

TITLE

**CARDIO-PROTECTIVE EFFECT OF ETHYLACETATE FRACTION OF *COLA*
HISPIDA LEAVES ON DOXORUBICIN-INDUCED CARDIOTOXICITY IN
WISTAR ALBINO RATS**

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NSUKKA**

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CERTIFICATION

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.....
EXTERNAL EXAMINER

DEDICATION

This work is dedicated to the Almighty God for making all things possible.

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My eternal gratitude goes to the Almighty God for his love, protections, benevolent blessings and graces to see the successful completion of this research work. My sincere gratitude goes to my amiable supervisor Prof. O.U. Njoku for his guidance and willingness to see to the overall success of this work.

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ABSTRACT

This study was undertaken to investigate the possible cardio-protective effect of ethyl acetate fraction of *Cola hispida* leaves on doxorubicin-induced cardiotoxicity in wistar rats. The percentage yield of the leaves was 11.9 % w/w. Qualitative and quantitative analysis of the plant secondary metabolites showed the presence of terpenoids (1.20 ± 0.21 mg/g), saponins (1.09 ± 0.20 mg/g), flavonoids (0.99 ± 0.50 mg/g), alkaloids (0.87 ± 0.20 mg/g), tannins (0.85 ± 0.13 mg/g) and glycosides (0.37 ± 0.16 mg/g). The result of proximate composition showed the presence of carbohydrate (68.45 ± 0.02 %), moisture content (16.71 ± 0.06 %), crude protein (8.44 ± 0.03 %), crude fibre (3.87 ± 0.21 %), fats (1.35 ± 0.02 %), and ash (1.18 ± 0.02 %). The result of mineral composition showed the presence of potassium (0.85 ± 0.01 %), iron (0.49 ± 0.00 %), zinc (0.14 ± 0.00 %) and copper (0.03 ± 0.02 %) while the result of vitamin composition revealed the presence of vitamin C (26.10 ± 0.03 mg/100g), vitamin E (3.87 ± 0.15 mg/100g), vitamin A (0.08 ± 0.01 IU). A total of thirty (30) adult male and female albino rats were divided into six (6) groups with five (5) animals in each group. Groups 1 received distilled water only, Group 2 received DOX only, Group 3 received 100mg/kg b. w of vitamin E while Groups 4, 5 and 6 received 100, 200 and 400 mg/kg b.w of *Cola hispida* leave fraction respectively. A single dose, 20 mg/kg b.w of Doxorubicin was administered intraperitoneally to all the groups except group I on the twenty-seventh day. The result showed that Doxorubicin intoxication caused a significant ($p < 0.05$) increase in serum level of cardiac biomarker enzymes (creatinine kinase, lactate dehydrogenase and aspartate aminotransferase) in DOX treated rats compared to the normal control. The serum levels of cardiac biomarker enzymes in the Vit E and fraction pre-treated groups were significantly ($p < 0.05$) lower compared to the group that received DOX only. The total cholesterol, triacylglycerol, LDL and atherogenic index were observed to be significantly ($p < 0.05$) lower while HDL was found to be non-significantly ($p > 0.05$) higher in the groups that received fraction and Vit E when compared to the group that received Dox only. The activities of antioxidant enzymes of the heart homogenate (SOD, CAT and GPx) showed a dose dependent significant ($p < 0.05$) increase with the groups that received fraction and vit E when compared to the group that received Dox only. The DPPH result showed that the standard drug (ascorbic acid) possess a better scavenging activities when compared with the fraction. A significant ($p < 0.05$) decrease in the concentration of malondialdehyde (MDA) was observed in groups that received vit E and fraction when compared to the group that received Dox only. Furthermore, the histological examination of the heart tissues showed significant multifocal areas of hyaline degeneration and myocardial necrosis indicating cardiotoxicity in the group that received Dox only while the normal control and groups that received Vit E and 400 mg/kg b.w extract respectively showed a normal histo-architecture of the heart. The observed cardio-protective effects of the *Cola hispida* leave extract were dose dependent hence could be attributed to its antioxidant properties.

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ABBREVIATIONS

CVD: Cardiovascular diseases

CHD: Coronary heart diseases

IHD: Ischemic heart diseases

PAD: Peripheral artery diseases

RVH: Right ventricular hypertrophy

SNP: Single nucleotide polymorphisms

HDL: High density lipoprotein

LDL: Low density lipoprotein

LVEF: Left ventricular ejection fraction

HF: Heart failure

BNP: B-type natriuretic peptide level

SACT: Systematic anticancer therapy

CHD: Coronary heart diseases

VEGF: Vascular endothelial growth factor

ROS: Reactive oxygen species

DNA: Deoxyribonucleic acid

DIC: Doxorubicin induced cardiotoxicity

DOX: Doxorubicin

ATP: Adenosine triphosphate

eNOS: Endothelial nitrogen oxygen species

NADPH: Nicotinamide dinucleotide phosphate

SNP: Single nucleotide polymorphism

Doxol: Doxorubicinol

LP: Lipid peroxidase

CK: Creatine kinase

AST: Aspartate transferase

MDA: Malondialdehyde

ROS: Reactive oxygen species

GPx: Glutathione peroxidase

CAT: Catalase

LDH: Lactate dehydrogenase

SOD: Superoxide dismutase

PMP: Pyridoxamine phosphate

ECG: Electrocardiography

EDTA: Ethylene diaminetetracetic acid

CHAPTER ONE

INTRODUCTION

Cardiovascular diseases (CVD) are a class of diseases and injuries that affect the cardiovascular system (the heart and blood vessels of the heart and brain). They include the coronary artery diseases (CAD), peripheral artery diseases, pulmonary heart diseases. Other CVD include cardiac dysthymias, cerebrovascular diseases, rheumatic heart diseases, aortic aneurysms, congenital heart diseases and venous thrombosis (Shaima *et al.*, 2016; Wang *et al.*, 2015). The sequential events leading to this CVD includes endothelial dysfunction, atherosclerosis plaque formation and rupture of atherosclerotic lesion. The underlying mechanisms vary depending on the diseases. The risk factors associated with cardiovascular diseases include age, sex, family history, high blood pressure, smoking, diabetes mellitus, lack of exercise, obesity, high blood cholesterol, poor diet, and excessive alcohol consumption, among others (Mendis *et al.*, 2011). High blood pressure is estimated to account for approximately 13% of CVD deaths, while tobacco accounts for 9%, diabetes 6%, lack of exercise 6% and obesity 5%. Rheumatic heart disease may follow untreated strep throat.

Cardiovascular diseases are the second leading cause of long-term morbidity and mortality among cancer survivors globally. This is true in all areas of the world except Africa. Together, CVD resulted in 17.9 million deaths (32.1%) in 2015, up from 12.3 million (25.8%) in 1990 (Naghavi *et al.*, 2015). Deaths, at a given age, from CVD are more common and have been increasing in much of the developing world, while rates have declined in most of the developed world since the 1970s (Moran *et al.*, 2014). Coronary artery disease and stroke account for 80% of CVD deaths in males and 75% of CVD deaths in females' hence cardiovascular disease affects older adults. In the United States 11% of people between 20 and 40 have CVD, while 37% between 40 and 60, 71% of people between 60 and 80, and 85% of people over 80 have CVD (Mozaffarian *et al.*, 2013). Conventional chemotherapy and targeted therapies are associated with an increased risk of myocardial dysfunction to irreversible heart failure or even death. Cancer and cardiovascular disease are the two most common causes of death in Northern Africa.

1.1 Cardiovascular Diseases

1.1.1 Types of Cardiovascular Diseases

There are many cardiovascular diseases involving the blood vessels. They are known as vascular diseases. They include:

i. Coronary Artery Disease

Coronary artery disease (CAD), also known as Ischemic heart disease (IHD), involves the reduction of blood flow to the heart muscle due to build-up of plaque in the arteries of the heart (Donnell *et al.*, 2016). It is the most common of the cardiovascular diseases. A common symptom is chest pain or discomfort which may travel into the shoulder, arm, back, neck, or jaw. Occasionally it may feel like heartburn. Usually symptoms occur with exercise or emotional stress, last less than a few minutes, and improve with rest. Shortness of breath may also occur and sometimes no symptoms are present. In many cases, the first sign is a heart attack while other complications include heart failure or an abnormal heartbeat (Wang *et al.*, 2016).

ii. Peripheral Arterial Disease

Peripheral artery disease (PAD) is an abnormal narrowing of arteries other than those that supply the heart or brain (Finks *et al.*, 2012). When narrowing occurs in the heart, it is called coronary artery disease, and in the brain, it is called cerebrovascular disease. Peripheral artery disease most commonly affects the legs, but other arteries may also be involved. The classic symptom is leg pain when walking which resolves with rest, known as intermittent claudication. Other symptoms include skin ulcers, bluish skin, cold skin, or abnormal nail and hair growth in the affected leg (Wang *et al.*, 2015). Complications may include an infection or tissue death which may require amputation; coronary artery disease, or stroke. Up to 50% of people with PAD do not have symptoms. The greatest risk factor for PAD is cigarette smoking. Other risk factors include diabetes, high blood pressure, kidney problems, and high blood cholesterol (Sutcliffe *et al.*, 2013). The most common underlying mechanism of peripheral artery disease is atherosclerosis, especially in individuals over 40 years old.

iii. Cerebrovascular Disease

Cerebrovascular disease includes a variety of medical conditions that affect the blood vessels of the brain and the cerebral circulation. Arteries supplying oxygen and nutrients to the brain are often damaged or deformed in these disorders (Mozaffarian *et al.*, 2013). The most common presentation of cerebrovascular disease is an ischemic stroke or mini-stroke and

sometimes a hemorrhagic stroke. Hypertension (high blood pressure) is the most important contributing risk factor for stroke and cerebrovascular diseases as it can change the structure of blood vessels and the affected area at an increased risk of sudden cardiac death. Types of cardiomyopathy include hypertrophic cardiomyopathy, dilated cardiomyopathy, restrictive cardiomyopathy, arrhythmogenic right ventricular dysplasia, and takotsubo cardiomyopathy ie: broken heart syndrome (Wang *et al.*, 2015). In hypertrophic cardiomyopathy the heart muscle enlarges and thickens, in dilated cardiomyopathy the ventricles enlarge and weaken while in restrictive cardiomyopathy the ventricle stiffens (Wang *et al.*, 2015).

iv. Pulmonary Heart Disease

Pulmonary heart disease, also known as cor pulmonale, is the enlargement and failure of the right ventricle of the heart as a response to increased vascular resistance (such as from pulmonic stenosis) or high blood pressure in the lungs. Chronic pulmonary heart disease usually results in right ventricular hypertrophy (RVH), whereas acute pulmonary heart disease usually results in dilatation (Mozaffarian *et al.*, 2013). Hypertrophy is an adaptive response to a long-term increase in pressure. Individual muscle cells grow larger (in thickness) and change to drive the increased contractile force required to move the blood against greater resistance. Dilatation is a stretching (in length) of the ventricle in response to acute increased pressure (Mozaffarian *et al.*, 2013).

v. Cardiac Dysrhythmias

Heart arrhythmia (also known as arrhythmia, dysrhythmia or irregular heartbeat) is a group of conditions in which the heartbeat is irregular, too fast or too slow (Wang *et al.*, 2015). A heart rate that is too fast above 100 beats per minute in adults is called Tachycardia, and a heart rate that is too slow below 60 beats per minute is called Brady-cardia.

1.1.2 Risk Factors of Cardiovascular Diseases

There are many risk factors for heart diseases: age, sex, tobacco use, physical inactivity, excessive alcohol consumption, unhealthy diet, obesity, genetic predisposition and family history of cardiovascular disease, raised blood pressure (hypertension), raised blood sugar (diabetes mellitus), raised blood cholesterol (Ciaccio *et al.*, 2017).

i. Genetic Factor

This influences the development of cardiovascular disease in men who are less than 55 years-old and in women who are less than 65 years old. Cardiovascular disease in a person's parents increases their risk by 3 fold. Single nucleotide hyperlipidemia), undiagnosed celiac disease, psychosocial factors, poverty and low educational status, and air pollution (Finks *et al.*, 2012; Micha *et al.*, 2012; Ciaccio *et al.*, 2017). While the individual contribution of each risk factor varies between different communities or ethnic groups the overall contribution of these risk factors is very consistent. Some of these risk factors, such as age, sex or family history/genetic predisposition, are immutable; however, many important cardiovascular risk factors are modifiable by lifestyle changes, social changes, drug treatment for example prevention of hypertension, hyperlipidemia, and diabetes (McPhee, 2012). People with obesity are at increased risk of atherosclerosis of the coronary arteries. polymorphisms (SNP) have been found to be associated with cardiovascular disease in genetic association studies (Nikpay *et al.*, 2015; MacRae and Vasan 2016) but usually their individual influence is small, and genetic contributions to cardiovascular disease are poorly understood.

ii. Age

Age is the most important risk factor in developing cardiovascular or heart diseases, with approximately a tripling of risk with each decade of life. Coronary fatty streaks can begin to form in adolescence (Adamo *et al.*, 2015). It is estimated that 82 percent of people who die of coronary heart disease are 65 and older. Simultaneously, the risk of stroke doubles every decade after age. Multiple explanations are proposed to explain why age increases the risk of cardiovascular/heart diseases. One of them relates to serum cholesterol level. In most populations, the serum total cholesterol level increases as age increases. In men, this increase levels off around age 45 to 50 years. In women, the increase continues sharply until age 60 to 65 years. Aging is also associated with changes in the mechanical and structural properties of the vascular wall, which leads to the loss of arterial elasticity and reduced arterial compliance and may subsequently lead to coronary artery disease (Adamo *et al.*, 2015).

iii. Sex

Men are at greater risk of heart disease than pre-menopausal women. Once past menopause, it has been argued that a woman's risk is similar to a man's although more recent data from the WHO and UN disputes this (Ciaccio *et al.*, 2017). If a female has diabetes, she is more likely to develop heart disease than a male with diabetes. Coronary heart diseases are 2 to 5 times more common among middle-aged men than women. In a study done by the World Health Organization, sex contributes to approximately 40% of the variation in sex ratios of coronary

heart disease mortality (Adamo *et al.*, 2015). Another study reports similar results finding that sex differences explains nearly half the risk associated with cardiovascular diseases. One of the proposed explanations for sex differences in cardiovascular diseases is hormonal differences (MacRae and Vasan 2016). Among women, estrogen is the predominant sex hormone. Estrogen may have protective effects on glucose metabolism and hemostatic system, and may have direct effect in improving endothelial cell function. The production of estrogen decreases after menopause, and this may change the female lipid metabolism towards a more atherogenic form by decreasing the HDL cholesterol level while increasing LDL and total cholesterol levels (Ciaccio *et al.*, 2017). Among men and women, there exist differences in body weight, height, body fat distribution, heart rate, stroke volume, and arterial compliance. In the very elderly, age-related large artery pulsatility and stiffness is more pronounced among women than men. This is as a result in the women's smaller body size and arterial dimensions which are independent of menopause.

iv. Tobacco

Cigarettes are the major form of smoked tobacco. Risks to health from tobacco use results not only from direct consumption of tobacco, but also from exposure to second-hand smoke. Approximately 10% of cardiovascular disease is attributed to smoking; however, people who quit smoking by age 30 have almost as low a risk of death as never smokers (Hooper *et al.*, 2015).

v. Physical Inactivity

According to Hooper *et al.*, 2015, insufficient physical activity (defined as less than 5 x 30 minutes of moderate activity per week, or less than 3 x 20 minutes of vigorous activity per week) is currently the fourth leading risk factor for mortality worldwide. In 2008, 31.3% of adults aged 15 or older (28.2% men and 34.4% women) were insufficiently physically active. The risk of ischemic heart disease and diabetes mellitus is reduced by almost a third in adults who participate in 150 minutes of moderate physical activity each week (or equivalent). In addition, physical activity assists weight loss and improves blood glucose control, blood pressure, lipid profile and insulin sensitivity (Hooper *et al.*, 2015). These effects may, at least in part, explain its cardiovascular benefits.

vi. Diet

High dietary intakes of saturated fat, trans-fats, salt, low intake of fruits, vegetables and fish are linked to cardiovascular risk, although whether all these associations are a cause is disputed. The World Health Organization attributes approximately 1.7 million deaths

worldwide to low fruit and vegetable consumption (Morenga *et al.*, 2014). The amount of dietary salt consumed is also an important determinant of blood pressure levels and overall cardiovascular risk. Frequent consumption of high-energy foods, such as processed foods that are high in fats and sugars, promotes obesity and may increase cardiovascular risk. A Cochrane review found that replacing saturated fat with polyunsaturated fat (plant based oils) reduced cardiovascular disease risk. Cutting down on saturated fat reduced risk of cardiovascular disease by 17% including heart disease and stroke.

