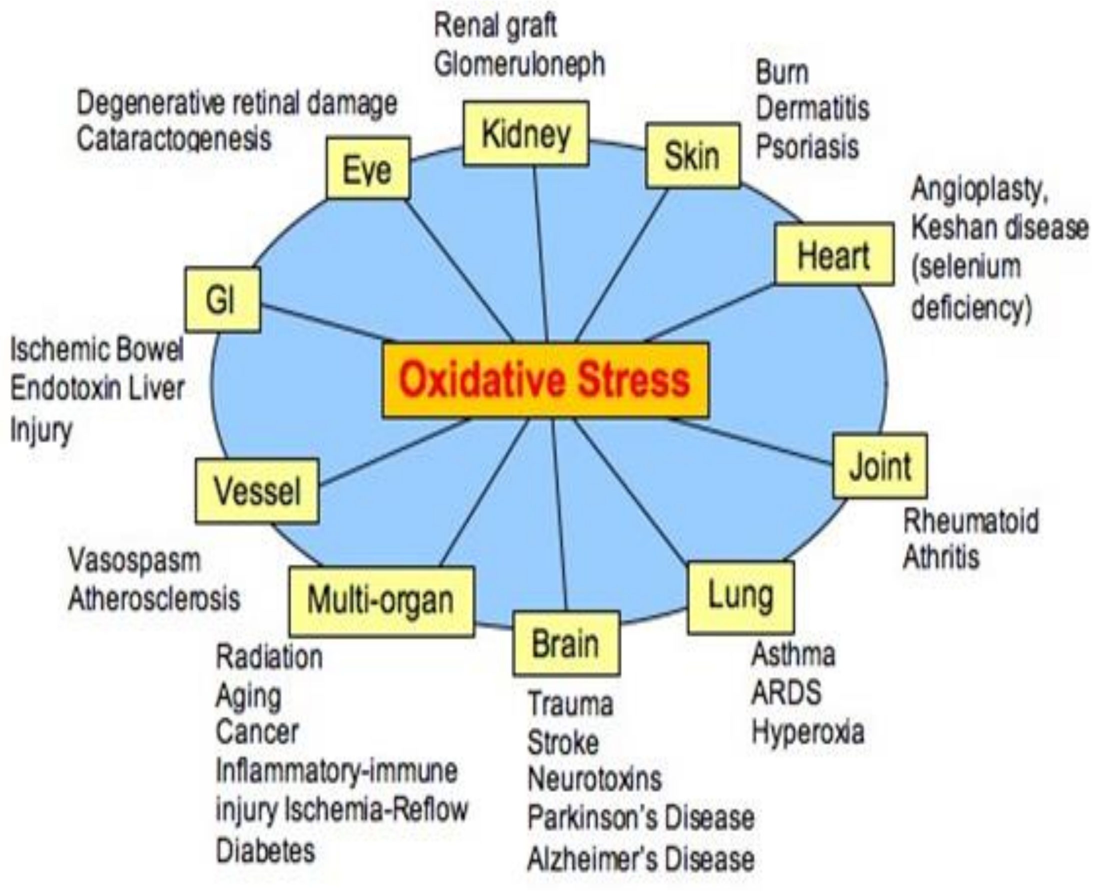


Fig 2: Disorders caused by oxidative stress

Source: National Institute of Standards and Technology (NIST)

Figure 2 showed the disorders caused by oxidative stress. Petroleum hydrocarbon pollution leads to the introduction of pro-oxidants into the environment (air, soil, water) and these in-turn leads to the generation of free radicals when they come in contact with both plant and animal tissues. And where there is an impairment or deficiency in the antioxidant defense system it leads to a condition called oxidative stress. Oxygen free radicals and other reactive species can react with the main cellular components, thus damaging tissues and causing cell injuries. This include oxidation of biomolecule such as proteins, nucleic acids (DNA and RNA), peroxidation of lipids in cells and cell membranes, as well as obstruction of proper functioning of some cell organelles e.g. mitochondria. All these affect the integrity of cells, tissues, organs or whole organism.

2.4.1 Free Radicals

Free radicals are atoms or group of atoms that have an unpaired electron. Chemical compounds and free radical reactions capable of generating potential toxic reactive species are referred to as pro-oxidants.

Oxygen molecule (O_2) can behave like a radical owing to the presence of two unpaired electrons of parallel spin. Its electronic structure result in formation of water by reduction with four electrons, i.e.

$$O_2 + 4H^+ + 4e \longrightarrow 2H_2O$$

In the sequential univalent process by which O_2 undergoes reduction, several reactive intermediates are formed, such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), and the extremely reactive hydroxyl radical (°OH), collectively termed as the reactive oxygen species, the process can be represented as:

$$O_2 \xrightarrow{e-} O_2^- \xrightarrow{e-} H_2O_2 \xrightarrow{e-} OH \xrightarrow{e-} H_2O$$

Pro-oxidants include reactive oxygen species (ROS) and reactive nitrogen species (RNS). These reactive species participate in a variety of biochemical reactions with biomolecules such as nucleic acids (DNA and RNA), proteins, lipids etc., leading to a pathological condition known as oxidative stress. Examples of reactive oxygen species include nitric oxide ($NO^{\cdot}$), superoxide radicals ($O_2^{\cdot -}$), hydroxyl radicals (OH.), alkoxy radicals (RO.), peroxy radicals (ROO.), hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl). The reactive nitrogen species are derived from nitric oxide and superoxide produced via the enzymatic activity of nitric oxide synthase 2 and NADPH oxidase respectively. Examples are peroxynitrite (ONOO-), nitrogen dioxide ($.NO_2$), dinitrogen trioxide (N_2O_3), nitrosoperoxycarbonate ($ONOOCO_2^-$) etc. The RNS act together with ROS to damage cells, causing nitrosative stress. Nitrosative stress leads to nitrocylation reaction of proteins, thereby altering their structure and so inhibit their functions as shown in figure 3.

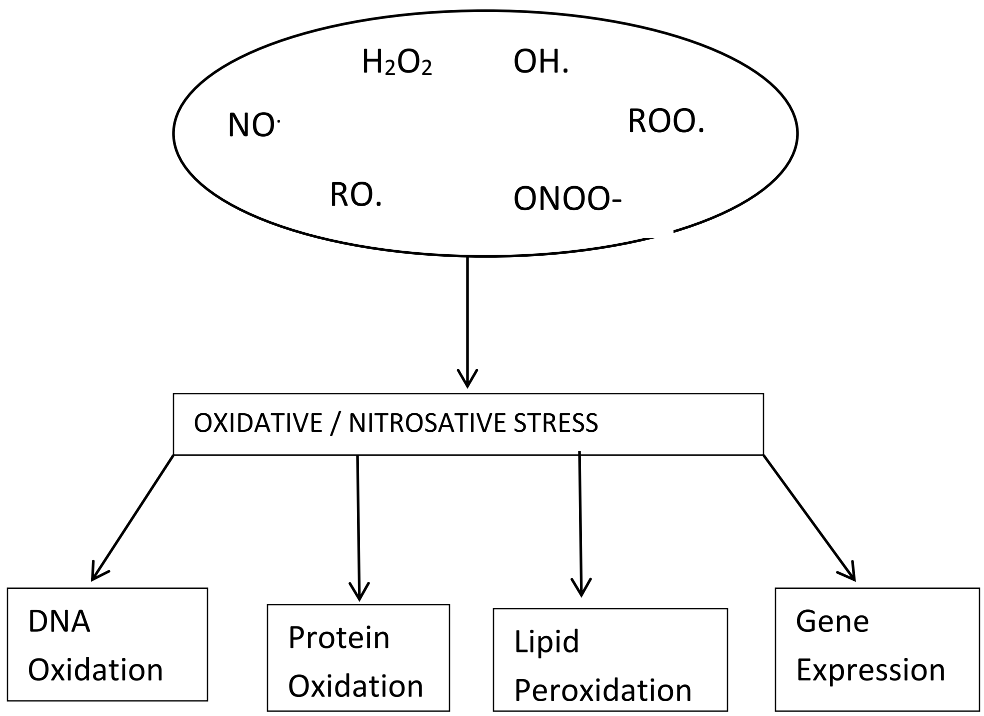


Fig. 3: Free radicals and their effects on biomolecules

2.4.2 Antioxidants

Antioxidants are molecules that inhibit the oxidation of other molecules. Oxidation is a chemical reaction that can produce free radicals, leading to chain reactions that may damage cells. Antioxidants such as thiols or ascorbic acid (vitamin C) terminate these chain reactions. To balance the oxidative state, plants and animals maintain complex systems of overlapping antioxidants, such as glutathione and enzymes e.g. catalase and superoxide dismutase, produced internally or the dietary antioxidants, vitamin A, vitamin C and vitamin E.

When pollutants from gas flaring and oil spillage gain entry into living organism either by inhalation from air or ingestion from dietary sources, they generate lots of free radicals which attack cell components causing damage to tissues and organs. The biological oxidative effects of free radicals on macromolecules are therefore controlled by a spectrum of antioxidants which can be classify into non-enzyme antioxidants e.g ascorbic acid, glutathione, vitamin E, α-lipoic acid etc. and enzyme antioxidants which include superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR), catalase (CAT), xanthine oxidase (XO) etc. These enzymes have been proposed as biomarkers of oxidative stress in a variety of aquatic and terrestial organisms (Borković, 2005). Superoxide dismutase is an oxido-reductase which catalyses the dismutation of the superoxide anion into molecular oxygen and hydrogen peroxide (Fridovich, 1989). This enzyme (SOD) exists as several isotypes, characterized by their redox-active metals at the catalytic sites. Catalase detoxifies hydrogen peroxide and has no electron donor requirement. Although catalase is a well-known antioxidative enzyme and has been implicated in protection against hydrogen peroxide, its localization is limited to the peroxisome (Kono and Fridovich, 1982).

The antioxidant defense system is therefore necessary for the maintenance of redox homeostasis in organisms. They act as primary defense against excessive generation of harmful reactive species. Where there is an impaired antioxidant defense system, it leads to a condition termed oxidative stress.

2.4.3 Oxidation of Proteins

Oxidative modification of protein leads to increased recognition and degradation by proteases and loss of enzymatic activity (Rivett and Levine, 1990). Modification of different intracellular proteins including key enzymes and structural protein lead to degeneration of neurons in the Alzheimer's disease brain (Aksenov *et al*., 2001). It could also contribute to secondary impairment of biomolecules, for instance inactivation of DNA polymerases in replicating DNA (Evans, 1999).

Mutant hemoglobins (Hb) provide opportunity to study structure–function relationships in proteins because hemoglobin is a readily isolated protein of known structure that has a large number of well-characterized naturally occurring variants. The examination of individuals with physiological disabilities, together with the routine electrophoretic screening of human blood samples, has led to the discovery of over 1000 variant hemoglobins, 90% of which result from single amino acid substitutions in a globin polypeptide. Changing an internal residue by ROS/RNS often destabilizes the hemoglobin molecules. The degradation products of these hemoglobins, particularly those of heme, form granular precipitates (known as Heinz bodies) that hydrophobically adhere to the erythrocyte cell membrane. The membrane’s permeability is thereby increased, causing premature cell lysis. Carriers of unstable hemoglobins therefore suffer from hemolytic anemia of varying degrees of severity.