High trans-fat intake has adverse effects on blood lipids and circulating inflammatory markers, and elimination of trans-fat from diets has been widely advocated (Remig *et al.*, 2010). In 2018 the World Health Organization estimated that trans fats were the cause of more than half a million deaths per year. There is evidence that higher consumption of sugar is associated with higher blood pressure and unfavorable blood lipids, (Morenga *et al.*, 2014) and sugar intake also increases the risk of diabetes mellitus. High consumption of processed meats is associated with an increased risk of cardiovascular disease, possibly in part due to increased dietary salt intake. The relationship between alcohol consumption and cardiovascular disease is complex, and may depend on the amount of alcohol consumed. There is a direct relationship between high levels of drinking alcohol and cardiovascular disease. Drinking at low levels without episodes of heavy drinking may be associated with a reduced risk of cardiovascular disease (Mukamal *et al.*, 2010). At the population level, the health risks of drinking alcohol exceed any potential benefits (Mendis *et al.*, 2011).

1.1.3 Common Cardiovascular Adverse Effects

Cardiac dysfunction and Heart failure (HF) are among the most serious cardiovascular consequences of systemic cancer treatment (Aleman *et al.*, 2014). Conventional chemotherapeutics, such as anthracyclines, antimetabolites, and cyclophosphamide, can induce permanent myocardial cell injury, leading to acute or chronic LVD. Anthracyclines, commonly used in the treatment of solid tumors (ie, breast cancer, osteosarcoma etc.) and hematologic malignancies (Hodgkin/non-Hodgkin lymphoma, acute lymphoblastic leukemia), can trigger significant Left ventricular dysfunction (LVD). Anthracycline-related LVD has historically been considered to be dose-dependent, cumulative, and progressive, which manifest as decreased left ventricular ejection fraction (LVEF) and, ultimately, symptomatic HF in up to 5% of patients.

1.2 Diagnosis of Heart Failure (HF)

Biomarkers of cardiac injury (e.g. cardiac troponin I concentration) have been shown to identify patients at risk for anthracycline cardiotoxicity and predict a poor response to cardiac therapy. The finding of a significantly raised B-type natriuretic peptide level (BNP) provides evidence of heart failure with high sensitivity (Plana *et al.*, 2014). Whilst not useful in an unselected cohort of patients receiving systematic anticancer therapy (SACT), or B-type natriuretic peptide trends can identify at-risk patients treated with anthracycline (Moran *et al.*, 2014).

- i. Echocardiography is an excellent tool for identifying a patient with heart failure. A reduction in left ventricular ejection fraction (LVEF) is a late finding and may occur only after a significant amount of myocardial damage has occurred. It uses sound waves to create a moving picture of the heart. The test provides information about the size and shape of the heart and how well the heart chambers and valves are working. Echo also can show areas of poor blood flow to the heart, areas of heart muscle that are not contracting normally, and previous injury to the heart muscle caused by poor blood flow (NHLBI, 2017). Advanced echocardiographic techniques such as global longitudinal strain measurement may detect left ventricular impairment earlier than conventional LVEF. Deteriorations of 13.7% or more from baseline warrant continued increased clinical vigilance with fair sensitivity (88%) and specificity (71%), (Gulati *et al.*, 2016).
- ii. Electrocardiogram (ECG): An ECG is a simple, painless test that detects and records the heart's electrical activity. The test shows how fast the heart is beating and its rhythm (steady or irregular). An ECG also records the strength and timing of electrical signals as they pass through the heart. An ECG can show signs of heart damage due to coronary heart disease and signs of a previous or current heart attack (NHLBI, 2017).
- iii. Stress Testing: Stress testing involves undergoing exercise to make the heart work hard and beat fast while heart tests are done. When the heart is working hard and beating fast, it needs more blood and oxygen. Plaque-narrowed coronary (heart) arteries cannot supply enough oxygen-rich blood to meet the heart's needs. A stress test can show possible signs and symptoms of coronary heart disease, such as: abnormal changes in the heart rate or blood pressure, shortness of breath or chest pain and abnormal changes in the heart rhythm or heart's electrical activity (NHLBI, 2017).
- iv. Chest X-ray: A chest x-ray creates pictures of the organs and structures inside the chest, such as the heart, lungs, and blood vessels. A chest x-ray can reveal signs of heart failure, as

well as lung disorders and other causes of symptoms not related to coronary heart disease (NHLBI, 2017).

v. **Blood Tests:** Blood tests check the levels of certain fats, cholesterol, sugar, and proteins in the blood. Abnormal levels may be a sign that there is a risk of coronary heart disease (CHD). During a heart attack, heart muscle cells die and release proteins into the bloodstream. Blood tests can measure the amount of these proteins in the bloodstream. High levels of these proteins are a sign of a recent heart attack (NHLBI, 2017).

1.3 Cardiotoxicity

Cardiotoxicity is defined as “any toxicity that affects the heart” resulting to cardiac dysfunction and heart failure by the US (National Cancer Institute, 2014). A large body of research has been generated on chemotherapy and in particular anthracycline-induced cardiotoxicity. In this field of research, cardiotoxicity generally refers to cardiac dysfunction and failure. The largest body of research that has been done on the cardiotoxicity of cancer treatments has involved anthracycline-induced cardiac effects. Anthracycline-induced cardiotoxicity was first described in the 1970s and was initially thought of as a cumulative dose-related form of congestive heart failure that occurred during treatment (Renu *et al.*, 2017). Subsequently, both acute reversible cardiotoxicity during treatment and late progressive cardiotoxicity were also identified. The direct cardiac specific effects of anthracyclines are quite pleiotropic, however, heart failure is the most recognized. The effects of anthracyclines have been further categorized based on whether complications present during treatment (acute), within a year of treatment discontinuation (early), or subsequently (late). Acute and late complications are believed to arise from different pathological mechanisms (Renu *et al.*, 2017).

In general, the most confusing aspect of the definition of cardiotoxicity is whether only clinical and subclinical, or only subclinical events are considered. In the case of heart failure, these subclinical events can include histological changes, changes in left ventricular ejection fraction (LVEF), changes in subtler parameters of cardiac injury and function such as myocardial deformation indices (strain) and serum biomarkers. The degree to which the subclinical events are included in the definition of cardiotoxicity represents the most flagrant difference in the definition of cardiotoxicity adopted by the cardiology and oncology societies (Renu *et al.*, 2017).

1.3.1 Types of Cardiotoxicity

With respect to ventricular dysfunction, two categories of cardiotoxicity have been previously proposed and conventionally accepted thus far. They are:

- i) Type I Cardiotoxicity. This seen classically with anthracyclines, it is thought to be irreversible, dose related, and caused by free radical formation, oxidant stress, and myofibrillar disarray.
- ii) Type II Cardiotoxicity. This traditionally with the use of trastuzumab, has been described as reversible and not dose related, with no accompanied ultrastructural abnormalities (Ewer and Lippman, 2005).

The distinction between type I and type II may be more complicated than once perceived. Studies have shown improvement in anthracycline-induced cardiac dysfunction with heart failure (HF) therapy, while other studies have shown irreversible scar formation on magnetic resonance imaging in patients treated with trastuzumab (Wells and Lenihan, 2010). The cardiotoxicity occurring with anthracyclines can be acute, occurring in less than 1% of patients and characterized by a transient decline in myocardial contractility or chronic, occurring in 1.6% to 5% of patients, occurring at least 1 year after the completion of therapy (Curigliano *et al.*, 2010).

The mechanism of anthracycline-induced cardiac injury has been studied extensively and is still not clearly understood, (Hahn *et al.*, 2014). Structural cardiomyocyte alterations and cell death induced by anthracyclines are mediated in part by reactive oxygen species (ROS) generated in iron-dependent chemical reactions. ROS lead to the peroxidation of myocyte membranes and, after calcium influx, into the intracellular space, which can ultimately lead to permanent myocyte damage (Zhang *et al.*, 2012). The risk of doxorubicin-induced HF (which can occur within hours, weeks, or years after exposure) increases with cumulative dose of anthracycline: 3% to 5% with 400 mg/m², 7% to 26% at 550 mg/m², and 18% to 48% at 700 mg/m². High-risk patients include those at the extremes of age (<5 or > 65 years), those who received prior or concurrent chest radiation, and those with preexisting cardiac disease or established CV risk factors. The risk of heart failure remains higher for patients who receive anthracycline compared with those who receive other agents (Hooper *et al.*, 2015).

1.3.2 Symptoms of Cardiotoxicity

Symptoms of a potential heart issue related to cardiotoxicity may include:

Shortness of breath, Chest Pain, Heart palpitations, Fluid retention in the legs, Distention of the stomach, Dizziness and Indigestion (NHLBI, 2017).

1.3.3 Mechanisms of Cardiotoxicity

Despite almost 60 years of research, the mechanisms to explain Doxorubicin-induced cardiotoxicity (DIC) are not completely understood. It seems to be a multistep process, with different potential pathways involved that leads to cardiomyocyte death (Salazar *et al.*, 2014; Ghigo *et al.*, 2016). Until now, the main mechanisms that have been proposed by various research groups include oxidative stress, iron metabolism, Ca^{2+} Doxorubicin-Induced Cardiotoxicity:

i. Oxidative Stress

Since the discovery of Doxorubicin (DOX), its result has led to the increase of the reactive oxygen species (ROS) production, including superoxide anion (O_2^-) and hydroxyl radicals ($\text{OH}^\cdot$) as well as other non-radicals such as hydrogen peroxide (H_2O_2), singlet oxygen (O_2), (Raj *et al.*, 2014; Ghigo *et al.*, 2016; Varricch *et al.*, 2018). Unlike other tissues, the heart is extremely prone to oxidative damage, at least in part, due to lower levels of antioxidant enzymes such as peroxidase, catalase, and superoxide dismutase. In addition, the chemical structure of DOX contains quinone groups that can be reduced to a semi-quinone, an unstable metabolite which can react with molecular oxygen (an electron acceptor) and rapidly revert to the parent compound. This redox cycle leads to the formation of superoxide anion radicals within mitochondria, causing cardiotoxicity (Salazar *et al.*, 2014; Ichikawa *et al.*, 2014; McGowan *et al.*, 2017).

The mitochondria have been identified as the main subcellular organelles injured in the heart by Doxorubicin induced cardiotoxicity (Ghigo *et al.*, 2016). DOX is a cationic drug that binds with high affinity to cardiolipin (a phospholipid) forming nearly irreversible complex in the mitochondrial inner membrane. Cardiolipin is required for the proper functioning of the electron transport chain proteins hence evidence suggests that DOX disrupts the cardiolipin protein interface, causing more superoxide anion radicals formation. As a result, ROS can induce different forms of cardiomyocyte death (Qinggong *et al.*, 2019). Furthermore, the reduction of mitochondrial function causes energetic metabolism change evidenced by a decrease of the adenosine triphosphate (ATP) production, which may contribute to abnormal contraction and relaxation in the failing heart (Raj *et al.*, 2014).

Nitric oxide synthases (NOS) and Nicotinamide adenine dinucleotide phosphate (NADPH) oxidases pathways is also another form of DOX-induced ROS generation. These enzymes interact with DOX and induce oxidative stress (Ghigo *et al.*, 2016). NOS are a group of enzymes responsible for the nitric oxide (NO) production from L-arginine and oxygen

(Elnakish *et al.*, 2013). The NO generation is altered by the direct binding of DOX to endothelial NOS (eNOS) reductase domain, lead to the reduction of the DOX semiquinone radical, which reacts with oxygen and produces superoxide. There is evidence to suggest that, in low DOX concentrations, eNOS signaling is the main pathway for DOX reduction. In addition, the increase of DOX-eNOS interaction completely modifies normal functioning of the enzyme (NO production) and transforms it into a potent superoxide generator. DOX also affects NOS signaling by increasing eNOS transcription and protein activity in bovine aortic endothelial cells. BAEC (Qinggong *et al.*, 2019).

ii. Iron Metabolism

It is reported that DOX is able to alter iron metabolism due to its strong affinity for this metal, thereby forming iron-DOX complexes which, in turn, react with oxygen and trigger ROS production (Ma *et al.*, 2013). Thus, researchers believed that only oxidative stress was responsible for the cardiotoxicity induced by iron-DOX complexes. However, in physiological conditions, there would not be enough free iron to interact with DOX to the extent necessary to cause cardiomyopathy. On the other hand, another theory suggests that the effect of DOX on iron metabolism occurs due to the interference of this drug in the activity of proteins that transport and bind intracellular iron (Octavia *et al.*, 2017). One of the mechanisms involves the doxorubicinol (DOXol), a metabolite of DOX, which removes iron from the catalytic Fe-S cluster of the cytoplasmic aconitase (also called iron regulatory protein 1; IRP-1), converting this enzyme to a null protein (Octavia *et al.*, 2017). Consequently, there is an increase in the stability of transferrin mRNA and preventing translation of iron sequestration proteins. As a result, reduction of IRP-1 causes an increase in free iron, which can lead to free radical production.

iii. Calcium Homeostasis Dysregulation

Normal calcium homeostasis is altered by ROS and hydrogen peroxide via disruption of normal sarcoplasmic reticulum function. This is accomplished by inhibiting the Ca^{2+} ATPase pumps, caused by reducing the expression of SERCA2a mRNA levels and the direct activation of the ryanodine calcium-release channels themselves (Mitry *et al.*, 2016). In addition, a study suggests that DOX induces calcium release from the sarcoplasmic reticulum due to increasing the frequency of opening of these channels. At the same time, DOX induced the inhibition of sodium-calcium channels in the plasma membrane as well as increased L-type calcium channel activation. DOX has also been shown to decrease the

calcium storage capacity of mitochondria by specifically activating the selective CsA-sensitive calcium channel, exacerbating the calcium-overload. As a result, an increase of calcium cytoplasmic concentrations occurs, leading to mitochondrial dysfunction and apoptosis (Mitry *et al.*, 2016). Therefore, the preservation of calcium homeostasis is essential to prevent DOX-induced cardiomyopathy. With all these theories in view, the role of free radicals occupies the central position because it has been hypothesized that the oxidative stress not only causes myocardial death but directly affects excitation-contraction properties of cardiac muscles. Free radicals mainly nitrite free radicals are the major culprit of oxidative stress leading to investigation to determine better pharmacotherapies and interventions for the impediment of cardiotoxicity (Renu *et al.*, 2017). DOX cardiotoxicity still occurs, and its prevention and management remains unconcern to both cardiologists and oncologists.