All known methemoglobins arise from substitutions that provide the iron (Fe) atom with an anionic oxygen atom ligand. Changes at the oxygen-binding site that stabilize the heme in the Fe (III) oxidation state eliminate the binding of oxygen (O_2) to the defective subunits. Such methemoglobins are designated HbM and individuals carrying them are said to have methemoglobinemia. These individuals usually have bluish skin, a condition known as cyanosis, which results from the presence of deoxyhemoglobin (deoxyHb) in their arterial blood (Voet and Voet, 2011).

2.4.4 Oxidation of Deoxyribonucleic Acid (DNA) by Reactive Species

Oxidative DNA damage is an inevitable consequence when cellular metabolism is affected by ROS/RNS. Reactive species produced during oxidative stress can cause direct damage to the DNA and are therefore mutagenic, and it may also suppress apoptosis and promote proliferation, invasiveness and metastasis (Evans and Cooke, 2004). Exposure to nitrogen oxides, especially N_2O has been associated with increased oxidative DNA damage and the level of exposure plays a critical role in this regard. Increased oxidative stress represents a mechanistic link between chronic N_2O exposure and genotoxicity (Wrońska-Nofer *et al*., 2012).

The oxidation of DNA by hydroxyl radicals ($OH^{\cdot}$) takes place mainly at the guanine nucleobase of the nucleotide, leading to the conversion of guanine to 8- hydroxydeoxyguanosine. The consequences of this oxidation by hydroxyl radical include mismatch in base pairing, while normally guanine would bind three hydrogen bonds to pair with cytosine (G = C), the oxidized form (8-hydroxydeoxoguanosine) pairs with adenine via two hydrogen bonds (G = A). This mismatch in base pairing can lead to base pair transversion in the next cycle of DNA replication, which can further lead to point mutation. The presence of 8-hydroxydeoxyguanosine therefore serves as an indicator of DNA oxidation in the system.

2.4.5 Oxidation of lipids

This is considered to be the most damaging process known to occur in living organisms (García *et al*., 2004). It includes a number of reactions leading to the development of oxidized lipids and fatty acids that give rise to free radicals. Oxidation products of lipids, particularly 4-hydroxyalk-2-enals and aldehydes such as malondialdehyde, as well as alkanes, lipid epoxides and alcohols, react with proteins and nucleic acids. The overall effects of lipid oxidation are a decrease in membrane fluidity, an increase in the leakiness of the membrane to substances that do not normally cross it except through specific channels and damage to\ membrane proteins, and inactivation of receptors, enzymes, and ion channels. The most common oxidation of fatty acids is by $^{3}O_2$ from the air. Oxidation of unsaturated fatty acids only occurs in three stages at normal temperatures. In the initiation stage, free hydrogen (H•) and fatty acid (R•) emerge as the C-H covalent bond of the hydrocarbon chain split. The energy required to split bonds can come from ultraviolet radiation, radioactivity and also visible light. In the latter case, it is a two-electron oxidation of $^{1}O_2$. A reaction also exists to break any binding with other free radicals or transition metals. During the second propagation stage the reactive R• quickly merges with O_2, and produces a peroxyl radical (R-O-O•). As the hydrogen atom splits from the hydrocarbon chain, another molecule of unsaturated fatty acid forms hydroperoxide (R-O-OH) and another R•. The initiation rate of oxidation for the production of R-O-OH is slow (induction period) leading to a gradual accumulation of R-O-OH, followed by the creation of other radicals. As long as there is enough oxygen, the reaction takes place spontaneously, sharply rising to reach the maximum speed of reaction, in which reactive groups are diminished. The rate of this reaction then slows and starts to be overtaken by the degradation of R-O-OH. R-O-OH is very fragile and H• splits from the molecule, leaving R-O-O• or HO•. According to the current knowledge, R-O-OH degradation with conjugated double bonds leads preferentially to formation of the alkoxyl radical (R-O•) (Cleveland *et al*., 2007).

2.4.6 Biomarkers In Oxidative Stress Assessment

The concentrations or activities of antioxidants and other oxidative stress parameters are usually investigated during petroleum hydrocarbon pollution in order to assess cellular damage and its effect on individual cells, tissues, organs or whole organism. The free radical scavenging ability of antioxidants, directly or indirectly, facilitates reactive species neutralization from the system. ROS/RNS can be detoxified by non-enzyme antioxidants with scavenging action eg. ascorbic acid, glutathione, vitamin E etc. or the enzyme defense system comprising of catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR) and xanthine oxidase (XO) etc. as seen in figure 4.

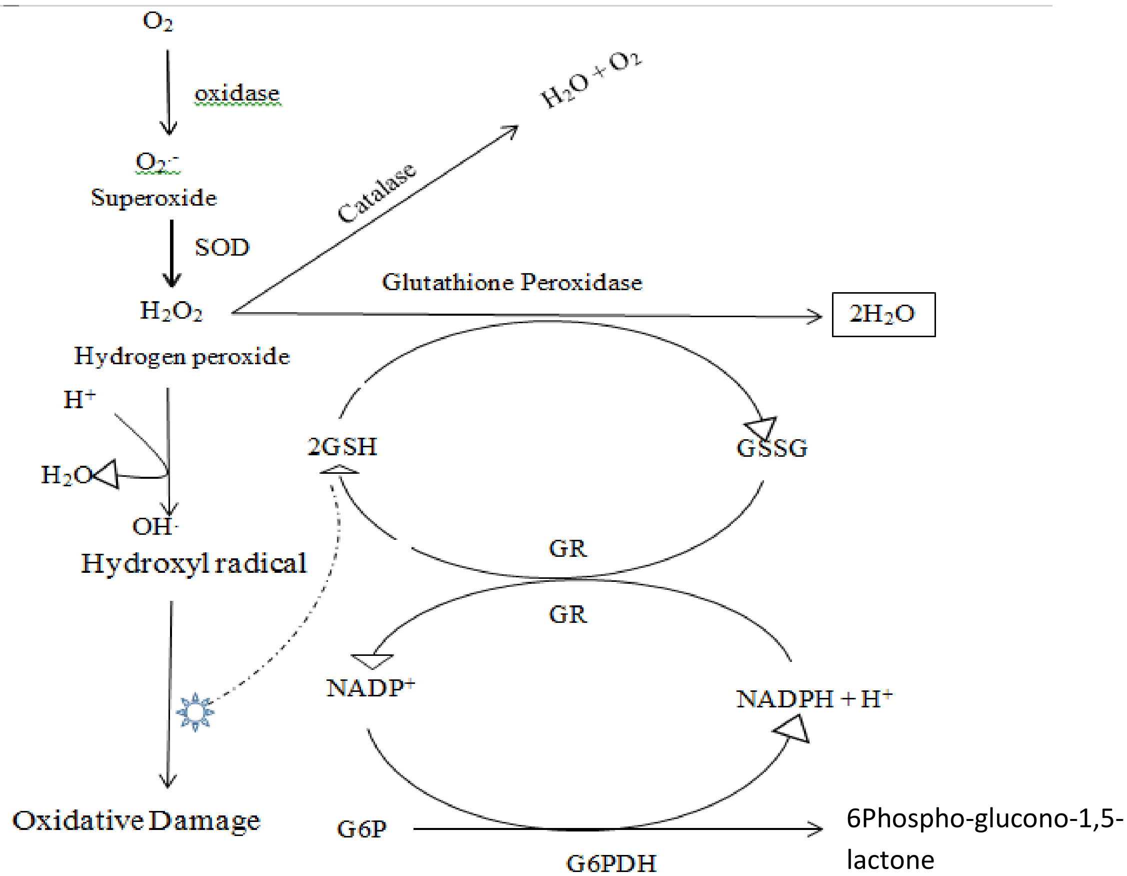


Fig. 4: The role of antioxidants and NADPH in protecting cells against oxidative damage.

Key: G6P = Glucose 6-phosphate, G6PDH= Glucose 6-phosphate dehydrogenase

GR= Glutathione reductase, NADPH = Nicotiamide Adenine Dinucleotide Phosphate

Defence mechanisms against free radical-induced oxidative damage include the following:

i. Catalytic removal of free radicals and reactive species by factors such as CAT, SOD, GPx and thiol-specific antioxidants;

ii. Binding of proteins (e.g., transferrin, metallothionein, haptoglobins, ceruloplasmin) to pro-oxidant metal ions, such as iron and copper;

iii. Protection against macromolecular damage by proteins such as stress or heat shock proteins; and

iv. Reduction of free radicals by electron donors, such as GSH, vitamin E (α- tocopherol), vitamin C (ascorbic acid), bilirubin, and uric acid (Halliwell and Gutteridge, 1999).

2.5 MECHANISM OF ACTION OF ANTIOXIDANTS

2.5.1 Mechanism of Action of Non-Enzyme Antioxidants

(i) Ascorbic Acid (AA)

Ascorbic acid is a water-soluble antioxidant which scavenges reactive intermediates in the cytoplasm and intracellular environment thereby reducing its concentration in living organisms. As a cellular reductant, it readily donates electrons to reduce free radicals in the cellular compartment and it is converted to dehydroascorbic acid (DHAA), its oxidized form. The decrease of this antioxidant explains its utilization in the neutralization of free radicals in order to prevent cellular damage (Luay and Tareq, 2014). In the process of scavenging free radicals in order to prevent free radical-induced damage of lipoproteins and other macromolecules, ascorbic acid is first oxidized to ascorbyl radical and then to dehydroascorbate. Ascorbic acid is found to be far more effective in inhibiting lipid peroxidation initiated by a peroxyl radical initator than other plasma components, such as bilirubin and alpha-tocopherol (Chavez *et al*., 2007).

(ii) Glutathione (GSH)

Glutathione is a major soluble antioxidant located in the cytosol and mitochondria (Masella *et al*., 2005). It reacts with singlet oxygen, superoxides and hydroxyl radicals and by so doing function directly as free radical scavenger. Glutathione (GSH) participates in the detoxification of xenobiotics as a substrate for the enzymes glutathione-S-transferase and glutathione peroxidase. In the process of quenching free radicals GSH becomes oxidized.

$$\underset{\text{Glutathione}}{GSH + H_2O_2} \xrightarrow{\text{Glutathione Reductase}} \underset{\text{Glutathione Disulfide}}{GSSG} + H_2O$$

Therefore investigating the concentration of this antioxidant in living organisms located close to the petroleum hydrocarbon pollution (gas flaring) site is one of the major assessments that determine the state of oxidative stress. A decrease in glutathione concentration was observed in petrol station workers (Luay and Tareq, 2014), which could be as a result of exposure to the petroleum hydrocarbon fumes.

(iii) Vitamin E

Vitamin E, is the collective name for a set of eight related tocopherols and tocotrienols, which are fat-soluble vitamins with antioxidant properties (Herrera and Barbas, 2001). Of these, α-tocopherol has been most studied as it has the highest bioavailability, with the body preferentially absorbing and metabolising this form (Brigelius and Traber, 1999).

It has been claimed that the α-tocopherol form is the most important lipid-soluble antioxidant, and that it protects membranes from oxidation by reacting with lipid radicals produced in the lipid peroxidation chain reaction. This removes the free radical intermediates and prevents the propagation reaction from continuing. This reaction produces oxidised α-tocopheroxyl radicals that can be recycled back to the active reduced form through reduction by other antioxidants, such as ascorbate, retinol or ubiquinol (Traber and Atkinson, 2007). Vitamin E is a powerful chain-breaking antioxidant, which primarily prevents lipid peroxidation by breaking the chain of events leading to the formation of hydroperoxides. Vitamin E decreased significantly in petroleum station workers, and this was believed to be due to the effects of pollutants from the petroleum hydrocarbons (Luay *et al*., 2014**).**

2.5.2 Mechanism of Action of Enzyme Antioxidants

(iv) Catalase (EC 1.11.1.6)

Catalase is a common enzyme that catalyzes the decomposition of hydrogen peroxide to water and oxygen molecule. Mammals, including humans and mice, express catalase in all tissues. One of the most efficient ways of removing peroxide is through the activity of catalase, which is encoded by a single gene, and is highly conserved among species and a high concentration of catalase can be found in the liver, kidneys and erythrocytes. The expression is regulated at transcription, post-transcription and post-translation levels. High catalase activity is detected in peroxisomes. Catalase is very important in protecting the cell from oxidative damage. More recently, short wavelength UV radiation has been shown to produce reactive oxygen species (ROS) through the action of catalase. This response is thought to act as a mechanism to protect DNA by converting damaging UV radiation into ROS species that can be metabolized and detoxified by cellular antioxidant enzymes.

$$2H_2O_2 \xrightarrow{\text{catalase}} 2H_2O + O_2$$

Catalase consists of four subunits, each of which contains a ferric (Fe^{+3}) heme group bound to its active site (Chih and Pei, 2012); however, Fe deficiency causes a significant decrease of catalase activity in the presence of large amounts of H_2O_2 and thereby diffusing to the cytosol from the mitochondria, catalase along with GPx becomes the most important scavenger in the cytosol. Various studies have reported lower plasma and erythrocyte catalase activity and increased oxidative stress in patients suffering from mitochondria related diseases (Guo *et al.*, 2011) and individuals exposed to xenobiotics (Ashraf and Hoda, 2008; Ambica *et al*., 2016). The variable response of CAT activity has been observed under metal stress (Ghafourifar and Cadenas, 2005), while all ions of heavy metals are non-competitive inhibitors of CAT. Cyanides are strong inhibitors of CAT as they form a strong bond with the heme of CAT and stop its catalytic activity (Vlasits *et al.*, 2010). Studies have shown that CAT is effective in the degradation of H_2O_2 present only, while glutathione peroxidase is effective in peroxide degradation at concentrations lower than 100 μmol. (Dale *et al.*, 2000).

(v) Superoxide dismutases (EC 1.15.1.1)

Superoxide dismutase, SOD, (EC 1.15.1.1) belongs to the group of oxido reductase. In living systems, O_2•- is capable of reacting with another molecule of O_2•- (dismutation), it is also able to react with another radical, such as NO•. The formation of HO• from O_2•- via the metal-catalyzed Haber Weiss reaction has a reaction rate 10 000 times faster than that of spontaneous dismutation, therefore, SOD provides the first line of defense against ROS (Ghafourifar and Cadenas, 2005),

$$2O_2\bullet^- + 2\,H^+ \xrightarrow{\text{SOD}} H_2O_2 + O_2$$

These enzymes are present in almost all aerobic cells as well as in anaerobic organisms, in all subcellular compartments. The active site of the enzyme contains one or two different atoms of a transition metal in a certain oxidation state. Antioxidant, superoxide dismutase (SOD), plays critical roles in scavenging superoxide ($O_2^{-\cdot}$). They are classified by their metal cofactors into known types (i) copper-zinc superoxide dismutase (Cu/ZnSOD) (ii) manganese superoxide dismutase (MnSOD) and (iii) extracellular superoxide dismutase (EC-SOD). The Cu/ZnSOD and MnSOD, are localized in different cellular compartments. The major intracellular SOD is a 32-kD copper and zinc containing homodimer (Cu/Zn SOD). The mitochondrial SOD (MnSOD) is a manganese-containing 93-kD homotetramer that is synthesized in the cytoplasm and translocated to the inner matrix of mitochondria, while EC-SOD is the primary extracellular SOD enzyme and is highly expressed in many organs. Studies have shown that SOD acts as both an antioxidant and anti-inflammatory in the body, neutralizing the free radicals that can lead to wrinkles and precancerous cell damages (Elchuri *et al*., 2005). The enzyme has also been seen to decrease in its activity in many disease conditions such as and in individuals who are exposed to xenobiotics such as benzene, Ashraf and Hoda (2008) and lead (Ambica *et al*., 2016), but on the contrary few reports revealed increase in the enzyme's activity (Ahamed *et al*., 2006; Shrirang *et al*., 2017. When superoxide dismutase is deficient or absent, this will result to elevated free radical (superoxide). Therefore, its assay will help in the assessment of oxidative stress. MnSOD is a mitochondrial enzyme which contributes significantly to protecting the organism from the toxic effects of O_2•- (Schaich, 1992).

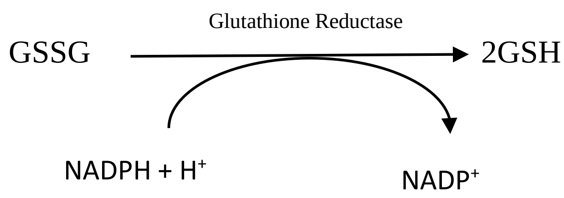
(vi) Glutathione Peroxidase (EC1.11.1.9)

Glutathione peroxidase (GPx) is an enzyme that catalyses the reduction of peroxides by using glutathione as the reducing agent. Glutathione acts as an electron donor in the reduction reaction, producing GSSG (oxidized form) as an end product. Glutathione peroxidase (GPx) has several types of isoenzymes that are encoded by different genes, which vary in cellular location and substrate specificity. Glutathione reductase (GR) plays a critical role by regenerating reduced glutathione (GSH) from the oxidized form (GSSG). Thus both the glutathione peroxidase and reductase are enzyme antioxidants and can be used as an indicator for oxidative stress in living organisms. CAT is found in many types of cells and scavenges hydrogen peroxide as its sole substrate while GPx scavenges various peroxides. The expression of CAT in most cells is lower than that of GPx, with the exception of hepatocytes and erythrocytes. A significant decrease in activities of the enzymes i.e., GPx, CAT and Cu/Zn-SOD were expressed in different types of cancer patients (Asaduzzaman *et al*., 2010; Janina *et al.*, 2014).

(vii) Glutathione Reductases (EC1.6.4.2)

Glutathione reductase (GR) plays an indirect but essential role in the prevention of oxidative damage within the cell. Glutathione reductase is an important enzyme which act in aerobic glycolysis and has been found not only in animal tissues but also in microorganisms, yeasts and higher plants. Glutathione (GSH) plays an important role in oxido-reduction processes and detoxification of H_2O_2 and organic peroxides, which are substances, produced in large quantities during inflammatory processes in living cells.

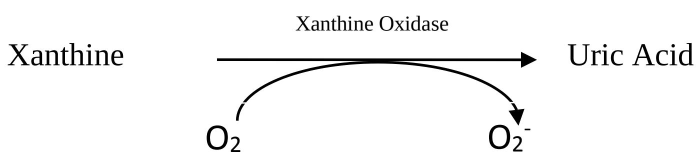
Glutathione is an active reductant responsible for minimizing harmful hydrogen peroxide in the cell; thus helping to maintain appropriate level of intracellular glutathione (GSH) is made possible by the presence of glutathione peroxidase, in the process, glutathione is oxidized to glutathione disulfide (GSSG). The regeneration of GSH is catalyzed by glutathione reductase (GR) (Andersen 1997). Glutathione reductase is one of the enzymes requiring riboflavin for its activation. Investigations have shown that erythrocyte glutathione reductase is present in at least two forms, an active form associated with flavin adenine dinucleotide (FAD) and an inactive form not bound to FAD, which can be activated.



Oxidative stress set in when the activity of glutathione reductase is inhibited. This will result in accumulation of glutathione disulfide, and this implies that glutathione (GSH) cannot be regenerated, leading to reduction in its concentration and finally its deficiency. Deficiency or total absence of glutathione will give room for the free radicals to exact their effect.

(viii) Xanthine Oxidase (EC 1.17.3.2)

Xanthine oxidase is recognized as the terminal enzyme of purine catabolism, catalyzing the hydroxylation of hypoxanthine to xanthine and then to uric acid. Xanthine oxidase (XO) is a highly versatile enzyme. It is present in nearly all species, in mammalian tissues, xanthine oxidase is found predominately in the liver and intestine. Human xanthine oxidase activity is almost exclusively to these tissues. This enzyme is known for its ability to serve as a source of oxidizing agent for radicals such as hydrogen peroxide and superoxide radicals. When acting as an NADH oxidase, xanthine oxidase is a generator of superoxide.



CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Area

The study was conducted in two communities in two different states in the East Geopolitical Zone of Nigeria. Imo State is one of the nine states in the Niger Delta. Ebocha, an oil and gas field run by Nigeria Agip Oil Company constitute the test area, while Nsukka in Enugu state which has no history of petroleum hydrocarbon pollution constitute the control area.

3.2 Selection of Participants

One hundred and five (105) apparently healthy volunteers (Ebocha = 45 females and 30 males; Nsukka = 15 males and 15 females) were used. Making it a total of seventy residents of Ebocha and thirty were resident in Nsukka communities for over twenty years and were selected for sample collection as test and control samples respectively excluding pregnant women, HIV patients, alcoholics, and hypertensive patients.

3.3 Equipment and Chemicals

The instruments used included:

Spectrophotometer (Gallen kamp and compec M210), centrifuge (ROVSUN 110v), incubator (Nhybrid VX), other apparatus and materials used were usual laboratory apparatus such as syringe, sample bottles, pipettes, and test tubes. The chemicals used for the various determination of the concentration and assay of biochemical parameters were of analytical grade. They included 2, 4-dinitrophenyl hydrazine, sodium azide, 5-5^{1} dithiobis-2-nitrobenzoic acid (DTNB) etc.

3.4 METHODS

3.4.1 Sample Preparation

Four millilitres (4ml) of blood sample was collected and dispensed into a test tube with no anticoagulant. The sample was allowed to clot at room temperature for 30 minutes, and then centrifuged at 2000 xg for 15 minutes at $4^{o}C$. The yellow serum supernatant was collected and used for various analyses.

3.4.7 Determination of Ascorbic Acid Concentration

The measurement of the concentration of ascorbic acid was chosen in this study because it has been known as a powerful, potent water soluble antioxidant. Ascorbic acid concentration was determined according to Roe and Kuether (1961).