1.3.4 Serum Biomarkers of Cardiotoxicity

The serum biomarkers of cardiotoxicity include:

i. Lipid Peroxidation

Lipid Peroxidation Process: Lipid peroxidation can be described generally as a process under which oxidants such as free radicals or non-radical species attack lipids containing carbon-carbon double bond(s) especially polyunsaturated fatty acids (PUFAs) that involve hydrogen abstraction from a carbon, with oxygen insertion resulting in lipid peroxy radicals and hydroperoxides as described previously (Ayala *et al.*, 2014; Volinsky and Kinnunen, 2013). Glycolipids, phospholipids (PLs), and cholesterol (Ch) are also well-known targets of damaging and potentially lethal per oxidative modification. Lipids also can be oxidized by enzymes like lipoxygenases, cyclooxygenases, and cytochrome P₄₅₀. In response to membrane lipid peroxidation, and according to specific cellular metabolic circumstances and repair capacities, the cells may promote cell survival or induce cell death. Under physiological or low lipid peroxidation rates (sub-toxic conditions), the cells stimulate their maintenance and survival through constitutive antioxidants defense systems or signaling pathways activation that upregulate antioxidants proteins resulting in an adaptive stress response (Antonio *et al.*, 2014).

By contrast, under medium or high lipid peroxidation rates (toxic conditions) the extent of oxidative damage overwhelms repair capacity, and the cells induce apoptosis or necrosis programmed cell death; both processes eventually lead to molecular cell damage which may facilitate development of various pathological states and accelerated aging. The impact of lipids oxidation in cell membrane and how these oxidative damages are involved in both

physiological processes and major pathological conditions have been analysed in several reviews.

The overall process of lipid peroxidation consists of three steps: Initiation, propagation, and termination, (Michael and Brent, 2017).

In the lipid peroxidation initiation step, pro-oxidants like hydroxyl radical abstract the allylic hydrogen forming the carbon-centered lipid radical ($L\cdot$). In the propagation phase, lipid radical ($L\cdot$) rapidly reacts with oxygen to form a lipid peroxy radical ($LOO\cdot$) which abstracts a hydrogen from another lipid molecule generating a new L (that continues the chain reaction) and lipid hydroperoxide ($LOOH$). In the termination reaction, antioxidants like vitamin E donate hydrogen atom to the $LOO\cdot$ species and form a corresponding vitamin E radical that reacts with another $LOO\cdot$ forming non radical products. Once lipid peroxidation is initiated, a propagation of chain reactions will take place until termination products are produced (Njoku *et al.*, 2017).

ii. Creatine Kinase

Creatine kinase (CK), also known as creatine phosphokinase (CPK) or phosphocreatine kinase, is an enzyme expressed by various tissues and cell types. CK catalyses the conversion of creatine and uses adenosine triphosphate (ATP) to create phosphocreatine (PCr) and adenosine diphosphate (ADP). This CK enzyme reaction is reversible and thus ATP can be generated from PCr and ADP. In tissues and cells that consume ATP rapidly, especially skeletal muscle, but also brain, photoreceptor of the retina, hair cells of the inner ear, spermatozoa and smooth muscle, PCr serves as an energy reservoir for the rapid buffering and regeneration of ATP *in situ*, as well as for intracellular energy transport by the PCr shuttle or circuit (Moghadam *et al.*, 2016). Thus creatine kinase is an important enzyme in such tissues.

Clinically, creatine kinase is assayed in blood tests as a marker of damage of CK-rich tissue such as in myocardial infarction (heart attack), rhabdomyolysis (severe muscle breakdown), muscular dystrophy, autoimmune myositides, and acute kidney injury (Moghadam *et al.*, 2016). In the cells, the "cytosolic" CK enzymes consist of two subunits, which can be either *B* (brain type) or *M* (muscle type). There are, therefore, three different isoenzymes: CK-MM, CK-BB and CK-MB. The genes for these subunits are located on different chromosomes: *B* on 14q32 and *M* on 19q13. In addition to those three cytosolic CK isoforms, there are two mitochondrial creatine kinase isoenzymes, the ubiquitous and sarcomeric form. The mitochondrial creatine kinase (CK_m) is present in the mitochondrial intermembrane space,

where it regenerates phosphocreatine (PCr) from mitochondrial generated ATP and creatine (Cr) imported from the cytosol. Apart from the two mitochondrial CK isoenzyme forms, that is, ubiquitous mtCK (present in non-muscle tissues) and sarcomeric mtCK (present in sarcomeric muscle), there are three cytosolic CK isoforms present in the cytosol, depending on the tissue. Whereas MM-CK is expressed in sarcomeric muscle, that is, skeletal and cardiac muscle, MB-CK is expressed in cardiac muscle, and BB-CK is expressed in smooth muscle and in most non-muscle tissues (Moghadam *et al.*, 2016).

Mitochondrial mtCK and cytosolic CK are connected in a so-called PCr/Cr-shuttle or circuit. PCr generated by mtCK in mitochondria is shuttled to cytosolic CK that is coupled to ATP-dependent processes, e.g. ATPases, such as acto-myosin ATPase and calcium ATPase involved in muscle contraction, and sodium/potassium ATPase involved in sodium retention in the kidney. The bound cytosolic CK accepts the PCr shuttled through the cell and uses ADP to regenerate ATP, which can then be used as energy source by the ATPases. PCr is not only an energy buffer but also a cellular transport form of energy between subcellular sites of energy (ATP) production (mitochondria and glycolysis) and those of energy utilization (ATPases). Thus, CK enhances skeletal, cardiac, and smooth muscle contractility, and is involved in the generation of blood pressure (Njoku *et al.*, 2017).

iii. Aspartate Aminotransferase

Aspartate aminotransferase (AST), also known as (serum) glutamic oxaloacetic transaminase (GOT, SGOT), is a pyridoxal phosphate (PLP)-dependent transaminase enzyme. AST catalyzes the reversible transfer of an α -amino group between aspartate and glutamate and, as such, is an important enzyme in amino acid metabolism (Njoku *et al.*, 2017). AST is found in the liver, heart, skeletal muscle, kidneys, brain, and red blood cells. Aspartate transaminase catalyzes the interconversion of aspartate and α -ketoglutarate to oxaloacetate and glutamate (Njoku *et al.*, 2017).



As a prototypical transaminase, AST relies on PLP (Vitamin B6) as a cofactor to transfer the amino group from aspartate or glutamate to the corresponding ketoacid. In the process, the cofactor shuttles between PLP and the pyridoxamine phosphate (PMP) form. The amino group transfer catalyzed by this enzyme is crucial in both amino acid degradation and biosynthesis. In amino acid degradation, following the conversion of α -ketoglutarate to glutamate, glutamate subsequently undergoes oxidative deamination to form ammonium ions,

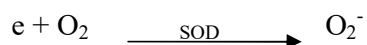
which are excreted as urea. In the reverse reaction, aspartate may be synthesized from oxaloacetate, which is a key intermediate in the citric acid cycle (Njoku *et al.*, 2017).

1.4 Reactive Oxygen Species

Reactive oxygen species (ROS) are highly reactive molecules that are generated from oxygen metabolism. They can be free radicals or non-radicals. Free radicals are molecules that contain at least one unpaired valence electron at their outer shell, making them highly reactive and short lived (Nunes and Freitas 2014). Among all the ROS, superoxide anion ($\bullet\text{O}_2^-$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($\bullet\text{OH}$) are the most known examples. Reactive nitrogen species (RNS) is the subclass of ROS that contain nitrogen compound (Doshi, 2012). Both ROS and RNS, when present in physiological amount, have important roles in normal cellular functions such as fighting against infection, regulating different intercellular signaling pathways and facilitating normal maturation and fertilization in reproductive systems (Nunes and Freitas 2014; Agarwal, 2014). However, when ROS is present in high concentration, overwhelming of antioxidant defense system and oxidative stress may result leading to cellular dysfunction via lipid peroxidation, protein and DNA damages (Doshi, 2012).

i. Superoxide Anion ($\text{O}_2^\bullet$)

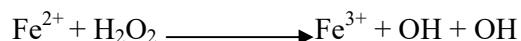
Univalent reduction of oxygen will result in the formation of superoxide anion ($\text{O}_2^\bullet$). This reaction can happen in the mitochondrial electron transport chain (ETC) without the involvement of any enzymatic activity by the action of oxidation reduction reactive intermediates such semi-ubiquinone (Nunes and Freitas, 2014). On the other hand, enzymes such as nicotinamide adenine dinucleotide phosphate (NADPH) oxidases and xanthine oxidase (XO) can also mediate this reaction. High amounts of superoxide anions are usually released from neutrophils and macrophages by the action of different isoforms of NADPH oxidase. These superoxide anions can then be converted into non-radical species, hydrogen peroxide (H_2O_2) and singlet oxygen ($^1\text{O}_2$), enzymatically with oxidative enzymes such as superoxide dismutase (SOD) or non-enzymatically (Nunes and Freitas 2014).



ii. Hydroxyl Radical ($\bullet\text{OH}$)

The interaction between hydrogen peroxide (H_2O_2) and reduced transition metals such as copper and iron results in Fenton reaction, which leads to the formation of hydroxyl radical

(•OH). In addition, the exposure of water molecules to ionizing radiation can also result in the production of hydroxyl radical (Nunes and Freitas 2014).



1.5 Antioxidants

Antioxidant is defined as any substance able to eliminate ROS and derivatives (RNS, or reactive sulfur species, RSS), directly or indirectly, acting as an antioxidant defense regulator, or reactive species production inhibitor (Mut-Salud *et al.*, 2016). They either stabilize free radicals or reduce their functions. Antioxidants are compounds that inhibit oxidation. Oxidation is a chemical reaction that can produce free radicals, thereby leading to chain reactions that can damage the cells of organisms. Antioxidants, such as thiols or ascorbic acid (vitamin C) end 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 vitamins C and E (Pawar *et al.*, 2016). Antioxidant defenses induction or endogenous ROS/RNS levels reduction is a rapid and clear oxidative stress indicator. Indeed, ROS/RNS production and accumulation is a common denominator in many disorders and environmental insults, at the same time that can cause serious cell damage leading to physiological dysfunction and cell death in almost all aerobes (Maes *et al.*, 2018). Antioxidant therapy has long been investigated as a means of reducing the extent of injury resulting from an ischemic stroke with varying degrees of success. Antioxidants can be classified as enzymatic and non-enzymatic, both of which modulate free radical reactions. The non-enzymatic include both natural and synthetic. Natural antioxidants are contained in fruits, vegetables, legumes and medicinal plants. The mechanisms of actions of non-enzymatic antioxidants involve the interruption of free radical chain reactions (Keshari *et al.*, 2015). The endogenous antioxidants include the enzymes superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx). These endogenous defense enzymes function interactively and synergistically with diet-derived antioxidants, lipid-soluble vitamin E, carotenes, and water-soluble vitamin C to increase their effectiveness by either stabilizing the free radicals or by reducing their function. These helps to minimize oxidative stress and reduce the risk of cancer, cardiovascular diseases and aging. For example, some antioxidants display a more important role in preventing certain diseases, while other ones work better fighting degenerative diseases (Kumar *et al.*, 2016).

Antioxidants are said to have been overwhelmed by the free radicals when the production of free radicals exceed the level which the body's natural antioxidant defense mechanisms can cope with; consequently, creating a cellular oxidative environment which triggers the oxidation of essential biomolecules like DNA, protein and lipids, leading to multiple disease conditions. The interplay between the trio of free radical's antioxidants, and diseases is important in maintaining health, aging and age-related diseases (Ighodaro and Akinloye 2017). Antioxidants such as polyphenols, ascorbic acid, vitamin A, alpha-lipoic acid, thioredoxin, glutathione, melatonin, coenzyme Q, beta carotenoids, alpha-tocopherols as well as antioxidant enzymes including superoxide dismutase, catalase and glutathione peroxidases have been widely investigated for the prevention and treatment of diseases resulting from oxidative damage (Yuan *et al.*, 2010; Dysken *et al.*, 2014).

1.5.1 Levels of Action of Antioxidants

According to Kumar *et al.* (2016), the antioxidants molecules that constitute the antioxidant defense grid in living systems act at different levels. These levels may be radical preventive, radical scavenging and radical induced damage repair. On the basis of line of defense, antioxidants can be categorized as first line defense antioxidants, second line defense antioxidants, third line defense antioxidants and fourth line defense antioxidants though this study is focused on the first line defense antioxidants.

1.5.2 First Line Defense Antioxidants

These are a collection of antioxidants that act to suppress or prevent the formation of free radicals or reactive species in cells (Kumar *et al.* 2016). These enzymes respectively breakdown hydrogen peroxides to harmless molecules alcohol and O₂, (Dysken *et al.*, 2014). This class also includes metal ion binding proteins like transferrin and caeruloplasmin which chelate or sequester iron and copper respectively, consequently preventing them from free radical formation. There are several enzyme systems that catalyze reactions to neutralize free radicals and reactive oxygen species. These enzymes include: Superoxide dismutase, Glutathione peroxidase and Catalases. These form the body's endogenous defense mechanisms to help protect against free radical-induced cell damage (Mironczuka-Chodakowska *et al.*, 2018). The antioxidant enzymes metabolize oxidative toxic intermediates.

1.5.3 Antioxidant Biomarkers

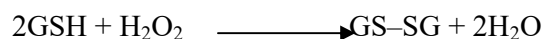
The Antioxidant biomarkers include: Superoxide Dismutase, Glutathione Peroxidase and Catalase.

i. Superoxide Dismutase

According to Ighodaro and Akinloye (2018), superoxide dismutase (SOD) are a class of enzymes that catalyzes the breakdown of the superoxide anion into oxygen and hydrogen peroxide. These enzymes are present in almost all aerobic cells and in extracellular fluids. Superoxides contain metal ion cofactors which, depending on the isozyme, could be copper, zinc, manganese or iron. For example, in human's copper/zinc SOD is present in the cytosol, while manganese SOD is present in the mitochondrion. The mitochondrial SOD is most biologically important of these three. Mitochondrial Mn-SOD2 has been described as a homo tetramer with a molecular weight of 96 kDa and contains one manganese atom per subunit (Aoyama and Nakaki, 2015). This SOD isoform cycles from Mn^{3+} to Mn^{2+} and back to Mn^{3+} during the two-step dismutation of superoxide. In plants, SOD isozymes are present in the cytosol and mitochondria while iron SOD are found in chloroplasts (Ighodaro and Akinloye, 2018).

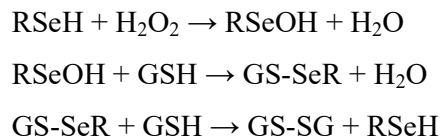
ii. Glutathione Peroxidase

Glutathione peroxidase (GPx) is the general name of an enzyme family with peroxidase activity whose main biological role is to protect the organism from oxidative damage (Nachiappan *et al.*, 2010; Pisoschi and Pop 2015). The biochemical function of glutathione peroxidase is to reduce lipid hydroperoxides to their corresponding alcohols and to reduce free hydrogen peroxide to water. Several isozymes are encoded by different genes, which vary in cellular location and substrate specificity. Glutathione peroxidase 1 (GPx1) is the most abundant version, found in the cytoplasm of nearly all mammalian tissues, whose preferred substrate is hydrogen peroxide. Glutathione peroxidase 4 (GPx4) has a high preference for lipid hydroperoxides; it is expressed in nearly every mammalian cell, though at much lower levels. Glutathione peroxidase 2 is an intestinal and extracellular enzyme, while glutathione peroxidase 3 is extracellular, especially abundant in plasma (Cassia *et al.*, 2018). So far, eight different isoforms of glutathione peroxidase (GPx1-8) have been identified in humans. The main reaction that glutathione peroxidase catalyzes is:



where GSH represents reduced monomeric glutathione, and GS-SG represents glutathione disulfide (Reczek and Chandel 2015). The mechanism involves oxidation of the selenol of a selenocysteine residue by hydrogen peroxide. This process gives the derivative with a

selenenic acid (RSeOH) group. The selenenic acid is then converted back to the selenol by a two-step process that begins with reaction with GSH to form the GS-SeR and water. A second GSH molecule reduces the GS-SeR intermediate back to the selenol, releasing GS-SG as the by-product (Liu *et al.*, 2018a). A simplified representation is shown below.



iii. Catalases

Catalases are enzymes that catalyze the conversion of hydrogen peroxide to water and oxygen, using either an iron or manganese cofactor. This is found in peroxisomes in most eukaryotic cells. Its only substrate is hydrogen peroxide. It follows a ping-pong mechanism because of the high turnover rates for all enzymes. Here, its cofactor is oxidized by one molecule of hydrogen peroxide and then regenerated by transferring the bound oxygen to a second molecule of substrate (Aoyama and Nakaki, 2015).

1.5.4 Other Antioxidants

1.5.4.1 Dietary Antioxidants

Vitamin C, vitamin E, and beta-carotene are among the most widely studied dietary antioxidants. Vitamin C is considered the most important water-soluble antioxidant in extracellular fluids. It scavenges free radicals and reactive oxygen species (ROS), which are produced during metabolic pathway (Wang *et al.*, 2018). Vitamin C or ascorbic acid (AA) are naturally present in some foods and available as a dietary supplement. Humans, unlike most animals, are unable to synthesize vitamin C endogenously, so it is an essential dietary component. It is capable of neutralizing ROS in the aqueous phase before lipid peroxidation is initiated (Manita *et al.*, 2019).

Vitamin E, a major lipid-soluble antioxidant, is transported and distributed in the plasma through lipophilic carriers and localizes to lipid rich components within the cells and tissues. In the plasma the major lipoprotein carrier of vitamin E is LDL, while in the cells is localized mainly at the cell membrane. The pattern of distribution enables vitamin E to protect against LPO wherever it occurs across the cell (Patrice *et al.*, 2016). Vitamin E is comprised of eight isoforms collectively known as tocopherols and tocotrienols, of which α -tocopherol is the most active antioxidant. α -tocopherol functions as a chain breaking antioxidants that prevent the propagation of free radicals in membrane and plasma lipoproteins. Peroxyl radicals

(ROO) have a much greater affinity for α -tocopherol (100 folds) than for polyunsaturated fatty acids. Vitamin E is the most effective chain-breaking antioxidant within the cell membrane where it protects membrane fatty acids from lipid peroxidation. Vitamin C has been cited as being capable of regenerating vitamin E. Beta-carotene and other carotenoids are also believed to provide antioxidant protection to lipid-rich tissues (Patrice *et al.*, 2016). Research suggests that beta-carotene may work synergistically with vitamin E. The same mechanism is also responsible for the ability of carotenoids to physically quench singlet oxygen without degradation and for the chemical reactivity of carotenoids with free radicals (Kurutas, 2015). They also scavenge peroxy radical hence preventing lipophilic damage. A diet that is excessively low in fat may negatively affect beta carotene and vitamin E absorption, as well as other fat-soluble nutrients. Fruits and vegetables are major sources of vitamin C and carotenoids, while whole grains and high quality, properly extracted and protected vegetable oils are major sources of vitamin E (Patrice *et al.*, 2016).

1.6 Doxorubicin Cellular Effects

Doxorubicin (DOX) is a broad-spectrum anti-tumor drug, but its cardiotoxicity limits its clinical application (Wang, 2016). DOX associates strongly with cellular nuclei by intercalating with DNA base pairs resulting to inhibition of Top 2 β , which is active in quiescent non-proliferating cells hence is thought to be the key mediator of anthracycline-induced cardiotoxicity. Inhibition of Top 2 β anthracycline in cardiomyocytes causes double-stranded DNA breaks hence prevent DNA and RNA synthesis (Cheung *et al.*, 2015; Henriksen, 2018). This is required for activation of the p53-mediated apoptotic cell death pathway which interferes with the mitochondrial biogenesis. Mice embryonic fibroblasts with the Top 2 β deletion are resistant to doxorubicin toxicity and conditional knockout mice with the cardiac Top 2 β deletion showed fewer double-stranded breaks, no decline in LVEF and less mitochondrial dysfunction following doxorubicin treatment (Levis *et al.*, 2017). Mechanistic understanding of anthracycline cardiomyocyte injury has focused on the formation and targeting of Top 2 β in cardiomyocytes, iron accumulation within the mitochondria is associated with futile redox cycling and increased ROS production (Ichikawa *et al.*, 2014).

Cardiomyocytes exposed to DOX are ATP deficient and exhibit increased proteotoxic load, suggesting that protein degradation pathways are compromised, overwhelming the ER and mitochondria and contributing to cardiac injury (Lv *et al.*, 2012; Li *et al.*, 2014). Indeed, numerous studies have demonstrated that DOX-induced cardiac injury is associated with

dysregulation in autophagy function (Bartlett *et al.*, 2016; Ge *et al.*, 2016; Ghigo *et al.*, 2016; Park *et al.*, 2016 and Sun *et al.*, 2014).

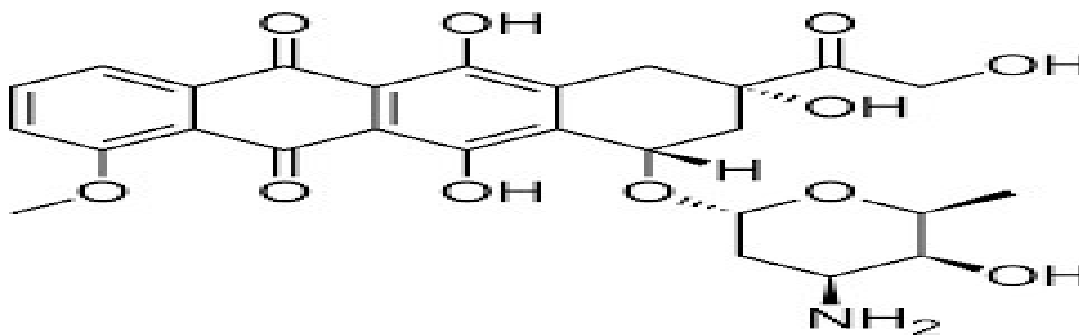


Fig 1: Structure of Doxorubicin

Source: (Henriksen, 2018)

1.6.1 Doxorubicin Induced Cardiotoxicity

Doxorubicin (DOX) is utilized for anti-neoplastic treatment for several decades. DOX (also known as adriamycin) was isolated in the early 1960s from the pigment-producing bacterium *Streptomyces peucetius* var. *caesius*, along with daunorubicin (DAU, also known as daunomycin and rubidomycin), and belongs to the family of anthracyclines. Until now, DOX remains among the most largely prescribed and effective antineoplastic agents ever developed for the treatment of a variety of adult and pediatric cancers (Levis *et al.*, 2017; Gorini *et al.*, 2018). Whereas DAU has been used against acute lymphoblastic and myeloblastic leukemias, DOX has been used against breast cancer, soft tissue sarcomas, childhood tumors (e.g., Wilms' tumor), leukemias, Hodgkin's and non-Hodgkin's lymphoma, and many other cancers (Ewer and Ewer, 2015).

The minor differences in the chemical structure between DOX and DAU are responsible for the different spectrums of activity of these drugs. The side chain of DOX terminates with primary alcohol, while that of DAU terminates with a methyl group. Unfortunately, in addition to its potent antitumor effect, the use of DOX has been hampered by conventional toxicities (hematopoietic suppression, nausea, vomiting, extravasation, and alopecia), development of resistant tumor cells or toxicity in healthy tissues, especially with serious cardiac toxicity manifested by congestive cardiomyopathy (Ewer and Ewer, 2015). Over time, more than 2000 analogs were developed in an attempt to reduce the adverse effects of DOX and DAU. However, few analogs have reached the stage of clinical development and approval, such as epirubicin (EPI) and idarubicin (IDA), with DOX- and DAU-like spectrums, respectively. Despite the development of new components, replacing DOX does

not eliminate the risk of developing cardiotoxicity (Ewer and Ewer, 2015) thus, DOX continues to be considered as a first line antineoplastic drug.

Since doxorubicin induces cardiotoxicity, it is thought to involve the excessive generation of ROS followed by oxidative stress leading to the determination of representative tissue index of oxidation, lipid peroxidation, and antioxidant, glutathione (GSH), as well as the parameters of heart injury. Doxorubicin treatment successfully induced cardiotoxicity as evidenced by histological examination and serum parameters (levels of LDH and CK activities) in the treated albino rats. It was accompanied by increased lipid peroxidation and a decrease in both cysteine and GSH, further supporting previous reports that oxidative stress is a potential mechanism of doxorubicin-induced cardiotoxicity (Kim *et al.*, 2017). Moreover, increased oxidative stress and release of reactive oxygen radicals, including super oxide anion (O_2^-) and other reactive oxygen intermediates as well as antioxidant deficits, have been suggested to play a major role in dox-induced cardiomyopathy and heart damage. In recent years, it has been observed that there is a growing interest in uses of natural antioxidants as a protective strategy against the cardiovascular-related problems such as ischemia-reperfusion and Dox-induced cardiotoxicity (Ricordi *et al.*, 2015).

1.6.2 Doxorubicin Clinical Aspects

Since the late 1970s, DOX-induced cardiotoxicity (DIC) has been recognized as a complication of chemotherapy. The first case report in the literature was that of a 23-year-old patient with osteosarcoma, who was treated for 9 months with DOX. One month after the end of treatment, the patient died due to development of congestive heart failure (Simunek *et al.*, 2009) also a case of an 11-year-old patient, also with osteosarcoma, who died 9.5 years' after the end of chemotherapy with DOX as a result of progressive heart failure with late severity of Cardiotoxicity. In 1991, long term cardio-toxic effects were identified in patients with acute lymphoid leukemia in childhood. Patients with childhood cancer and those treated with DOX have a high risk of developing symptomatic cardiac events at an early stage, and this risk remains high within 30 years after treatment. In addition, it is estimated that one in eight DOX-treated patients will be afflicted with severe cardiac disease (Raj *et al.*, 2014). Doxorubicin induced cardiotoxicity manifests in several forms, ranging from asymptomatic electrocardiography (ECG) changes to decompensated cardiomyopathy characterized by decreased left ventricular ejection fraction. According to their clinical manifestation, these cardio toxic events can be classified into three types:

- i. Acute, occurring during or immediately after treatment;

- ii. Early-onset chronic progressive cardiotoxicity, occurring within 1 year after exposure to chemotherapeutic treatments.
- iii. Late-onset chronic progressive cardiotoxicity, occurring 1 or more years after the end of treatment (Raj *et al.*, 2014).

Acute cardiotoxicity is characterized by depression of myocardial contractility that may be reversible within 1 week when discontinuing the DOX treatment. In some patients, complications have already been described, such as hypertension; pericarditis; myocarditis; supraventricular, ventricular, or sinus (more common) tachycardia; decrease in QRS complex; and increase in serum levels of brain natriuretic peptide and cardiac troponin (Geiger *et al.*, 2010). However, this type of cardiotoxicity is very rare and affects less than 1% of patients.

Early-onset chronic progressive cardiotoxicity is characterized by systolic or diastolic ventricular dysfunction within 1 year after the completion of DOX treatment. It can be progressive and occurs in 5–35% of the cases (Raj *et al.*, 2014; Kalil *et al.*, 2011). In the majority of adult patients, early cardiotoxicity is related to the development of a chronic dilated cardiomyopathy, with a decrease in the mass and wall of left ventricle. In the pediatric patient, in addition to chronic dilated cardiomyopathy, restrictive cardiomyopathy characterized by increase in the wall stiffness of the left ventricle cavity may also occur in isolated moments (Geiger *et al.*, 2010). The typical manifestation of these cardiomyopathies is the progressive reduction of the ejection fraction. Other events, including severe electrical conduction changes, damage to cardiac valves, and/or depression of contractility may also be observed.

Finally, late-onset chronic progressive cardiotoxicity is characterized by cardiac dysfunction after a latency period of 1 or more years following the completion of DOX treatment. In this type of cardiotoxicity, there is a period during which the patient is asymptomatic (normal cardiac function). After that, chronic dilated and/or restrictive cardiomyopathy can be manifested with subsequent development of congestive heart failure. In this case, mortality rate is more than 50% (Geiger *et al.*, 2010; Kalil *et al.*, 2011).

Table 1: List of Clinically Used Drugs That Exert Cardiotoxic Effects.

	Drugs	Class	Mode of action	Uses
1	Doxorubicin	Anticancer	Inhibit progression of topoisomerase II	Breast-cancer, bladder-cancer, lymphoma, Kaposi's sarcoma.
2	Daunorubicin	Anticancer	Inhibit progression of topoisomerase II	Kaposi's sarcoma, lymphoma, myelogenous leukemia.
3	Idarubicin	Anticancer	Inhibit progression of topoisomerase II	Acute myeloid leukemia.
4	Cyclophosphamide	Anticancer	Bind at 7 guanine residue and inhibit cell division	Lymphoma, multiple myeloma, ovarian, breast and lung cancer.
5	5 fluorouracil	Anticancer	Thymidylate synthase inhibitor, inhibit DNA replication.	Colon, stomach, esophageal, pancreatic, breast and cervical cancer.
6	Chloroquine	Antimalarial	Prevent biocrystallization of hemozoin	Arthritis, malaria, lupus erythematosus
7	Cytarabine	anticancer immunosuppressant	Inhibit DNA and RNA polymerase	AML, CML and non-Hodgkin's lymphoma
8	Paclitaxel	Anticancer	Act on tubulin and causes instability of cytoskeleton causes cell cycle arrest	kapski sarcoma, ovarian, breast, lung, cervical, and pancreatic cancer
9	Mitomycin	Anticancer	Inhibit DNA cross linking	Adenocarcinoma, anal, bladder, lungs, cervical, colorectal.
10	Sunitinib	Anticancer	Tyrosine-kinase inhibitor	Renalcell carcinoma and gastrointestinal stromal tumors.
11	Trastuzumab	Monoclonal antibodies	Act-through PI3K/Akt pathway	Breast cancer
12	Zidovudine	Antiretroviral	Inhibit-HIV's reverse transcriptase enzyme	HIV and HAART

Source: (Raj *et al.*, 2014).

1.6.3 Strategies for Heart Protection

Since several mechanisms are involved in the development of cardiac toxicity, different strategies are being performed to prevent DOX-induced cardiomyopathy. One of these strategies is the use of dexrazoxane (also known as ICRF-187), an adjunctive agent derivative of ethylene diaminetetraacetic acid (EDTA), which acts as a free radical scavenger. In this case, dexrazoxane is an EDTA-like chelator that interferes with iron-mediated oxygen free radical generation and, consequently, lipid peroxidation. Further, a meta-analysis of six randomized trials that included 1013 adult and pediatric patients demonstrated significantly reduced incidence of heart failure after dexrazoxane treatment, confirming its beneficial effects. In addition, cardio-protective effects have been observed in children with high-risk acute lymphoblastic leukemia (ALL) receiving chronic DOX (10 doses of 30 mg/m²) (Lipshultz *et al.*, 2010). The studies concluded that treatment with dexrazoxane is justified individually when the risk of cardiac dysfunction is expectedly high.

Unfortunately, according to the current (FDA) approval statement and European Medicines Agency (EMA), the use of this drug as a cardio-protective is limited to women with metastatic breast cancer who have received cumulative doses of 300 mg/m² DOX (Lipshultz *et al.*, 2010).