3.4.2.1 Principle of the Test

Ascorbic acid determination by Roe and Kuether's method (1961) is based on the conversion of ascorbic acid to dehydroascorbic acid by shaking with norit and this is coupled with 2, 4-dinitrophenyl hydrazine in the presence of thiourea as a mild reducing agent. Sulphuric acid (85%) then converts the dinitrophenyl hydrazine to a red coloured solution. The absorbance was read at 540nm

3.4.2.2 Procedure

One millilitre (1ml) of 2, 4-dinitrophenyl hydrazine and 20ul of thiourea were added to the test tube containing 0.02ml of serum, the mixture was then treated with 85% sulphuric acid. The standard solution was prepared by adding 1ml of 2, 4-dinitrophenyl hydrazine into a test tube containing 0.4ml ascorbate concentration. The absorbance readings were read at 540nm.

Calculation:

$$\text{Ascorbic Acid Concentration (mg/dl)} = \frac{\text{Absorbance of Test}}{\text{Absorbance of Standard}} \times \text{Conc. Of Standard}$$

3.4.8 Assay for Catalase Activity

One of the most efficient ways of scavenging peroxide is through the activity of catalase. This formed the basis for its inclusion in this study. Serum catalase activity was assayed according to the method of Beer and Sizer (1952).

3.4.3.1 Principle of Assay

The enzyme catalase catalyzes the decomposition of hydrogen peroxide to form water and oxygen.

$$2H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O + O_2$$

In the reaction, catalase induced decomposition of hydrogen peroxide H_2O_2 into water and oxygen. The rate of disintegration of hydrogen peroxide into water and oxygen is proportional to the activity of catalase.

3.4.3.2 Procedure

The reaction mixture (3ml) contained 0.1ml of serum in phosphate buffer (50mM, pH 7.0) and 2.9ml of 30mM H_2O_2 in phosphate buffer. An extinction coefficient for H_2O_2 at 240nm was used to calculate the enzyme activities.

Calculation:

$$\text{Catalase Activity (U/L)} = \frac{\log A1/A2 \times 0.23}{6.93 \times 10^{-3}}$$

Where A1 = initial absorbance

A2 = absorbance after 15sec

3.4.4 Assay for Superoxide dismutase Activity

The deficiency or absence of superoxide dismutase in the body will result to elevated free radical (superoxide) (Schaich, 1992). Therefore, its assay will help in the assessment of oxidative stress. Serum superoxide dismutase activity was assayed according to the method of Sun and Sigma as described by Ogbunugafor *et al.,* (2010).

3.4.4.1 Principle

The assay of superoxide dismutase (SOD) activity was based on the ability of the enzyme to inhibit the auto-oxidation of adrenaline at pH 10.2. Superoxide anion ($O_2^{\cdot-}$) generated by the xanthine oxidase reaction is known to cause oxidation of adrenaline to adrenochrome. The yield of adrenochrome produced per superoxide anion introduced, increases with increasing pH and concentration of adrenaline.

3.4.4.2 Procedure

The method of Sun and Sigma as described by Ogbunugafor *et al.* (2010) was adopted. The reaction mixture (3ml) contained 2.95ml sodium carbonate buffer (0.05mM, pH 10.2), 0.02ml of serum and 0.03ml of epinephrine in 0.005N HCl was used to initiate the reaction. The reference cuvette contained 2.95ml buffer, 0.03ml of substrate (epinephrine) and 0.02ml of water. The reaction mixture was read at 30sec interval for 150sec. An extinction coefficient for epinephrine at

480nm was used in calculating the activity. The % inhibition of auto oxidation of epinephrine by SOD was calculated and the serum SOD activity was expressed as U/ml. One unit of SOD activity was equivalent to the amount of SOD that can cause 50% inhibition of epinephrine.

Calculation:

Increase in absorbance per time = (Af – Ao)/ 2.5

Where: Ao = Absorbance after 30sec,

Af = Absorbance after 150secs

$$\% \text{ Inhibition} = 100 - \left(\frac{\text{Increase in absorbance for substrate} \times 100}{\text{Increase in absorbance for Blank}}\right)$$

One unit of superoxide dismutase activity was given as the amount of the SOD necessary to cause 50% inhibition.

3.4.5 Assay for Glutathione Peroxidase Activity

The activity of glutathione peroxidase was assayed using the method of Rotruck *et al.* (1973).

3.4.5.1 Principle

Glutathione peroxidase in the presence of H_2O_2 oxidizes glutathione to form water and GSSG (oxidized glutathione). The amount of glutathione consumed is directly proportional to the activity of the enzyme, glutathione peroxidase and it is expressed as U/ml. The glutathione remaining after the reaction is allowed to react with 5-5[1] dithiobis-2-nitrobenzoic acid (DTNB) to form a yellow complex that absorbs maximally at 412nm.

3.4.5.2 Procedure

The reaction mixture contained 0.4ml of phosphate buffer (pH 7.0), 0.1ml sodium azide and 0.2ml of serum sample, 0.2ml of glutathione and 0.1ml of H_2O_2. The mixture was incubated at 37°C for 10min and the reaction was arrested by the addition of 0.4ml of 10% trichloroacetic acid (TCA). The tubes were centrifuged at 4000rpm for 5min, then 0.5ml of the supernatant, 2ml of phosphate buffer and 0.5ml of 40nM DTNB was mixed and the resulting yellow colour was read at 412nm wavelength. The blank was treated the same way except that it contained 0.2ml of distilled water instead of the sample.

Calculation:

$$\text{GPx Activity (U/L)} = \Delta \text{ Absorbance / min} \quad \times F$$

$$F = TV/SV \times 10^3/6.22$$

Where F= dilution factor; TV = Total Volume in ml; SV = Sample Volume in ml

3.4.6 Assay for Glutathione Reductase Activity

Serum glutathione reductase activity was assayed according to the method of Stahl *et al.* (1969). The assay of glutathione reductase was analysed considering the fact that the enzyme helps to maintain appropriate level of glutathione (GSH) in the system.

3.4.6.1 Principle

Glutathione reductase catalyses the reduction of oxidised glutathione (GSSG) in the presence of reduced nicotiamide adenine dinucleotide phosphate (NADPH), which is oxidised to $NADP^+$. The activity of glutathione reductase was calculated by measuring the rate of NADPH oxidation.

$$NADPH + GSSG + H^+ \xrightarrow{\text{Glutathione reductase}} NADP^+ + 2GSH$$

3.4.6.2 Procedure

The reaction mixture containing 1ml of phosphate buffer, 0.5ml of EDTA, 0.5ml GSSG (oxidized glutathione) and 0.2ml of NADPH was made up to 3ml with distilled water. After addition of 0.1ml of sample. The change in optical density at 340nm was monitored for 2min at 30seconds interval.

Calculation:

$$\text{GR Activity (U/L)} = 4983 \times \Delta \text{ Absorbance/min}$$

3.5 Statistical Analysis

All readings from this study were obtained in quadruplicate. The data generated were expressed as mean ± standard deviation and analyzed using the student t-test and statistical software package, SPSS. The values were considered significant at $p < 0.05$.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.0 RESULTS

4.1 Ascorbic Acid Concentrations (Female Participants)

The results are presented as mean ± standard deviation. The mean values obtained for ascorbic acid concentration of adult female individuals from Ebocha (n = 45) and Nsukka (n=15) were 2.06 ± 0.81 mg/dl and 3.39 ± 0.95 mg/dl respectively, as shown in fig. 4.1.

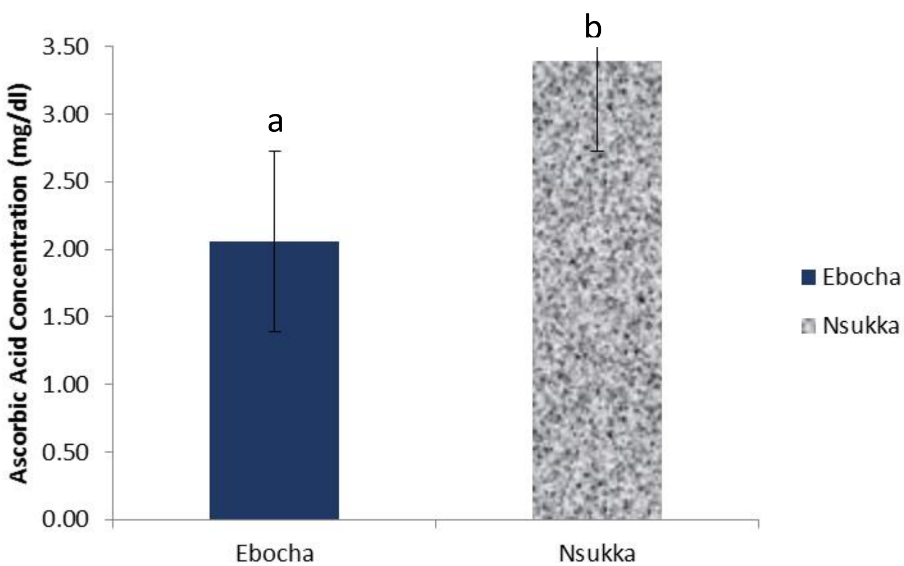


Fig.4.1: Serum ascorbic acid concentrations of female individuals native to Ebocha and Nsukka.

The means were found to be significantly different ($p < 0.05$) with difference in mean of 1.33± 0.14mg/dl. This indicates that chronic exposure to petroleum hydrocarbon pollution to adult females of Ebocha community has adversely reduced the ascorbic acid concentration in the body.

4.2 Catalase Activity (Female Participants)

The result from adult female individuals from Ebocha gave a mean value of 1.75 ± 0.85 U/L for serum catalase activity as against the mean value of 4.04 ± 1.19 U/L obtained for the individuals from Nsukka as shown in figure 4.2. (Ebocha n=45; Nsukka n=15)

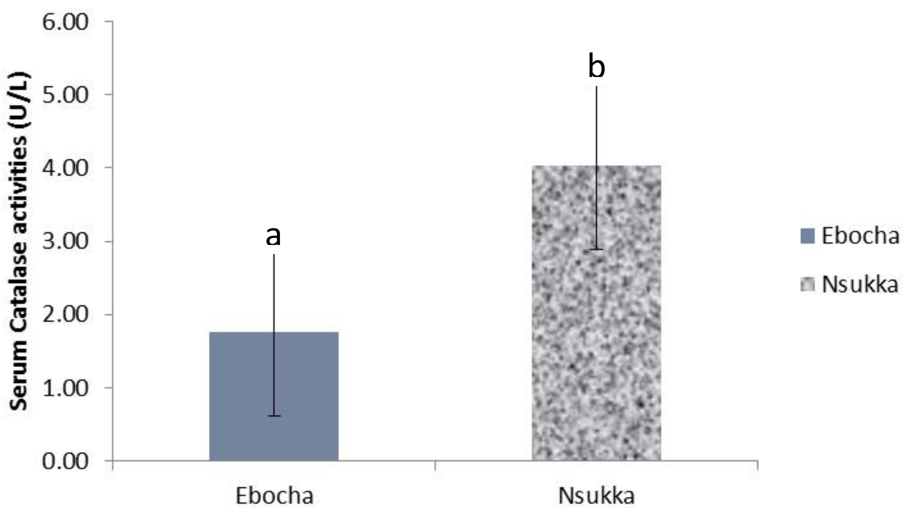


Fig. 4.2: Serum catalase activities of female individuals native to Ebocha and Nsukka.