Another strategy is the use of angiotensin-converting enzyme (ACE) inhibitors, including enalapril, zofenopril, and lisinopril. ACE inhibitors are commonly used in patients with heart failure as afterload-reducing agents. In addition to their features as an effective ACE inhibitor, these drugs act as antioxidant and, thus, may contribute to prevent cardiac toxicity. In support of this view, recent preclinical study demonstrated that administration of enalapril attenuated DOX-induced cardiac dysfunction via preservation of mitochondrial respiratory efficiency and reduction in DOX-associated free radical generation (Hiona *et al.*, 2011). Unluckily, in long-term survivors of childhood cancer treated with DOX, the beneficial effect of enalapril-induced improvement in left ventricle structure and function was lost after 6 or 10 years. Another antioxidant that has already been tested against DOX-induced cardiomyopathy is vitamin E. A study has shown that vitamin E only prevents the acute effects of DOX cardiotoxicity in mice (Jhorawat *et al.*, 2016).

On the other hand, probucol, a lipid-lowering agent and potent antioxidant, provided complete protection against DOX-induced cardiomyopathy and heart failure in animal experiments without interfering with the antitumor properties of this antibiotic. In this case, it is extremely important that clinical trials using DOX therapy in combination with probucol

are performed to determine a new preventive cardiotoxicity strategy. This approach was recently tested using a β -adrenergic receptor blocker and also an antioxidant agent called carvedilol. As a result, this drug protected systolic functions of the left ventricle due to reducing DIC (Jhorawat *et al.*, 2016). Therefore, the use of carvedilol may become a promising strategy to improve DIC. In addition to the antioxidant agents, accumulating evidence indicates that cardiac α 1-adrenergic receptors (α 1-ARs) protect cardiomyocytes from DIC (Beak *et al.*, 2017).

1.7 Cola Plants

The Cola nut is the fruit of the cola tree, a genus (*Cola*) of trees that are native to the tropical rainforests of Africa. It is a tree native to the rainforests of tropical West Africa. Common names include kola nut, cola, kola and bitter kola (Dewole *et al.*, 2013). Cola is a genus of tropical trees of the chocolate family (Sterculiaceae, order Malvales) that bear fruits enclosing large kola, or cola, nuts containing caffeine, tannin, and theobromine. Though native to Africa, two species especially, *Cola acuminata* and *Cola nitida*, are grown commercially in various tropical regions around the world, the dried kola nuts being used in manufacturing the popular soft drink called cola. The cola drink consists of flavoring extracts of kola nut, spice oils, and other aromatics caramel coloring, sugar and other sweeteners, singly or in combination; phosphoric acid or citric acid; carbon dioxide for effervescence; and water (86 to 92 percent by volume). In diet cola drinks, artificial flavorings and sweeteners may predominate, and the water content may near 100 percent (Dewole *et al.*, 2013). In medicine the refined extract is used as a cardiac and central-nervous-system stimulant.

1.7.1 Importance of Cola Plants

Human use of the kola nut, like the coffee berry and tea leaf, appears to have ancient origins. It is chewed in many West African cultures, in both private and social settings, to restore vitality (Odebunmi *et al.*, 2009). Cola nuts are an important part of the traditional spiritual practice of culture and religion in West Africa, particularly Niger, Nigeria, Sierra Leone and Liberia. The nuts contain caffeine, theobromine tannin and other bioactive compounds. Cola nuts are used as a religious object and sacred offering during prayers, ancestor veneration, and significant life events, such as naming ceremonies, weddings, and funerals. They are also used in a traditional divination system called Obi divination. For this use, only cola nuts divided into four lobes are suitable. They are cast upon a special wooden board and the

resulting patterns are read by a trained diviner (Dewole *et al.*, 2013). They were used as a form of currency in such West African groups as the Malinke and Bambara of Mali and Senegal. They are still used as such today in certain situation such as in negotiation over bride prices or as a form of a respect or host gift to the elders of a village should one move to a village or enter a business arrangement with the village (Osuagwu, 2003). The timber has a use in building work, boat construction, furniture and joinery, musical instruments, utensils and carving. The pods have been used to make fertilizer and soap, and they can be used as a substitute for up to 60% of the maize in poultry feed. In folk medicine, cola nuts are considered useful for aiding digestion when ground and mixed with honey, and are used for coughs (Odebunmi *et al.*, 2009).



FIG 2: Dried *Cola hispida* Leaves

SOURCE: (Dewole *et al.*, 2013).

1.7.2 Classification

Kingdom: Plantae

Subkingdom: Tracheobionta

Phylum: Tracheophyta

Class: Magnoliopsida

Subclass: Dilleniidae

Order: Malvales

Family: Sterculiaceae

Genus: *Cola*

Species: *hispida*

Source: Dewole *et al.*, 2013.

1.7.3 Other *Cola* Species (*Cola acuminata* and *Cola nitida*).

Cola acuminata

This kola nut is a caffeine-containing nut of evergreen trees of the genus *Cola*, primarily of the species *Cola acuminata* and *Cola nitida* (Burdock *et al.*, 2009). *Cola acuminata*, an evergreen tree about 20 metres in height, has long, ovoid leaves pointed at both the ends with a leathery texture. The trees have yellow flowers with purple spots, and star-shaped fruit. Inside the fruit, about a dozen round or square seeds develop in a white seed-shell. The nut's aroma is sweet and rose-like, the first taste is bitter, but it sweetens upon chewing. The nut can be boiled to extract the caffeine.

Cola nitida

This species of plant belonging to the family Malvaceae. *Cola nitida* is native to Sierra Leone, Liberia, Ivory Coast, Ghana and Nigeria. It is a lowland tree but can be found up to altitudes of about 300 metres (980 ft) in areas with deep, rich soils and evenly distributed rainfall. It has been cultivated in other parts of tropical Africa as well as India, Jamaica, Brazil and Hawaii. It can grow where there is around 1,200 millimetres (47 in) of rain but does better where the annual rainfall exceeds 1,700 millimetres (67 in), spread across eight months or more. Its temperature range is 23 to 28 °C (73 to 82 °F) and it grows on both light and heavy soils as long as they are deep. *Cola nitida* is an evergreen tree growing to a height of 12 to 20 metres (39 to 66 ft). The trunk is up to 1.5 m (4 ft 11.1 in) in diameter and older trees develop buttresses. When ripe, the pods split open to reveal the seeds which may be mottled white, reddish-grey or brown also the seeds are bitter when fresh but become more aromatic as they age (Dewole *et al.*, 2013).

1.7.4 Phytochemicals

Phytochemicals are chemical compounds produced by plants, generally to help them thrive or thwart competitors, predators, or pathogens. Phytochemicals (from Greek *phyto*, meaning "plant") are chemicals produced by plants through primary or secondary metabolism (Gonzales *et al.*, 2015). Phytochemicals under research can be classified into major categories, such as carotenoids and polyphenols, which include phenolic acids, flavonoids, and stilbenes/lignans. Phytochemists study phytochemicals by first extracting and isolating

compounds from the origin plant, followed by defining their structure or testing in laboratory model systems, such as cell cultures, *in vitro* experiments, or *in vivo* studies using laboratory animals. The phytochemical category includes compounds recognized as essential nutrients, which are naturally contained in plants and are required for normal physiological functions, so must be obtained from the diet in humans (Nascimento-Souza *et al.*, 2018). Some phytochemicals are known phytotoxins that are toxic to humans; for example aristolochic acid is carcinogenic at low doses. Some phytochemicals are anti nutrients that interfere with the absorption of nutrients. Others, such as some polyphenols and flavonoids, may be pro-oxidants in high ingested amounts. Non digestible dietary fibers from plant foods, often considered as a phytochemical, are now generally regarded as a nutrient group having approved health claims for reducing the risk of some types of cancer and coronary heart disease. The phytochemical screening showed that all leaves contain phenolics, flavonoid, hydroxyl anthraquinones and Saponins (Gonzales *et al.*, 2015). These are known to exhibit medicinal activity as well as physiological activity. Flavonoids have been shown to have antibacterial, anti-inflammatory, anti-allergic, antimutagenic, antiviral, antineoplastic, anti-thrombotic and vasodilatory activity. The potent antioxidant activity of flavonoids is their ability to scavenge hydroxyl radicals, superoxide anions and lipid peroxy radicals may be the most important function of flavonoids (Gonzales *et al.*, 2015).

1.7.4.1 Phytochemical Constituents of *Cola Hispida* Leaves

The phytochemical constituents of *Cola hispida* include the following:

i. Alkaloids

Alkaloids have traditionally been of great interest to humans because of their pronounced physiological and medicinal properties. From the beginning of civilization, alkaloid-containing plant extracts have been used in all cultures as potions, medicines, and poisons (Petrovska, 2012). Most alkaloids are very toxic and, therefore, have the potential to function in the chemical defense arsenal of plants against attack by herbivores and micro-organisms. For example, the nicotine present in tobacco leaves inhibits the growth of tobacco hornworm larvae; the purified compound is also applied as an effective insecticide in greenhouses (Petrovska, 2012). In addition, alkaloids have been suggested to serve as a storage form of nitrogen or as protectants against damage by ultraviolet light. Alkaloids can occur in all parts of the plant but frequently, depending on the plant species, they accumulate only in particular organs (e.g., in barks, roots, leaves, and fruits), whereas at the same time other organs are alkaloid-free. In potato plants, the edible tubers are devoid of alkaloids, whereas the green

parts contain the poisonous solanine (Ashok and Upadhyaya, 2012). The organ in which alkaloids accumulate is not always the site of their synthesis. In tobacco, nicotine is produced in the roots and translocated to the leaves where it accumulates. A single plant species may contain over one hundred different alkaloids, and the concentration can vary from a small fraction to as much as 10% of the dry weight.

ii. Flavonoids

Flavonoids, a group of natural substances with variable phenolic structures, are found in fruits, vegetables, grains, bark, roots, stems, flowers, tea and wine (Omame and Azeke, 2014). These products are well known for their beneficial effects on health and efforts are being made to isolate the ingredients so called flavonoids. Flavonoids are indispensable component in a variety of nutraceutical, pharmaceutical, medicinal and cosmetic applications. This is attributed to their anti-oxidative, anti-inflammatory, anti-mutagenic and anti-carcinogenic properties coupled with their capacity to modulate key cellular enzyme function (Panche *et al.*, 2016). The most reported activity of flavonoids is their protection against oxidative stress. Research on flavonoids according to Kurutas (2015) showed that flavonoids can scavenge peroxy radicals, and are effective inhibitors of lipid peroxidation. They also can chelate redox-active metals, and thus prevent catalytic breakdown of hydrogen peroxide (Fenton reaction). Further studies on flavonoids received an added impulse with the discovery of the low cardiovascular mortality rate and also prevention of coronary heart disease. However, it has widely been known for centuries that derivatives of plant origin possess a broad spectrum of biological activity. Current trends of research and development activities on flavonoids relate to isolation, identification, characterization and functions of flavonoids and their applications on health benefits (Panche *et al.*, 2015).

iii. Saponins

Saponins a class of chemical compounds found in particular abundance in various plant species. They are groups of secondary amphipathic glycosides grouped phenomenologically by the soap-like foam they produce when shaken in aqueous solutions, and structurally by having one or more hydrophilic glycoside moieties combined with a lipophilic triterpene or steroid derivative (Ashok and Upadhyaya, 2012). Saponins protect plants against attack by pest and pathogens. Saponins have substantial economic value and are processed as drugs and medicines, cosmetics, sweeteners, taste converters and foaming agents. Saponins also have stimulatory effects on the components of specific immunity and non-specific immune reactions such as inflammation. (Jeruto *et al.*, 2011).

iv. Tannins

Tannin, also called tannic acid, any of a group of pale-yellow to light-brown amorphous substances in the form of powder, flakes, or a spongy mass, widely distributed in plants and used chiefly in tanning leather, dyeing fabric, making ink, and in various medical applications. Tannin solutions are acid and have an astringent taste. Tannin is responsible for the astringency, colour, and some of the flavor in tea. Tannins occur normally in the roots, wood, bark, leaves, and fruit of many plants, particularly in the bark of oak species and in sumac and myrobalan. They also occur in galls, pathological growths resulting from insect attacks (Ashok and Upadhyaya, 2012).

Tannins are considered as anti-nutrient of plant origin due to its ability to precipitate proteins, inhibit the digestive enzymes and reduce the absorption of minerals and vitamins (Njoku *et al.*, 2017). In addition to their principal applications in leather manufacture and dyeing, tannins are used in the clarification of wine and beer, as a constituent to reduce viscosity of drilling mud for oil wells, and in boiler water to prevent scale formation. Because of its styptic and astringent properties, tannin has been used to treat tonsillitis, pharyngitis, hemorrhoids, and skin eruptions; it has been administered internally to check diarrhea and intestinal bleeding and as an antidote for metallic, alkaloidal, and glycosidic poisons, with which it forms insoluble precipitates. Soluble in water, tannins form dark blue or dark green solutions with iron salts, a property utilized in the manufacture of ink (Ashok and Upadhyaya, 2012).

v. Terpenoids

The terpenoids sometimes called isoprenoids have molecular structures comprising carbon backbones that are made up of isoprene units with ten carbon atoms with a large and diverse class of naturally occurring organic chemicals derived from terpenes (Moses *et al.*, 2013). Most are multicyclic structures with oxygen-containing functional groups. About 60% of known natural products are terpenoids. Although sometimes used interchangeably with "terpenes", terpenoids contain additional functional groups, usually O-containing. Terpenes are hydrocarbons. According to Yadav *et al.*, (2014), terpenoids possess medicinal properties such as anti-carcinogenic, antimalarial, anti-ulcer, antimicrobial or diuretic activity. Terpenoids are used for their aromatic qualities and play a role in traditional herbal remedies. Terpenoids contribute to the scent of eucalyptus, the flavors of cinnamon, cloves, and ginger, the yellow color in sunflowers, and the red color in tomatoes (Njoku *et al.*, 2017). Well-known terpenoids include citral, menthol, camphor in the plant *Salvia divinorum*, the

cannabinoids found in cannabis, ginkgolide and bilobalide found in *Ginkgo biloba*, and the curcuminoids found in turmeric and mustard seed.

vi. Steroids

Steroids and sterols in animals are biologically produced from terpenoid precursors. Sometimes terpenoids are added to proteins, e.g., to enhance their attachment to the cell membrane; this is known as isoprenylation. Sterols in plants are generally described as phytosterols with three known types occurring in higher plants: sitosterol, stigmasterol and campesterol (Harborne, 1973). These sterols occur both as free and as simple glucosides. Free sterols are integral components of the membrane lipid bilayer where they play significant role in the regulation of membrane fluidity and permeability. Cholesterol is the major sterol in animals where as a mixture of various sterols is present in higher plants. However, certain sterols are confined to lower plants such as ergosterol found in yeast and many fungi.

1.7.5 Cardio-protective Effect of Plant Extract on Doxorubicin

In a similar study Swamy *et al.*, 2012 on cardio-protective effect of curcumin against doxorubicin-induced myocardial toxicity in albino rats, albino rats of either sex weighing 150-200g were used for the study. They produced cardiotoxicity by cumulative administration of dox (15 mg/kg for two weeks). Curcumin (200 mg/kg, po) was administered as pretreatment for two weeks and then for two alternate weeks with DOX. The general observations, mortality, histopathology, biomarker enzymes were monitored after three weeks of last dose Swamy *et al.*, 2012. Their study showed that repeated administration of DOX induced cardiomyopathy associated with an antioxidant deficit and increased level biomarkers and observed that pretreatment with the curcumin (a yellow pigment obtainable from the rhizomes of the plant *Curcuma longa*, is a crystalline compound that has been traditionally used in medicine and culinary practices in India) significantly protected myocardium from the toxic effects of DOX by reducing the elevated level of biomarker enzymes like LDH and CPK and biochemical parameters such as AST, ALT and ALP back to normal. Curcumin increased the reduced level of GSH, SOD and CAT and decreased the elevated level of malondialdehyde (MDA) in cardiac tissue hence, the biochemical and histopathology reports of their study support the cardio-protective effect of curcumin which could be attributed to antioxidant.