The mean values proved to be significantly different ($p < 0.05$), with the mean difference of 2.29±0.34U/L. The value obtained for individuals from the petroleum hydrocarbon polluted area (Ebocha) was found to be substantially lower than that obtained for individuals from non-polluted area (Nsukka) as a result of chronic exposure to gas flaring.

4.3 Superoxide Dismutase (SOD) Activity (Female Participants)

The mean values of serum superoxide dismutase (SOD) activity for adult female individuals from Ebocha (n=45) and Nsukka (n=15) are shown in Figure 4.3.

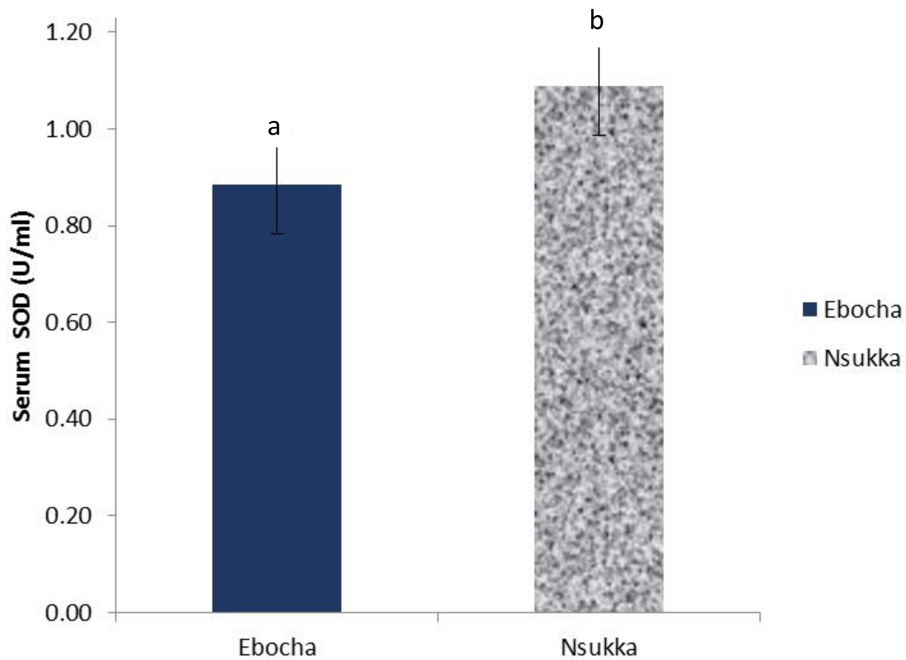


Fig. 4.3: Serum superoxide dismutase (SOD) activity of female individuals native to Ebocha and Nsukka.

The result showed a significant difference ($p < 0.05$) between the means obtained for the adult female individuals from Ebocha (0.89 ± 0.26 U/ml) and the ones from Nsukka (1.09 ± 0.07 U/ml).

The result indicates that chronic exposure to gas flaring by adult female individuals, native to Ebocha lowers superoxide dismutase activities when compared to Nsukka.

4.4 Glutathione Peroxidase (GPx) Activity (Female Participants)

Glutathione peroxidase activity was increased in the serum of adult female individuals native to Ebocha (n=45) when compared with that of individuals from Nsukka (n=15) as shown in figure 4.4.

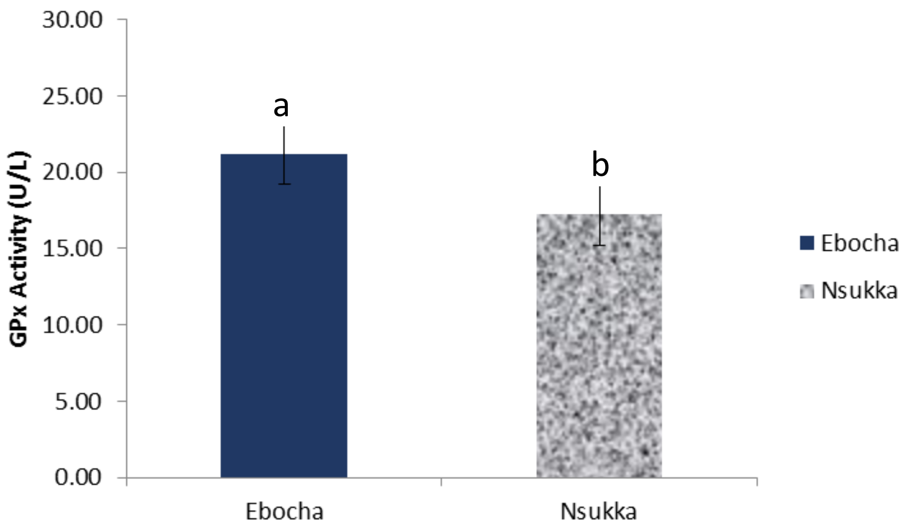


Fig. 4.4: Serum glutathione peroxidase (GPx) activity of female individuals native to Ebocha and Nsukka.

Thus the mean values 21.22 ± 7.19 U/L for individuals from Ebocha and 17.24 ± 3.55 U/L obtained for individuals from Nsukka were significantly different ($p < 0.05$) with the mean difference of 3.98±3.64U/L.

4.5 Glutathione Reductase (GR) Activity (Female Participants)

Glutathione reductase activity in the serum of adult female individuals from Ebocha (n=45) and Nsukka (n=15) showed a mean value of 26.84 ± 4.07 U/L and 24.08 ± 0.88 U/L respectively as indicated in figure 4.5.

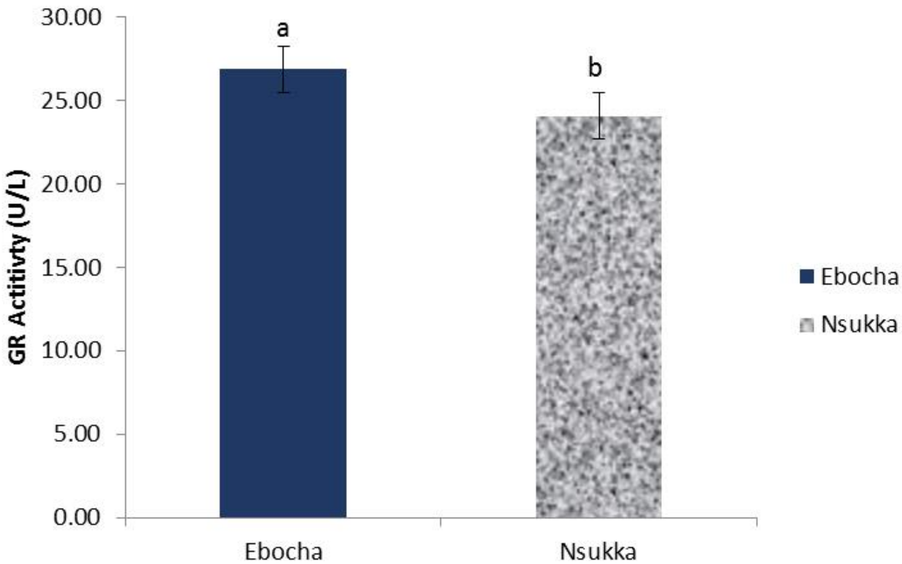


Fig. 4.5: Serum glutathione reductase activity of female individuals native to Ebocha and Nsukka.

At ($p < 0.05$), there was a significant difference between the means obtained from the two areas, namely Ebocha and Nsukka respectively with the mean difference of 2.76±3.19U/L. The result indicates that chronic exposure of adult female individuals, native to Ebocha to gas flaring increases the serum glutathione reductase activity than individuals from the non-polluted area (Nsukka).

4.6 Ascorbic Acid Concentrations (Male Participants)

The mean values obtained for ascorbic acid concentration for male individuals from Ebocha (n=30) and Nsukka (n=15) were 3.09 ± 0.11 mg/dl and 3.72 ± 1.11 mg/dl respectively as shown in figure 4.6.

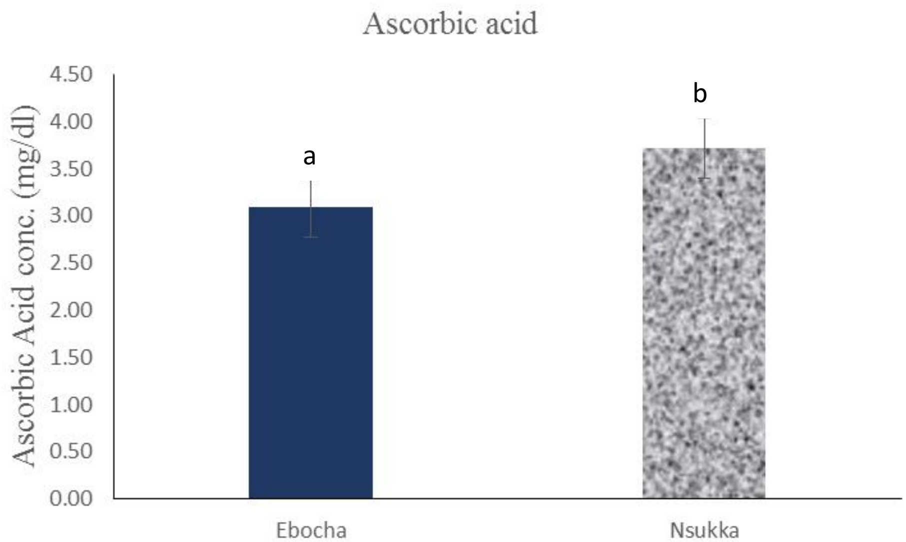


Fig. 4.6: Serum ascorbic acid concentrations of male individuals native to Ebocha and Nsukka.

These means were found to be significantly different ($p < 0.05$).with the mean difference of 0.63+1.00 mg/dl. From the result in figure 4.6, we can infer that chronic exposure to gas flaring by adult male individuals from Ebocha reduces their serum ascorbic acid concentrations than that of non-polluted area of Nsukka.

4.7 Catalase Activity (Male Participants)

The result from male individuals from Ebocha (n=30) gave a mean value of 1.94 ± 0.97 U/L for serum catalase activity as against the mean value of 2.67 ± 1.46 U/L obtained from Nsukka (n=15) as shown in figure 4.7.

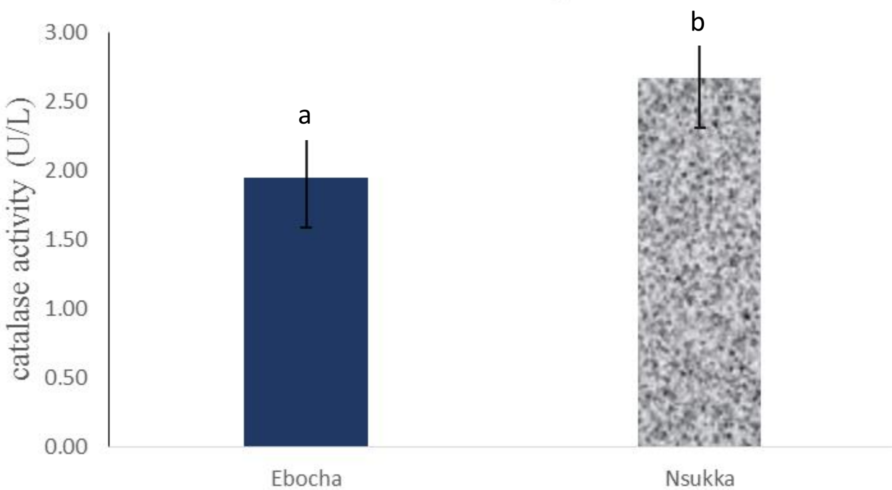


Fig. 4.7: Serum catalase activities of male individuals native to Ebocha and Nsukka.