Another study conducted by (Swamy *et al.*, 2012) on cardio-protective effects of sitagliptin against doxorubicin-induced cardiotoxicity in rats to elucidate the potential ability of sitagliptin to counteract the injurious effects of doxorubicin in cardiac tissue. The study was

conducted on 32 male Wistar rats weighing 150–180g and of 9-10 week's age. The male Wistar rats were pretreated with sitagliptin for 10 days then treated with a single dose of doxorubicin (20 mg/kg). Electrocardiography, biochemical estimation of serum and tissue markers, and histo and immunopathological examinations were done. They observed that supplementation with sitagliptin resulted in significant improvement of cardiac function with contaminant decrease in serum markers of doxorubicin-induced cardiotoxicity. These results were supported by the histopathological results of Swamy, 2012. Moreover, a marked protection against oxidative stress was also evident through reduction of lipid peroxidation and prevention of reduced glutathione content depletion and superoxide dismutase activity reduction in cardiac tissue of rats pretreated with sitagliptin in combination with doxorubicin hence, concluded that sitagliptin pretreatment could alleviate doxorubicin-induced cardiotoxicity by reducing oxidative damage and its subsequent inflammation and apoptosis (Swamy, 2012).

1.7.6 Rationale of the Study

Cardiotoxicity is any “toxicity that affects the heart” such as cardiac dysfunction and heart failure. The use of several strategies to prevent cardiotoxicity such as dexrazoxane an adjunctive agent derivative of EDTA, ACE inhibitors, probucol and vitamin E, which serves as free radical scavenger consequently, has adverse effect hence limit their wider acceptance. Recently, attention has been focused on the investigation of the efficacy of plants in the traditional medicine because they are less expensive, readily available and with little side effect. Hence, this study is designed to evaluate the cardio-protective effect of ethyl-acetate fraction of *Cola hispida* leaves on Doxorubicin-Induced cardiotoxicity in wistar albino rats.

1.8 Aim and Objectives of the Study

1.8.1 Aim of the Study

The aim of this study was to investigate the cardio-protective effect of ethyl-acetate fraction of *Cola hispida* leaves on doxorubicin-induced cardiotoxicity in wistar albino rats.

1.8.2 Specific Objectives of the Study

The specific objectives of the study were to:

1. Determine the qualitative and quantitative phytochemical compositions of methanol extract of *Cola hispida* leaves.
2. Determine the proximate compositions of methanol extract of *Cola hispida* leaves.

3. Determine the mineral and vitamin content of methanol extract of *Cola hispida* leaves.
4. Determine the acute toxicity of ethyl-acetate fraction of *Cola hispida* leaves in wistar rats.
5. Determine the effect of ethyl-acetate fraction of *Cola hispida* leaves on some cardiovascular diseases biomarkers.
6. Determine the effect of ethyl-acetate fraction of *Cola hispida* leaves on lipid profile of Dox induced cardiotoxicity wistar rats.
7. Determine the effect of ethyl-acetate fraction of *Cola hispida* leaves on the antioxidant enzymes of induced cardiotoxicity wistar rats.
8. Determine the effect of ethyl-acetate fraction of *Cola hispida leaves* on DPPH radical scavenging activity on wistar rats.
9. Determine the effect of ethylacetate fraction of *Cola hispida* leaves on heart structural alterations through histopathology of the heart tissues.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Materials

2.1.1 Animals

A total of thirty (30) male and female albino rats were used for the study while eighteen (18) albino mice were used for acute toxicity study on the plant extract. All the animals used were obtained from the animal house of the Department of Zoology, University of Nigeria Nsukka. The rats were fed standard grower's mash rat pellets (Grand Cereals LTD, Enugu) and water *ad libitum*.

2.1.2 Plant Materials

Fresh leaves of *Cola hispida* were collected from Ajuona Nsukka Local Government of Enugu State and were identified by the Taxonomist Mr. Alfred Ozioko of Bioresources. Development and Conservation Programme, Nsukka, Enugu State Nigeria.

2.1.3 Reagents and Chemicals

The chemicals used for this study were of analytical grade and products of Sigma Aldrich (USA), British Drug House (BDH, England) and Andalucia (Spain). They included methanol, hydrochloric acid, chloroform, Dragendorff's reagent, Mayers's reagent, ammonium sulphate, ferric chloride, Fehling solutions (A and B), ethanol, lead subacetate, bromine water, tetraoxosulphate (vi) acid, ethylacetate, olive oil, picric acid, ammonium solution, Wagners' reagent, acetic acid, concentrated ammonium hydroxide, diethyl ether, n-butanol, sodium chloride, petroleum ether, chloroform acetic anhydride, TCA-chloroform, potassium dichromate, thiamin, 2,6 dichlorophenol indophenol and α,α dipyridine. The reagents used for the biochemical assays were commercial kits and products of Randox (USA) and Teco (USA).

2.1.4 Instruments and Equipments

The equipments used were obtained from the Department of Biochemistry, University of Nigeria Nsukka and other scientific shops in Onitsha main market, Anambra State. They included: Weighing balance (Metler HAS, U.S.A), Water bath (Gallenkamp, England), Glass funnel (Pyrex, England), Spectrophotometer (Spectronic 20D, Germany), Refrigerator (Thermocool, England), centrifuge (Vickas Ltd, England) and Rotary evaporator.

2.2 METHODS

2.2.1 Preparation of Plant Material

The leaves were washed with distilled water, air-dried at room temperature and pulverized into powder for extraction. The powdered sample which weighed (750g) was macerated in 3.75 L of methanol and allowed to stand for 48 hours at room temperature. The mixture was filtered with Whatman No. 1 filter paper and the filtrate was concentrated using rotary evaporator to get a chocolate-like semi solid extract.

2.2.1.1 Solvent-Solvent Partitioning (Fractionation) of *Cola hispida* methanol Leaves Extract

Procedure

A quantity (20 g) of the crude extract was further re-extracted by the process of partition chromatography using 20 % methanol, ethyl-acetate and n-hexane in order of increasing polarity. 20 g of the crude ethanol extract was dissolved in 250 ml of 20 % methanol and the same volume of n-hexane added and shaken vigorously in a separating funnel and allowed to stand for 20 minutes to ensure complete separation into two partitions, then the n-hexane partition was collected in a flat bottom flask. This process was repeated three times with the remaining 250 ml of 20 % methanol until the n-hexane partition was colorless. 250 ml of ethylacetate was added to the remaining partition of the 250 ml of 20 % methanol, the ethylacetate partition was collected into flask. The process was repeated three times until the ethylacetate partition became colorless. The different solvents partition was collected, concentrated and the solvent (ethylacetate) which showed a better result in the pilot study was selected for further studies.

2.2.1.2 Experimental Design

The experiment was divided into two stages: Stage 1 consisting of mice were partitioned into phases 1 and 2 and used for the acute toxicity study while stage 2 partitioned into 6 groups with each group having 5 albino rats and used for the cardio-protective study.

Stage II

Group A: Distilled water

Group B: 20 mg/kg b.w DOX only

Group C: 100 mg/kg b.w vitamin E + 20 mg /kg b.w DOX

Group D: 100 mg/kg b.w of fraction + 20 mg/kg b.w DOX

Group E: 200 mg/kg b.w of fraction + 20 mg /kg b.w DOX

Group F: 400 mg/kg b.w of fraction + 20 mg /kg b.w DOX

2.2.2 Qualitative Phytochemical Composition of *Cola hispida* Leaves Extract

The screening for some chemical constituents of the plant leaves was carried out as described by Harbone (1973), and Trease and Evans (2002). Quantitative analysis was carried out as described by Harbone (1998) and Soni and Sosa (2013).

2.2.2.1 Test for Terpenoids and Steroids

Ethanol (9 ml) was added to 1 g of the sample and refluxed for 10 minutes and filtered. The filtrate was concentrated to 2.5 ml on a boiling water bath, and 5 ml of hot water was added. The mixture was allowed to stand for 1 hour, and the waxy matter filtered off. The filtrate was extracted with chloroform (2.5 ml) using a separating funnel. To 0.5 ml of the chloroform extract in a test tube was carefully added 1 ml of concentrated sulphuric acid to form a lower layer. A reddish-brown interface showed the presence of steroids.

Another 0.5 ml aliquot of the chloroform extract was evaporated to dryness on a water bath and heated with 3 ml of concentrated sulphuric acid for 10 minutes on water. A grey color indicated the presence of terpenoids.

2.2.2.2 Test for Saponins

A quantity of the sample (0.1 g) was boiled with 5 ml of distilled water for 5 minutes. The mixture was filtered while still hot. The filtrate was used for the following tests.

(i) Emulsion test: A quantity of the filtrate (1 ml) was added to two drops of olive oil. The mixture was shaken and observed for the formation of emulsion.

(ii) Frothing test: A quantity, 1 ml of the filtrate was diluted with 4 ml of distilled water. The mixture was shaken vigorously and then observed on standing for a stable froth.

2.2.2.3 Test for Flavonoids

A quantity of the sample (0.2 g) was heated with 10 ml ethyl acetate in boiling water for 3 minutes. The mixture was filtered, and the filtrate was used for the following tests:

Ammonium test: 4 ml of the filtrate was shaken with 1 ml of dilute ammonium solution to obtain two layers. The layers were allowed to separate. A yellow precipitate observed in the ammonium layer indicated the presence of flavonoids.

2.2.2.4 Test for Alkaloids

A quantity of the sample (0.2 g) was boiled with 5 ml of 2 % HCl on a steam bath for 3 minutes. The mixture was filtered and 1 ml of the filtrate was treated with 2 drops of the following reagents:

- i. Dragendorff's reagent: An orange precipitate indicated the presence of alkaloids.
- ii. Mayer's reagent: A creamy-white precipitate indicated the presence of alkaloids.
- iii. Wagner's reagent: A reddish-brown precipitate indicated the presence of alkaloids.

2.2.2.5 Test for Tannins

A quantity of the sample (2 g) was boiled with 5ml of 45 % ethanol for 5 minutes. The mixture was cooled and then filtered and the filtrate was treated with the following solutions.

- (i) Lead sub acetate solution: To 1 ml of the filtrate, 3 drops of lead sub acetate solution were added. A gelatinous precipitate indicated the presence of tannins.
- (ii) Bromine water: To 1 ml of the filtrate was added 0.5 ml of bromine water and then observed for a pale brown precipitate.
- (iii) Ferric chloride solution: A quantity, 1ml of the filtrate was diluted with distilled water and then 2 drops of ferric chloride solution were added. A transient greenish to black color indicated the presence of tannins.

2.2.2.6 Test for Glycosides

A quantity of the sample (2.0 g) was mixed with 30 ml of distilled water and 15 ml of dilute sulphuric acid respectively and heated in a water bath for 5minutes. The mixtures were filtered and the filtrates used for the following test.

To 5ml of each of the filtrate, 0.3 ml of Fehling's solutions A and B was added until it turned alkaline (tested with litmus paper) and heated on a water bath for 2 minutes. A brick-red precipitate indicated the presence of glycosides.

2.2.3 Quantitative Phytochemical Composition of *Cola hispida* Leaves Extract

2.2.3.1 Concentration of Terpenoids

The method reported by Harbone (1998) was used for the quantitative determination of terpenoids. Extract (2 g) was weighed and soaked in 50ml of 95% ethanol for 24 hrs. The mixture was filtered and the filtrate extracted with petroleum ether and concentrated to dryness. The dried ether extract was treated as total terpenoids. The concentrations of the various phytochemical constituents were calculated thus:

$$\text{Concentration (mg/100g)} = \frac{\text{absorbance of the sample}}{\text{slope of the standard}} \times \frac{\text{dilution factor}}{1}$$

$$\text{Dilution factor} = \frac{\text{total volume}}{\text{weight of extract}}$$

2.2.3.2 Concentration of Saponins

The method described in Soni and Sosa (2013) was used for saponin determination. A quantity of 20 g of each sample was placed into a conical flask and 100 ml of 20 % aqueous ethanol was added. The samples were heated over a hot water bath for 4 h with continuous stirring at 55 °C. The mixture was filtered and the residue re-extracted with another 200 ml 20 % ethanol. The combined extracts were reduced to 40 ml over water bath at 90 °C. The concentrate was transferred into a 250 ml separating funnel and 20 ml of diethyl ether was added and shaken vigorously. The aqueous layer was recovered while the ether layer was discarded. The purification process was repeated. 60 ml of n-butanol was added. The combined n-butanol extracts were washed twice with 10 ml of 5 % aqueous sodium chloride. The remaining solution was heated in a water bath. After evaporation the samples were dried in the oven to a constant weight and saponin content was calculated.

2.2.3.3 Concentration of Flavonoids

The method described in Soni and Sosa (2013) was used for flavonoid determination. 10 g of the plant sample was extracted repeatedly with 100 ml of 80 % aqueous methanol at room temperature. The whole solution was filtered through whatman filter paper No 4. The filtrate was allowed to evaporate to dryness over a water bath and weighed.

2.2.3.4 Concentration of Alkaloids

The method described by Harbone (1998) was used to determine the amount of alkaloids present in the extract. A quantity of 5 g of the dried powder of each sample was weighed into a 250 ml beaker and 200 ml of 10 % acetic acid in ethanol was added. The mixture was covered and allowed to stand for 4 hours. This was filtered and the extract was concentrated on a water bath until it reached one-quarter of the original volume. Concentrated ammonium hydroxide was added drop wise to the extract until the precipitation was complete. The whole solution was allowed to settle and the precipitate was collected and washed with dilute ammonium hydroxide and then filtered. The residue was the alkaloid, which was dried and weighed.

2.2.3.5 Concentration of Tannins

The method of Swain (1979) was used for the determination of the tannin content of *Carica papaya*. A quantity, 0.2 g of finely ground sample was measured into a 50 ml beaker. About 20 ml of 50% methanol was added and covered with paraffin and placed in a water bath at

77-80°C for 1 hr and stirred with a glass rod to prevent bumping. The extract was filtered using a double layer of Whatman No.1 filter paper into a 50 ml volumetric flask, then 20 ml distilled water, 2.5 ml Folin-Denis reagent and 10 ml of 17% Na₂CO₃ were added and mixed properly. The mixture was made up to mark with distilled water and allowed to stand for 20 min when a bluish-green colouration developed. Standard tannic acid solutions of range 0-10 ppm were treated similarly as 1 ml of sample above. The absorbance of the tannic acid standard solution and the samples were read after color development at 760 nm. The tannin content was calculated using the formula:

$$\text{Tannin (\%)} = \frac{\text{Absorbance of sample} \times \text{Average gradient} \times \text{Dilution factor}}{\text{Weight of sample} \times 10000}$$

2.2.3.6 Concentration of Glycosides

Spectrophotometric determination of glycoside content was carried out with a method described by Qasheesh (1937). A mixture of 2 g of the homogenate, 20 ml of water and 2.5 ml of lead acetate were macerated for 5 min, and 10 ml of chloroform was added. After 5 min of centrifugation, 2.5 ml of the lower layer was transferred into triplicate tubes, then evaporated to dryness. Acetic acid (3 ml) and 0.1 ml of ferric chloride in 0.2 ml of sulphuric acid were added. It was then kept in the dark for 2 hr after shaking before absorbance reading was taken at 530 nm.

2.2.4 Proximate Composition of *Cola hispida* Leaves Extract

The AOAC (1990) method was used to determine the proximate constituents of *Cola hispida* Extract.

2.2.4.1 Determination of Moisture Content

The method used involved measurement of weight loss due to evaporation of water. This method gives accurate result when considered on a comparative basis. Crucibles were washed and dried in an oven at 110 °C for 10 min and cooled in a desiccator. About 20 g of pulverized samples were placed in a hot oven and dried to a constant weight at 105°C for 2 hr. The moisture was calculated using the formular below:

$$\text{Weight of sample (g)} = 0.281$$

$$\text{Weight of dry matter (x)} = 0.234$$

$$\text{Loss in weight (g-x)} = 0.047$$

$$\% \text{ moisture content } \left(\frac{g-x}{g} \times \frac{100}{1} \right) = 16.73$$

2.2.4.2 Determination of Protein Content

Crude protein was determined by the method of the Association of Official Analytical Chemists (AOAC, 1990). Two grams (2.0 g) of the sample was weighed into a digestion flask and 0.5 g of selenium catalyst was added. Twenty-five (25) ml of concentrated H₂SO₄ was added and the flask was shaken to mix the contents. The flask was then placed on a digestion burner for 8 hr and heated until the solution turned green and clear. The sample solution was then transferred into a 100 ml volumetric flask and made up to the mark with distilled water. Twenty-five millilitres (25 ml) of 2 % boric acid was pipetted into a 250 ml conical flask and two drops of mixed indicator (20 ml of bromocresol green and 4 ml of methyl red) solution was added; and into the decomposition chamber of the distillation apparatus was added 15 ml of 40 % NaOH solution. Ten milliliters (10 ml) of the digested sample solution was then introduced into a Kjeldahl flask. The condenser tip of the distillation apparatus was then dipped into the boric acid contained in the conical flask. The ammonia in the sample solution was then distilled into the boric acid until it changed completely to bluish-green. The distillate was then titrated with 0.1 N HCl solutions until it became colourless. The percent total nitrogen and crude protein were calculated using a conversion factor of 6.25.

$$\text{Weight of sample (g)} = 0.504$$

$$\text{Titre value (T)} = 8.1$$

$$\%N = \frac{T \times M \times 0.014 \times DF \times 100}{g} = 1.350$$

$$\% \text{Protein} = 1.350 \times 6.25 = 8.44$$

2.2.4.3 Determination of Crude Fibre Content

In determining the fibre content of the sample by the method of the Association of Official Analytical Chemists (AOAC, 1990), the protein, starch, fat and other digestible carbohydrate was hydrolyzed and separated out of the sample. The residue was then weighed and ashed to determine the organic component. The removal of ash content from the original residue gives the fibre. A known weight of 2.0 g of the sample was weighed into a beaker and 180 ml preheated, 0.13 M H₂SO₄ was added and boiled for 30 min using a water pressure filter system. The moisture was filtered and residue washed 3 times with hot water. The residue was then collected and 150 ml preheated 0.22 M KOH was added and boiled for another 30 minutes. Finally, the mixture was filtered and residue washed on the water pressure system 3 times with acetone. The residue was collected in a crucible, dried at 130 °C for 1 hour and weighed.

$$\text{Weight of sample (g)} = 1.002$$

Weight of dry matter (x) =	0.042
Weight of residue (y)	0.003
% fibre ($\frac{x-y}{g} \times \frac{100}{1}$)	3.89

2.2.4.4 Determination of Crude Fat Content

Crude fat was determined based on the Soxhlet extraction method described by AOAC (1990). A 250 ml quick-fit round bottom flask was washed and dried in an oven at 103 °C for 25 min and allowed to cool to room temperature before it was weighed. Two grams (2.0 g) of the sample was weighed into an extraction thimble (double thickness 22 x 88 mm) and was inserted into the extraction column with the condenser connected. Two hundred milliliters (200 ml) of the extracting solvent (petroleum ether, boiling point 40 to 60°C) was poured into the round bottom flask and fitted into the extraction unit. The flask was then heated with the aid of electro thermal heater at 60 °C for 2 hr. Losses of solvent due to heating were checked with the aid of the condenser so that it cooled and refluxed the evaporated solvent. After extraction, the thimble was removed and the solvent salvaged by distillation. The flask containing the fat and residual solvent was placed on a water bath to evaporate the solvent, followed by a further drying in an oven at 103 °C for 30 minutes to completely evaporate the solvent. It was then cooled in a desiccator and weighed. The fat obtained was expressed as a percentage of the initial weight of the sample.

Weight of sample (g) =	2.000
Weight of fat (x)	0.027
% fat ($\frac{x}{g} \times \frac{100}{1}$) =	1.35

2.2.4.5 Determination of Ash Content

Ash was determined by the method of Association of Official Analytical Chemists (AOAC, 1990). 2.0 g sample was weighed into a previously dried and weighed porcelain crucible. The crucible with its content was placed in a furnace preheated to 600°C for 2 hr. The sample was allowed to cool in the furnace to 250 °C. The crucible and the ash were then transferred into an oven at 100 °C for 30 min cooling. After this period, the crucible with its content was cooled in a desiccator and weighed. The weight of the ash was expressed as a percentage of the initial weight of the sample.

Weight of sample (g) =	0.257
Weight of ash (x)	0.003

$$\% \text{ ash } \left(\frac{x}{g} \times \frac{100}{1} \right) = 1.17$$

2.2.4.6 Determination of Carbohydrate Content

This was determined by the method described by Onyeike *et al.* (1995). This involves finding the difference after adding the % crude protein, moisture, ash, crude fibre and fat content from 100 %. Percentage carbohydrate = 100 - (% moisture + % ash + % crude fat + % crude fibre + crude + % protein).

$$100 - (16.73 + 1.17 + 1.35 + 3.89 + 8.44) \% \\ = 68.42\%.$$

2.2.5 Determination of Mineral Content of *Cola hispida* Leaves Extract

2.2.5.1 Potassium

Potassium was determined as outlined in AOAC International Methods of Analysis (1990). The sample was determined by ashing accurately, weighed 1 g of dried and ground sample into glazed, high-form porcelain crucible for 2hr in a muffle furnace at 500°C. The ashed sample was allowed to cool and 10 drops of deionised water followed by 3 - 4 ml of nitric acid were added to the sample. Excess nitric acid was evaporated by placing the sample on a hot plate set at 100 – 120°C. The sample was returned to furnace and ashed for additional 1hr and after being cooled, the ash was dissolved in 10 ml hydrochloric acid and transferred quantitatively to 50 ml volumetric acid. Potassium is partially ionized in the air-acetylene flame and to suppress ionization, cesium nitrate (1000 µg/ml cesium) was added in all the solutions including the blank. Analysis of potassium was carried out using atomic absorption spectroscopy set at different wavelengths for the optimum working conditions of the mineral. The burner height was manually adjusted on the instrument to ensure maximum absorption.

$$\text{Mineral Weight} = \frac{\text{Absorbance (ppm)} \times \text{dry weight} \times \text{Dilution factor}}{\text{Weight of sample} \times 1000}$$

2.2.5.2 Iron

Iron content of the sample was determined using Association of Analytical Chemists (AOAC) (1990). One gram of each sample was converted to ash at 45° C and digested in 10 ml of 0.1 N HCl, heated for 30 minutes and cooled. The digest was filtered into a 25 ml standard flask and made up to the mark with distilled water. To 5 ml of the above solution duplicated in test tubes, 2 ml of acetate buffer (pH 7.4), 1 ml of hydroquinone and 1 ml of α α'-dipyridyl solution were added. The resultant solution was measured into a 1 cm micro-cuvette and the absorbance was read at 520 nm using a UV BIOCHROM 4049 spectrophotometer. The concentration was read

off using a standard curve prepared with standard Iron solution. The iron content of the sample was calculated using the formula:

$$\text{Mg Fe/100 g sample} = \frac{\text{Concentration} \times \text{dilution factor}}{\text{Weight of sample}}$$

2.2.5.3 Zinc

Zinc content of the sample was determined using Association of Analytical Chemists (AOAC) (1990). The sample was ashed and Zinc was extracted as coloured zinc dithizonate with CCl_4 for absorbance measurement. The solution was extracted immediately before use as follows: Slightly excess of dithizone was added and extraction was carried out with CCl_4 until solvent layer was clear bright green. Excess dithizone was entirely removed by repeated extraction with CHCl_3 , and finally with CCl_4 .

2.2.5.4 Copper

Copper content of the sample was determined using Association of Analytical Chemists (AOAC), (2003). To determine the copper content, 10ml of the digested sample was measured into 50ml of volumetric flask. 10ml of 0.01N NH_4OH was added to the solution and the resultant mixture made up to 50ml using distilled water. It was allowed to develop for 30mins, before absorbance reading was taking with aid of the spectrophotometer at wavelength of 620nm.

2.2.6 Determination of Vitamin Content of *Cola hispida* Leaves Extract

2.2.6.1 Vitamin C Content

The AOAC (2003) method was used. Five gram of sample was weighed into a flat-bottomed flask and 60 ml TCA/Acetic acid solution was added. The mixture was left for an hour before it was filtered. A volume of the filtrate (10 ml) was taken for titration with 0.05% and 2,6-dichlorophenol indophenols added and absorbance was taken at 15 seconds and 30 seconds interval at 520nm. The vitamin C standard was also prepared in the same way at varying concentrations, and a standard curve plotted. $Y = 0.67x + 0.0824$; $R^2 = 0.9714$

The vitamin C content was calculated as follows: $K = \frac{Y \times Z \times DF}{\text{Wt. of sample}}$

Wt. of sample

Where Y is the titre value; Z is the figure gotten when 50mg of the standard vitamin C is divided by its titre value; DF is the dilution factor.

2.2.6.2 Vitamin E Content

The method described by Baker *et al.* (1980) was used to determine the vitamin E content. One gram of the sample was macerated with 20 ml of ethanol and then filtered. A solution of ferric chloride (0.2 %) in ethanol and 1 ml of 0.5 % α - α -dipyridine was added to 1 ml of the filtrate. This was diluted to 5 ml with distilled water. Absorbance was taken at 520 nm. The standard solutions were prepared similarly and the concentration of vitamin E extrapolated from the standard curve.

$$Y = 0.00869 x + 0.0216; R^2 = 0.99$$

2.2.6.3 Vitamin A Content

The method described by Pearson (1976) was used to determine the vitamin A content of the extract. A quantity, 1.0 g of ground sample was macerated with 20 ml of petroleum ether. This was decanted into a test tube and then evaporated to dryness. 0.2 ml of chloroform-acetic anhydride (1:1 v/v) was added to the residue. 2 ml of TCA-chloroform in like (1:1 v/v) was added to the resulting solution and absorbance was measured at 620 nm. Vitamin A standards were prepared in the same way and the absorbance taken at 620 nm. The concentration of vitamin A in the sample was extrapolated from the standard curve.

$$Y = 0.001 x + 0.0018; R^2 = 0.9922$$

2.2.7 Assay for DPPH Radical Scavenging Activity

Procedure

The free radical scavenging capacity of *Cola hispida* leaves ethylacetate fraction was determined using Scavenging activity on DPPH free radicals according to the method by Gyamfi *et al.*, (1999). DPPH (200 μ M) solution was prepared in 95% methanol. From the plant fraction solution, 15.65, 31.25, 62.5, 125, 250, 500 and 1000 μ g/ ml were taken in seven test tubes. 0.5ml of freshly prepared DPPH solution was incubated with test drug and after 10 minutes, absorbance was taken as 517 nm using spectrophotometer. Standard ascorbic acid was used as reference. Calculation % scavenging of the DPPH free radical was measured using the following equation: DPPH Scavenging activity (%) = $\frac{\text{control} - \text{test}}{\text{control}} \times 100$

2.2.8 Inducement of Cardiotoxicity

Cardiotoxicity was induced according to the method described by Agbafor *et al.*, (2015). Briefly, on the twenty-seventh day, two hours after the extract administration to all groups and vitamin E (standard drug) administration to group C, groups B-F were treated with a single

dose of 2.5 ml/kg b.w of DOX intraperitoneally while group A was given distilled water and observed overnight.

2.2.9 Collection of Blood Sample from Experimental Animals

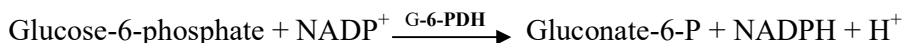
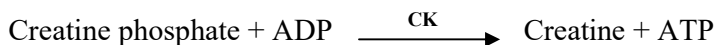
Briefly, blood samples were collected from the animals following an overnight fast through cardiac puncture under mild anesthesia using chloroform. The samples were measured into specimen bottles without anticoagulant. Hearts of the test animals were also quickly excised, perfused with cold normal saline and homogenized in 0.25 M sucrose in phosphate buffer (0.2 M, pH 7.4).

2.2.10 Cardiotoxicity Biomarkers

2.2.10.1 Assay for Creatine Kinase Activity

Creatine kinase (CK) is normally found in striated muscle, brain and heart tissues. The assay of creatine kinase activity in plasma or serum helps to identify the presence damage to tissues such as the heart tissues.

Principle



Procedure

The analysis was carried out using the standard technique described by Stein *et al.* (1985). A volume (2.5 ml) of the substrate was mixed with 0.05 ml of serum in a test tube, and was incubated for one minute at 37 °C. The initial absorbance was read at 0, 1, 2 and 3 minutes, and the CK activity was calculated.

2.2.10.2 Assay for Lactate Dehydrogenase

The enzyme lactate dehydrogenase (LDL) is densely distributed in the heart, liver, muscle and kidney. Elevated levels of LDH are found in serum during myocardial infarction and liver diseases.

Principle

Lactate dehydrogenase catalyses the oxidation of lactate to pyruvate in the presence of NAD^+ , which is subsequently reduced to NADH. The rate of NADH formation measured at 340 nm is directly proportional to serum LDH activity.

Procedure

The method described by Henry (1979) was used to assay for lactate dehydrogenase activity. The reagents for the assay (L-lactate 75mM, NAD^+ 5.5 mM and Buffer 80 mM; pH 9.0 ± 0.1) was pipetted into appropriate tubes and pre-warmed for three (3) minutes at 37°C . The spectrophotometer was zeroed with distilled water at 340 nm and subsequently. 0.25 ml of sample was added to the reagent, mixed and incubated for a minute at 37°C . After a few minutes, the absorbance was read and recorded. The procedure was repeated thrice times, and the average absorbance calculated.

2.2.10.3 Assay for Aspartate Aminotransferase Activity

Aspartate aminotransferase (AST) catalyses the transfer of amino group from aspartate to a keto acid to form a new amino acid and oxaloacetate. AST activity is measured by monitoring the concentration of oxaloacetate hydrazone formed with 2,4-dinitrophenylhydrazine.

Procedure

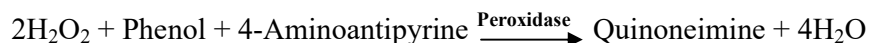
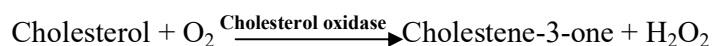
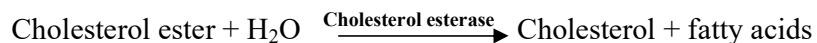
The standard method described by Reitman and Frankel (1957) was used. Sample (0.1 ml) was mixed in a test tube with 0.5 ml Reagent 1 (sodium azide buffer) and incubated for 30 minutes at 37°C . Subsequently, 0.5 ml of Reagent 2 (2, 4-dinitrophenylhydrazine) was added, mixed and allowed to stand for 20 minutes at $20-25^\circ\text{C}$. Sodium hydroxide (5 ml) was added and the absorbance of the sample was read against the blank at 546 nm.