From the figure 4.7, the mean values proved to be significantly different ($p < 0.05$) with the mean difference of 0.73±0.49U/L. The value obtained for individuals from the petroleum hydrocarbon polluted area (Ebocha) was found to be lower than that obtained for individuals from non-polluted area (Nsukka).

4.8 Superoxide Dismutase (SOD) Activity (Male Participants)

The mean values of serum superoxide dismutase (SOD) activity for male individuals from Ebocha (n=30) and Nsukka (n=15) are shown in Figure 4.8.

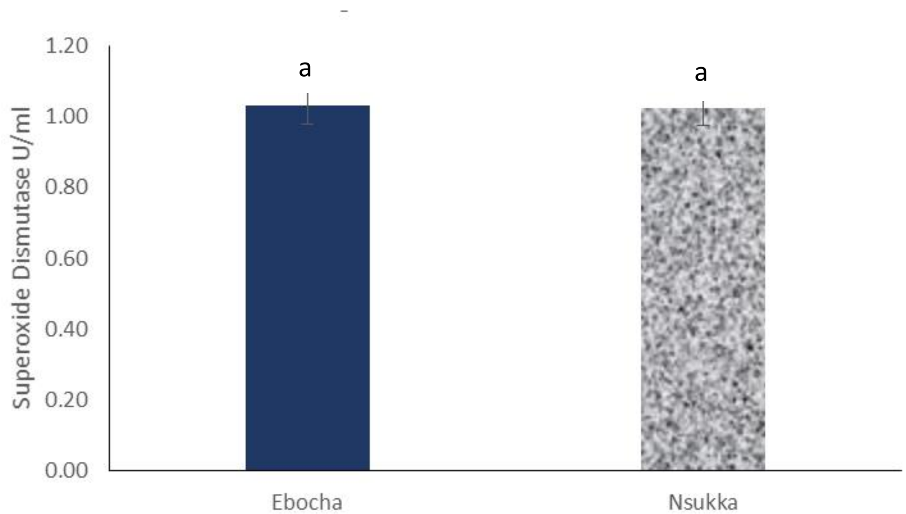


Fig. 4.8: Serum superoxide dismutase (SOD) activity of male individuals native to Ebocha and Nsukka.

The result showed no significant difference ($p < 0.05$) between the two means obtained for the males from Ebocha (1.03 ± 0.31 U/ml) and the ones from Nsukka (1.03 ± 0.15 U/ml) respectively were found to be the same.

4.9 Glutathione Peroxidase (GPx) Activity (Male Participants)

Glutathione peroxidase activity was found to be lower in the serum of male individuals from Ebocha (n=30) when compared with that of individuals from Nsukka (n=15) as indicated in Figure 4.9.

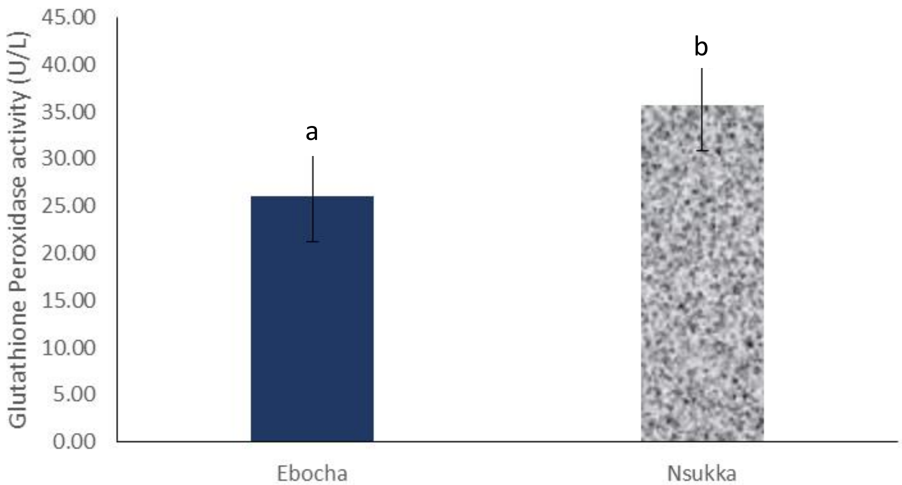


Fig. 4.9: Serum glutathione peroxidase (GPx) activity of male individuals native to Ebocha and Nsukka**.**

From the figure 4.9, the result showed 26.00 ± 8.11 U/L for males from Ebocha and 35.64 ± 11.97 U/L from Nsukka respectively and the mean values proved to be significantly different ($p < 0.05$) with the mean difference of 8.36±3.86U/L. From the result we can infer that chronic exposure to gas flaring substantially lowered the activities of serum glutathione peroxidase in adult males of Ebocha.

4.10 Glutathione Reductase (GR) Activity (Male Participants)

Glutathione reductase activity in the serum of male individuals from Ebocha (n=30) and Nsukka (n=15) showed mean values of 27.59 ± 1.83 U/L and 24.52 ± 0.91 U/L respectively as shown in figure 4.10.

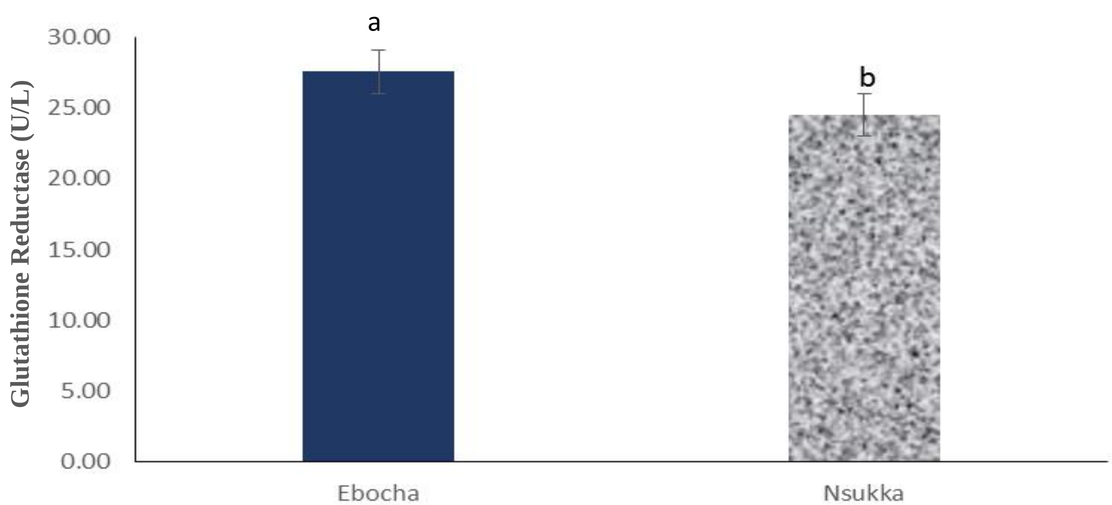


Fig. 4.10: Serum glutathione reductase activity of male individuals native to Ebocha and Nsukka.

From the figure 4.10, the mean values proved to be significantly different ($p < 0.05$) with the mean difference of 3.07±0.92U/L. From the result, increased serum glutathione reductase activity in adult male individuals of Ebocha is as a result of chronic exposure to gas flaring when compared to the non-polluted area of Nsukka.

4.11 Ascorbic Acid Concentrations across Different Age Groups

The result showed that there was a significant difference ($p < 0.05$) between the age groups 31-40yrs (3.22 ± 0.06 mg/dl) and 61-70yrs (1.83 ± 0.87 mg/dl) respectively with the mean difference of 1.39+0.81mg/dl.

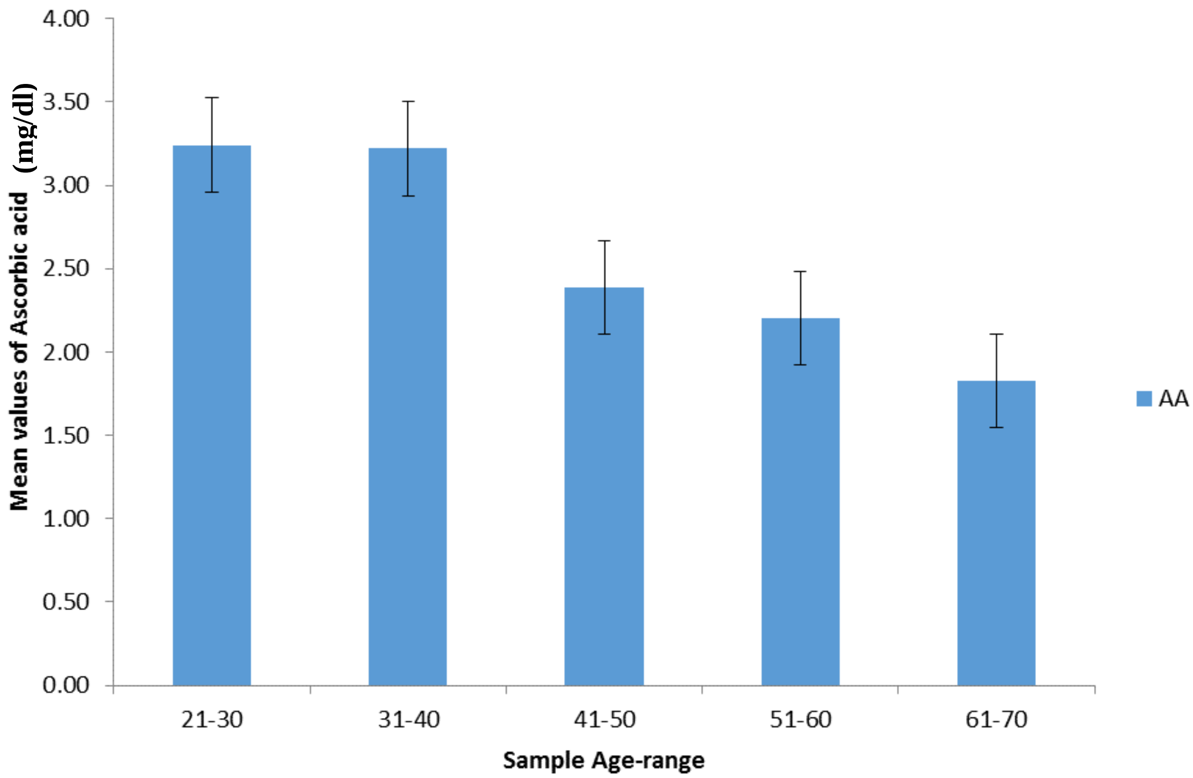


Fig. 4.11: Ascorbic acid concentrations across different age groups of individuals native to Ebocha**.**

The result further showed that ascorbic acid concentration decreased as age increased. That is, the concentration of ascorbic acid reduced as the duration of exposure increased.

4.12 Catalase Activity across Different Age Groups

The result showed that there was a significant difference between the mean values of ages 51-60yrs and 61-70yrs (1.82 ± 1.01 U/L and 1.24 ± 0.62 U/L respectively) while there was no significant difference between the mean values for ages 31-40yrs and 41-50yrs (2.01 ± 0.02 U/L and 2.00 ± 1.42 U/L respectively).

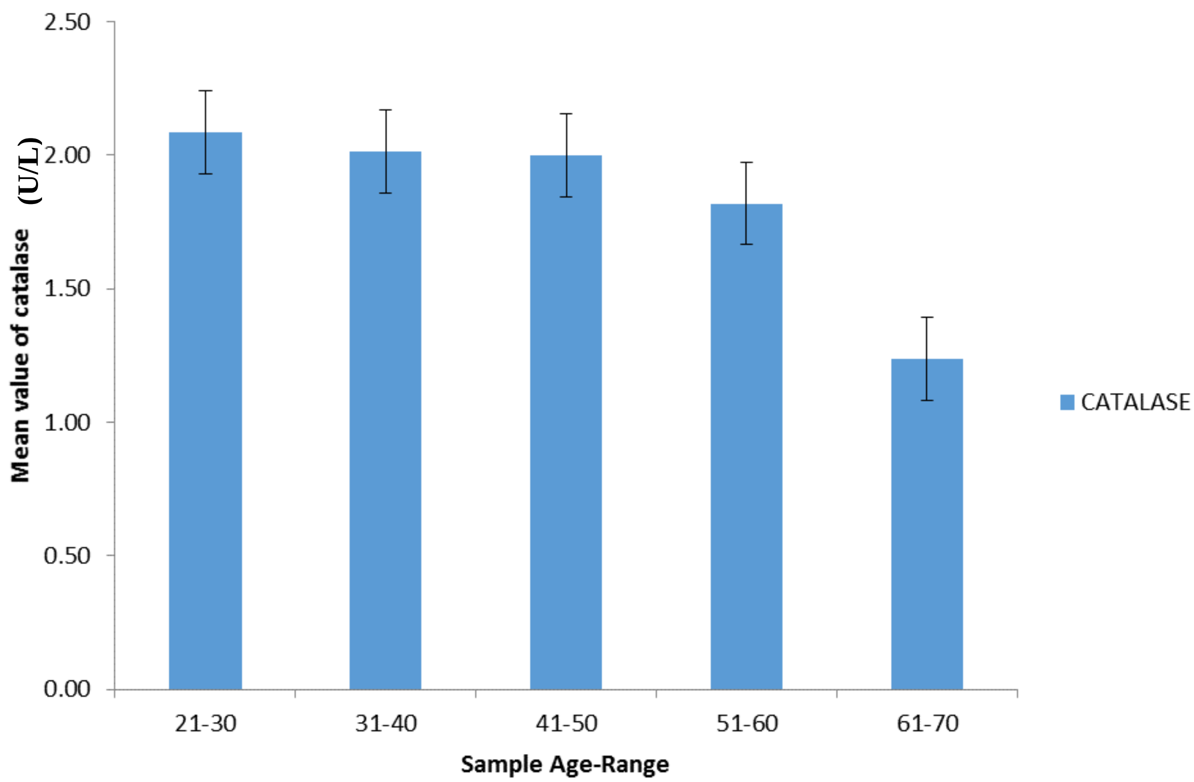


Fig. 4.12: Mean values of catalase activity across different age groups of individuals native to Ebocha.

This figure revealed that the mean serum catalase activity decreased across the age range, with age group 61-70yrs having the lowest mean value of 1.24 ± 0.62 U/L and followed by age group 51-60yrs with the mean value of 1.82±1.01U/L.

4.13 Superoxide Dismutase Activity across Different Age Groups

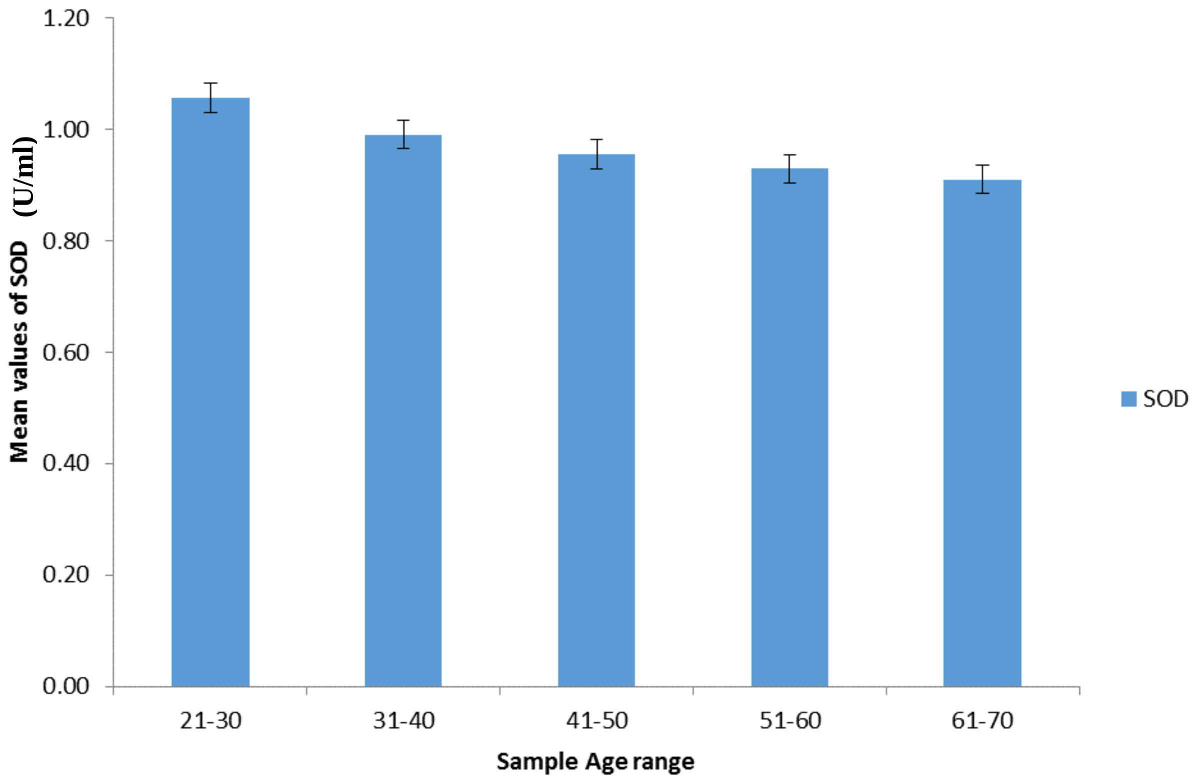


Fig. 4.13: Mean values of superoxide dismutase activity across different age groups of individuals native to Ebocha.

This result indicated that the mean values for serum superoxide dismutase activity of individuals across the age groups were 1.06 ± 0.05 U/ml, 0.99 ± 0.03 U/ml, 0.96 ± 0.08 U/ml, 0.93 ± 0.22 U/ml, and 0.91 ± 0.43 U/ml respectively which showed no significant difference ($p < 0.05$).

4.14 Glutathione Peroxidase Activity across Different Age Groups

The mean values obtained for serum glutathione peroxidase activity across the five age groups were 16.90 ± 2.44 U/L, 17.11 ± 1.29 U/L, 18.45 ± 7.16 U/L, 19.45 ± 5.58 U/L and 23.38 ± 5.74 U/L respectively.

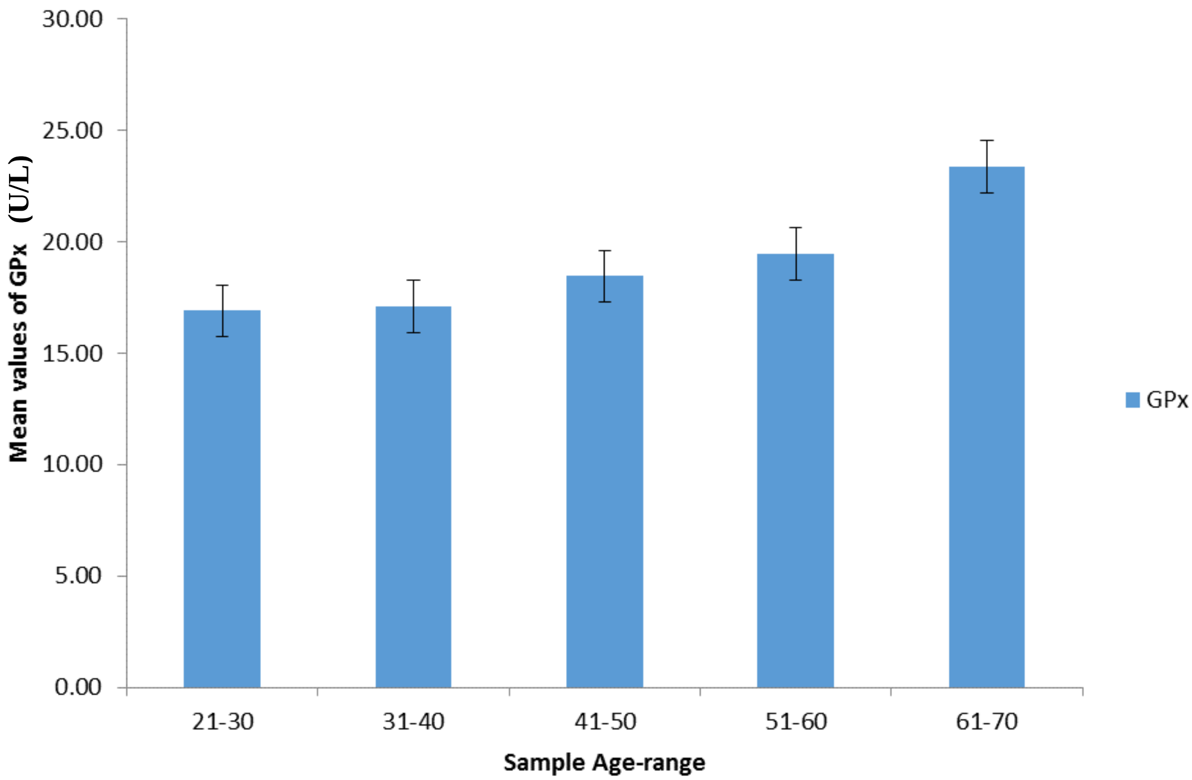


Fig. 4.14: Mean values of glutathione peroxidase (GPx) activity across different age groups of individuals native to Ebocha.

The result obtained further showed that the serum glutathione peroxidase activity increased across the age range with age group 61-70yrs having the highest mean value of 23.38 ± 5.74U/L, also increased as individuals were the more exposed to the pollution, that is with increasing age of participants.

4.15 Glutathione Reductase Activity across Different Age Groups

The result showed an increased serum glutathione reductase activity across the age range.

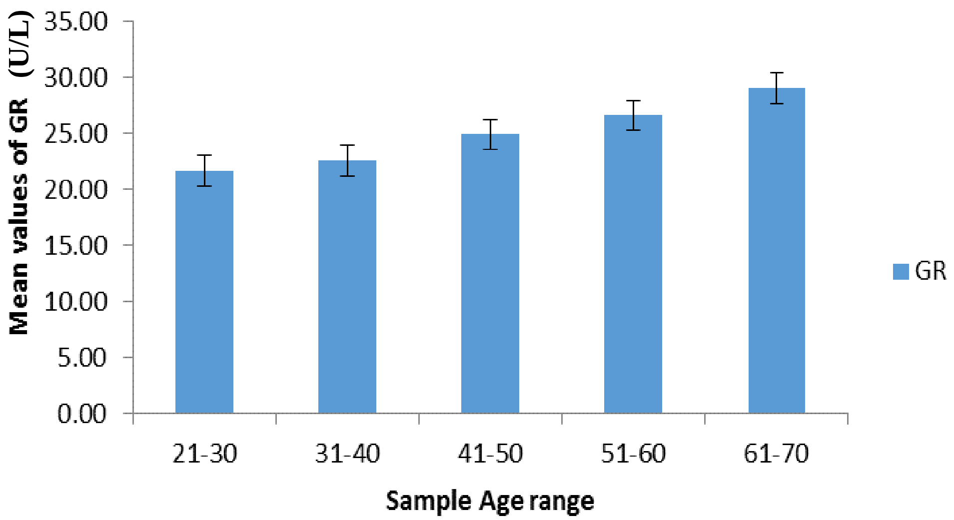


Fig. 4.15: Mean values of glutathione reductase (GR) activity across different age groups of individuals native to Ebocha and Nsukka respectively

The result showed that chronic exposure increased the glutathione reductase activities across the age range with age group 21-30 having the lowest mean value (21.65 ± 1.42 U/L.) and age group 61-70yrs having the highest mean value of 29.02 ± 2.19 U/L.

4.2 DISCUSSION

Combustion in flares and incinerators is seldom 100 per cent complete, as a result of this, gas flaring emits a number of substances that can affect human health, livestock and the environment (Robert, 2007). Individuals are exposed to the effects of gas flaring, particularly individuals closer to the flare sites. Local settlements are as close as 250m from the flare in some sites (Amanze, 2013). Plants and animals growing in such area have, over the years, taken in a large dose of harmful pollutants. Visually observed, these plants and animals including humans still physically appear as their identical ones in unpolluted (petroleum hydrocarbon-pollution free) areas in the same geographical region.

The mean value of the ascorbic acid concentration in female individuals native to Ebocha (2.06 ± 0.81mg/dl) is significantly ($p < 0.05$) lower than that of those from Nsukka (3.39 ± 0.95 mg/dl). The decrease was also observed in the concentration for the males. This is an indication that the water soluble vitamin was utilized by individuals to mop up the reactive oxygen species to prevent oxidative stress. Ascorbic acid is found to be far more effective in inhibiting lipid peroxidation initiated by a peroxyl radical initiator than other plasma components, such as bilirubin and alpha-tocopherol (Chavez *et al*., 2007).

The result of this study showed that the mean values obtained for serum catalase (CAT) activity in both females and males native of Ebocha (1.75 ± 0.85 U/L and 1.94 ± 0.79 U/L respectively) were significantly ($p < 0.05$) lower than those from Nsukka (4.04 ± 1.19 U/L and 2.67 ± 1.46 U/L). This could be attributed to pollution due to prolong exposure to gas flaring. Various studies have reported lower plasma and erythrocyte catalase activity and increased oxidative stress in patients suffering from mitochondria related diseases (Guo *et al.,* 2011). The decreased CAT activity in this study is also in agreement with Miguel *et al.* (2009) investigation on the effect of acute vs chronic H_2O_2 induced oxidative stress on antioxidant enzymes' activities. Accumulation of H_2O_2 due to low serum catalase activity can lead to early graying process (Wood *et al.,* 2009). On the contrary, Ishita *et al.*(2014) reported an increased catalase activities in the state of oxidative stress caused by RAS (Recurrent Aphthous Stomatitis), which is a common oral mucosal disorder characterized by recurrent, painful oral aphthae.

The result showed a reduction in the mean value with a significant difference ($p < 0.05$) between the mean values obtained for serum superoxide dismutase (SOD) activity of adult female individuals from Ebocha (0.89 ± 0.26 U/ml) and the ones from Nsukka (1.09 ± 0.07 U/ml) with a mean difference of 0.20 ± 0.19 U/ml. The result also revealed no significant difference in the mean value of the adult male counterpart. Catalase and SOD are metalloproteins and accomplish their antioxidant functions by detoxifying peroxides and superoxide anions. Previous investigations on this aspect have not put forth any conclusive answer as few of the studies revealed a significant decreased activities of SOD and CAT (Ambica *et al*., 2016), but on the contrary, few others (Ahamed *et al*., 2006; Shrirang *et al*., 2017) have showed increased activities of these enzymes. Furthermore, Ashraf and Hoda (2008) reported a decrease in superoxide dismutase (SOD) activity in the plasma of petrol pump workers, which corroborates this present study. A significant decrease in activities of these enzymes, GPx, CAT and Cu/Zn-SOD were expressed in different types of cancer patients (Asaduzzaman *et al*., 2010; Janina *et al.*, 2014). The observed decrease in SOD activities was seen in the female result, as there was no significant difference ($p < 0.05$) between the mean values obtained from serum superoxide dismutase activity in males from the two areas studied.

Furthermore, the present study was undertaken to assay the serum glutathione peroxidase activities in the individuals. In this study, the result reveals that the serum activity of glutathione peroxidase was significantly ($p<0.05$) lower in male individuals from the polluted area (26.00 ± 8.11 U/L) as against the control group (35.64 ± 11.97 U/L). Similar findings of low glutathione peroxidase activity in the state of oxidative stress have been previously reported by Ishita *et al.* (2014). The activity of GPx in the female counterparts were found to be significantly increased ($p < 0.05$) in the study group (21.22 ± 7.19 U/L) when compared to the control group (17.24 ± 3.55 U/L). The regeneration of glutathione (GSH) is catalyzed by glutathione reductase (GR) (Andersen, 1997). The study showed a significant increase in the activities of the enzyme antioxidant (glutathione reductase) of both female and male individuals across the study groups (26.87 ± 4.07 U/L and 27.59 ± 1.83 U/L) when compared with the control group (24.08 ± 0.88 U/L and 24.52 ± 0.91 U/L). Glutathione peroxidase and reductase activities were found to increase across the study age ranges.

Drastic reduction in the concentration of ascorbic acid is a clear indication that the antioxidant is being used up to scavenge and prevent free radical-induced damage to lipoproteins and other macromolecules (Luay and Tareq, 2014). Reduction in the concentration was also observed as the duration of exposure increased. There was a significant difference between the age range 21-30yr (3.24 ± 0.20mg/dl) and 61-70yr (1.83 ± 0.87mg/dl) respectively.

The result when analyzed based on duration of exposure of the individuals to gas flaring at Ebocha, results from the test area revealed a decline in the activities of the antioxidant enzymes, CAT and SOD as age increased. In line with this reduction in activities of antioxidant enzymes, reports had shown that during oxidative stress, the body uses its defense mechanism to minimize the process of lipid peroxidation by using antioxidant enzymes, thus the activity of this enzyme is higher in the early stage of oxidation, but when stress is overwhelming, the free radicals suppress antioxidant systems with resultant decrease in the antioxidant activities, the enzymes become depleted and unable to metabolize free radicals, which means in advanced stage of peroxidation due to prolong exposure, the activity of the enzymes decline (Abeer and Azza, 2017).

CHAPTER FIVE

5.0 CONCLUSION AND RECOMMENDATIONS

5.1 CONCLUSION

From the study, the mean value of ascorbic acid concentration (AA) obtained was lower in individuals native to Ebocha than in individuals from Nsukka. The mean value of serum catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase, GPx (male) activities of individuals from Ebocha were significantly ($p<0.05$) lower than that of individuals from Nsukka. In conclusion, residents of Ebocha, a community in the Niger Delta Region of Nigeria, have been exposed to pollutants from gas flaring, and the observed effects on the antioxidant parameters as seen from the results of this study may be due to the various pollutants present in the flared gas. Chronic exposure to petroleum hydrocarbon pollution (gas flaring) has been shown to exert adverse effect on both plants and domestic animals living in the polluted areas. Therefore, individual natives/residents of Ebocha, in the Niger Delta region have been exposed to reactive species (ROS/RNS) and xenobiotics from the flared gas, and prolonged exposure to these pollutants may expose them to various disease conditions such as cancer, hypertension, atherosclerosis, hyperlipidemia. This is supported by the observed changes in the antioxidants (of individuals) used as biomarkers in this study.

5.2 RECOMMENDATIONS FOR FURTHER STUDIES

There is yet a scanty information in literature about the effect of gas flaring on the biochemical parameters in individuals native to Ebocha, Niger Delta. In the light of research findings, the following recommendations are proposed:

1. Further research should be carried out to investigate the degree of the effect on individual’s sense organs such as eye and the skin,

2. Also, effects on vital organs such as kidney and liver should be investigated to ascertain their functionality.

3. Finally, research should be undertaken to investigate the effect of chronic exposure to gas flaring on lung and other cardiopulmonary parameters of the people.

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APPENDIX A

FEDERAL UNIVERSITY OF TECHNOLOGY OWERRI

DEPARTMENT OF BIOCHEMSITRY

QUESTIONNAIRE

Dear Sir/Madam,

I am a Postgraduate student of the above named University. I am conducting a research on "**Effect of Chronic Exposure to Petroleum Hydrocarbon Pollution on some Biochemical Parameters of Individuals Native to Ebocha, Niger Delta."** The questionnaire below is designed for this research. Please answer effectively and correctly. I promise that the answers will be confidentially treated for the above stated purpose.

Thanks.

Elewor, Ihunanya U.

1. Name (Optional) ……………………………………..:….

2. Sex: Male ☐ Female ☐

3. Age: 21-30 ☐ 31-40 ☐ 41-50 ☐ 51-60 ☐ 61-70 ☐

4. Occupation: Farmer ☐ Trader ☐ Teacher ☐ Others ☐

5. Highest Level of Education: Primary ☐ Secondary ☐ University ☐ Others ☐

6. Are you an indigene or resident in Ebocha? Indigene ☐ Resident ☐

7. How many years have you stayed in Ebocha? Less than 10yrs ☐ 10-15 ☐ 15-20 ☐ more than 20 ☐

9. Are you currently taking drugs? Yes ☐ No ☐

10. If yes, tick the sickness you are treating? Malaria ☐ Asthma ☐ HBP ☐ Diabetes ☐ Tuberculosis ☐

11. Do you drink alcohol? Yes ☐ No ☐

12. Have you travelled out of the community before? Yes ☐ No ☐

13. If yes, how long did you stay outside the community? Weeks ☐ months ☐ years ☐