2.2.11 Biochemical Studies

2.2.11.1 Determination of Cholesterol Content

Cholesterol was determined after enzymatic hydrolysis and oxidation. The indicator quinoneimine is formed from hydrogen peroxide and 4-aminoantipyrine in the presence of phenol and peroxidase. The method described by Abell *et al.* (1952) was used for the quantification of cholesterol.

Principle



$$\text{Conc. of cholesterol in sample} = \frac{\Delta A_{\text{sample}} \times \text{conc. of standard}}{\Delta A_{\text{standard}}}$$

Where ΔA_{sample} = change in sample absorbance $\Delta A_{\text{standard}}$ = change in the absorbance of standard.

Test Procedure

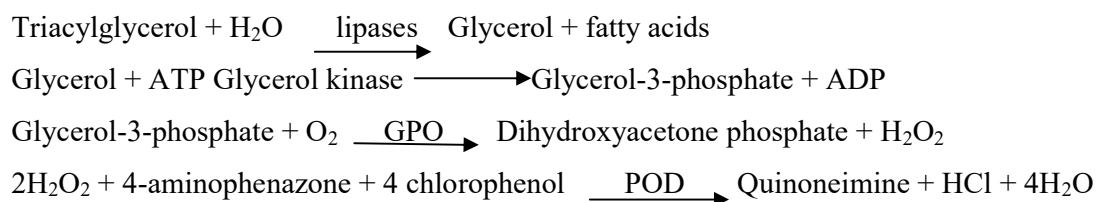
Three (3) test tubes were set up in a test tube rack and labeled blank, standard and sample respectively. To the blank test tube was added (10 µl) distilled H₂O while 10 µl of standard solution was added to the standard test tube and 10 µl of sample (serum) to the sample test tube. To each of these test tubes was added 1000 µl of the cholesterol reagent. Each was thoroughly mixed and incubated for 10 minutes at room temperature (20-25 °C). The absorbance of the sample (A sample) against the blank was taken within 60 minutes at 500 nm.

2.2.11.2 Determination of Triacylglycerol (TAG) Content

The method described by Abell *et al.* (1952) was used for the quantification of triacylglycerol.

Principle

The triacylglycerol was determined after enzymatic hydrolysis with lipases. The indicator is a quinoneimine formed from hydrogen peroxide, 4-aminophenazone and 4-chlorophenol under the catalytic influence of peroxidase.



Procedure: A quantity of the sample (0.1 ml) was pipetted into a clean labeled tube and 1.0 ml of trichloroacetic acid (TCA) was added to it, mixed and then centrifuged at 250 rpm for 10 minutes. The supernatant was decanted and reserved for use. The assay procedure was carried out as shown:

S/N		Blank	Standard	Sample
1.	Distilled water	0.5	-	-
2.	Standard Solution	-	0.5	-
3.	TCA (ml)	0.5	0.5	-
4.	Supernatant (ml)	-	-	1.0
5.	Reagent mixture (ml)	1.0	1.0	1.0

The mixtures were allowed to stand for 20 minutes at 25 °C and the absorbance of the sample and standards read against the blank at 540 nm.

Calculation: The concentration of triacylglycerol in serum was calculated as follows:

$$\text{TAG (mmol/l)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times \text{Standard concentration}$$

2.2.11.3 Determination of Low Density Lipoprotein (LDL) Content

Principle

LDL-Cholesterol can be determined as the difference between total cholesterol and the cholesterol content of the supernatant after precipitation of the LDL fraction by polyvinyl sulphate (PVS) in the presence of polyethylene glycol monomethyl ether.

Procedure

The concentration of the serum low density lipoprotein as described by Kameswara *et al.* (1999) was used. The serum samples were kept at 2-8 °C. The precipitant solution (0.1 ml) was added to 0.2 ml of the serum sample and mixed thoroughly and allowed to stand for 15 minutes. The mixture was centrifuged at 2,000 rpm for 15 min. The cholesterol concentration in the supernatant was determined.

Calculation: LDL-C (mmol/L) = TC (mmol/L) – 1.5 x Supernatant Cholesterol (mmol/L).

2.2.11.4 Determination of High Density Lipoprotein (HDL) Content

Principle

The concentration of the serum high density lipoprotein as described by Kameswara *et al.* (1999) was used. Low density lipoproteins (LDL and VLDL) and chylomicron fractions were precipitated quantitatively by the addition of phosphotungstic acid in the presence of magnesium ions. After centrifugation, the cholesterol concentration in the HDL (high density lipoprotein) fraction, which remained in the supernatant, was determined.

Procedure

About 1.0 ml of the precipitant solution was added to 0.3 ml of the serum sample and mixed thoroughly and allowed to stand for 15 minutes. The mixture was centrifuged at 2,000 rpm for 15 minutes. The cholesterol concentration in the supernatant was determined using the formula:

$$\text{Concentration of HDL-Cholesterol in sample} = \frac{\Delta A \text{ sample}}{\Delta A \text{ standard}} \times \text{Conc. of standard (mg/dL)}$$

Where ΔA sample = change in absorbance of the sample; ΔA standard = absorbance of standard. Atherogenic index (AI) = Log (TAG/HDL) .

2.2.12 Antioxidant Studies

Heart tissues were homogenized in KCl (10 mM) phosphate buffer (1.15 %) with ethylene diamine tetra acetic acid (EDTA, pH 7.4) and centrifuged at 12000 rpm for 20 minutes. Aliquot was used for biochemical estimation.

2.2.12.1 Assay for Catalase Activity.

This was done according to the method of Aebi (1983).

Principle

The ultra violet absorption of hydrogen peroxide can be easily measured at 240 nm. On the decomposition of hydrogen peroxide (H_2O_2) by catalase the absorption decreases with time and this decrease is a measure of catalase activity.

Procedure

Two milliliters of hydrogen peroxide and 2.5 ml of phosphate buffer were added to a beaker. The mixture was mixed with 0.5 ml of the sample. One milliliter (1 ml) portion of the reaction mixture was added to 2 ml of dichromate acetic acid reagent. The absorbance was read at 540 nm (at a minute interval in triplicates). Catalase activity was calculated using the following equation:

$$\text{Catalytic concentration (unit/l)} = \frac{\log (\text{abs 1/abs 2}) \times 0.23}{0.00693}$$

2.2.12.2 Assay for Superoxide Dismutase (SOD) Activity

Superoxide dismutase (SOD) activity was assayed as formerly described by Fridovich (1989).

Principle

The ability of superoxide dismutase to inhibit the autoxidation of adrenaline was the basis of the superoxide dismutase (SOD) assay. Superoxide generated by the xanthine oxidase reaction is known to cause the oxidation of adrenaline to adrenochrome. The yield of adrenochrome produced per superoxide introduced increased with increasing pH and also with increasing concentration of adrenaline. These led to the proposal that autoxidation of adrenaline proceeds by at least two distinct pathways; one of which is a free radical chain reaction involving superoxide radical and hence could be inhibited by superoxide dismutase (SOD).

Procedure

The post mitochondrial fractions were properly diluted. Each diluted sample (0.2 ml) was added to 2.5 ml of 0.05 M phosphate buffer, pH 7.8. The mixture was equilibrated in the spectrophotometer before adding adrenaline solution. The reaction started with the addition of 0.3 ml of freshly prepared adrenaline solution to the mixture followed by quick mixing by inversion in the cuvette.

Manual Calculation:

The increase in absorbance was taken at 480 nm for 150 seconds at 30 seconds intervals.

$$\text{Therefore, increased in absorbance per minute} = \left[\frac{Aa - A}{2.5} \right]$$

Where A_0 = absorbance after 30 seconds

A_a = absorbance after 150 seconds

$$\% \text{ Inhibition} = 100 - \frac{\text{increased in absorbance for substrate}}{\text{increased in absorbance for blank}} \times 100$$

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

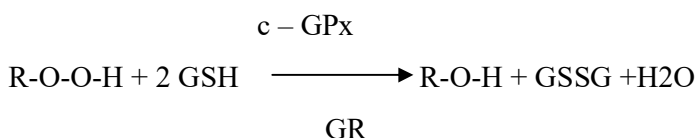
NOTE: A blank was prepared with 0.3 ml of adrenaline in 2.5 ml of buffer.

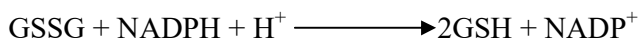
2.2.12.3 Assay for Glutathione Peroxidase Activity

Glutathione Peroxidase (GPx) enzymes belong to a family of seleno proteins whose function is to catalyze the reduction of various peroxides. Four types of GPx (GPx-1 to 4) have been identified in mammalian species. GPx-1 is the classic intracellular form while GPx-2 to 4 are gastrointestinal, plasma and phospholipid hydro peroxide metabolizing forms respectively.

Principle

This assay is an adaptation of the method of Paglia and Valentine (1967). Glutathione peroxidase (GPX) catalyzes the reduction of an organic peroxide (ROOH), oxidizing reduced glutathione (GSH) to form disulfide glutathione (GSSG). The oxidized glutathione is then reduced by glutathione reductase (GR) and b-nicotinamide adenine dinucleotide phosphate (NADPH) forming NADP^+ (resulting in decreased absorbance at 340 nm) and recycling the GSH. Because glutathione peroxidase (GPx) is limiting, the decrease in absorbance at 340 nm is directly proportional to the glutathione (GPx) concentration.





Procedure

The sample tissue homogenate was added to a solution containing glutathione, glutathione reductase, and NADPH. The enzyme reaction was initiated by adding the substrate, hydrogen peroxide and the A340 and recorded. The rate of decrease in the A340 is directly proportional to the GPx activity in the sample.

$$\text{Enzyme Activity} = \frac{\text{A340} / \text{min}}{0.00622} \times 121 \times \text{dil. Factor} \longrightarrow \text{(mU / mL)}$$

2.2.12.4 Determination of Malondialdehyde (MDA) Concentration

Lipid peroxidation was determined spectrophotometrically by measuring the level of the lipid peroxidation product, malondialdehyde (MDA) as described by (Wallin *et al.*, 1993).

Principle

Malondialdehyde (MDA) reacts with thiobarbituric acid to form a red or pink coloured complex which in acid solution, absorbs maximally at 532 nm.



Procedure

To a test tube, 0.1 ml of sample and 0.9 ml of distilled water were added, together with 0.5 ml of 25 % TCA + 0.5 ml of 1% TBA in 0.3 % NaOH. The test tube was incubated at 95°C for 40 minutes and cooled in water, and 0.1 ml of 20 % SDS (sodium dodecyl sulphate) was added.

Manual Calculation: The absorbance at 532 and 600 nm was determined against a blank.

$$\text{Therefore \% TBARS} = \left[\frac{A_{532} - A_{600} \times 100}{(0.5271 \times 0.1) \times 1} \right]$$

Slope from standard curve = y = 0.5208x (mg/ml).

2.2.13 Histological Examination

2.2.13.1 Tissue Preparation

The histopathological evaluation was carried out using the methods described by Drury *et al.* (1967). Tissue sections of the heart from all the experimental groups were collected for histopathological examination. The tissue samples were fixed in 10 % phosphate buffered formalin for a minimum of 48 hours. The tissues were subsequently trimmed, dehydrated in 4 grades of alcohol (70 %, 80 %, 90 % and absolute alcohol), cleared in 3 grades of xylene and embedded in molten wax. On solidifying, the blocks were sectioned, 5 µm thick with a rotary

microtome, floated in water bath and incubated at 60 °C for 30 minutes. The 5 µm thick sectioned tissues were subsequently cleared in 3 grades of xylene and rehydrated in 3 grades of alcohol (90 %, 80 % and 70 %). The sections were then stained with hematoxylin for 15 minutes. Blueing was done with ammonium chloride. Differentiation was done with 1 % acid alcohol before counterstaining with eosin. Permanent mounts were made on degreased glass slides using a mountant; DPX.

2.2.13.2 Slide Examination

The prepared slides were examined with a Motic™ compound light microscope using x4, x10 and x40 objective lenses. The photomicrographs were taken using a Motic™ 9.0 megapixels microscope camera at x400 magnification.

2.2.14 Statistical Analysis

The statistical analysis was carried out using Statistical Product and Service Solution (SPSS 15.0) version. Statistical differences were evaluated using a one way analysis of variance (ANOVA), followed by Duncan's Multiple Range Test to detect significant differences among the ($p > 0.05$) values of the different groups.

CHAPTER THREE

RESULTS

3.1. Preliminary Studies

3.1.1 Percentage Yield of Methanol Extract of *Cola hispida* Leaves

The extraction of 750g of *Cola hispida* Leaves with methanol gave a methanol extract yield of 89 g which represented 11.9% of the starting plant material.

3.1.2 Qualitative Composition of Methanol Extract of *Cola hispida* Leaves

The qualitative analysis carried out on *Cola hispida* leaves revealed the presence of terpenoids saponins, flavonoids, alkaloids, tannins and glycosides as shown in Table 1.

3.1.3 Quantitative Phytochemical Composition of the Methanol Extract

The amount of phytochemicals found in the leaves extract was quantitatively determined by standard procedures, and is presented in Table 2. However, the analysis revealed that the phytochemical content of *Cola hispida* leaves were in the following order: high levels of terpenoids (1.20 ± 0.21 mg/g), saponins (1.09 ± 0.20 mg/g), flavonoids (0.99 ± 0.50 mg/g), alkaloids (0.87 ± 0.20 mg/g), tannin (0.85 ± 0.13 mg/g) and Glycosides (0.37 ± 0.16 mg/g).

3.1.4 Proximate composition of *Cola hispida* Leaves Extract

The proximate compositions of *Cola hispida* leaves extract are presented in Table 3. The result of the proximate contents revealed the following: Carbohydrate content ($68.45 \pm 0.02\%$), Moisture content ($16.71 \pm 0.06\%$) > crude protein ($8.44 \pm 0.03\%$) > crude fiber ($3.87 \pm 0.21\%$) > crude fats ($1.35 \pm 0.02\%$) > ash content ($1.18 \pm 0.02\%$).

Table 1: Qualitative Phytoconstituents of *Cola hispida* Leaves Methanol Extract

Phytochemical Constituents	Leaves Extract
Terpenoids	+++
Saponins	+++
Flavonoids	++
Alkaloids	++
Tanins	++
Glycosides	+

Key:

+++ = Highly present.

+ + = Moderately present.

+ = Slightly present

Table 2: Quantitative Phytoconstituents of *Cola hispida* Leaves Methanol Extract

Phytochemical Constituents	Leaves Extract (mg/100g)
Terpenoids	1.20± 0.21
Saponins	1.09± 0.20
Flavonoids	0.99± 0.50
Alkaloids	0.87± 0.20
Tannins	0.85±0.13
Glycosides	0.37±0.16

Values are Mean±SD, (n= 3).

Table 3: Proximate Composition of *Cola hispida* Leaves Methanol Extract

PARAMETERS	QUANTITY (%)
Carbohydrate content	68.45 $\pm$ 0.02
Moisture content	16.71 $\pm$ 0.06
Crude protein	8.44 $\pm$ 0.03
Crude fibre	3.87 $\pm$ 0.21
Crude Fats	1.35 $\pm$ 0.02
Ash content	1.18 $\pm$ 0.02

Values are Mean $\pm$ SD, (n= 3).

3.1.5 Mineral Composition of *Cola hispida* Leaves Extract

The concentrations of selected mineral elements/contents of *Cola hispida* leaves methanol extract are presented in Table 4. The results showed the presence of potassium iron, zinc and copper.

3.1.6 Vitamin Content of *Cola hispida* Leaves Extract

The vitamin content of the leaves extract was presented in Table 5. However, the analysis revealed that the vitamin content of *Cola hispida* leaves extract were in the following order: vitamin C ($26.10 \pm 0.03 \mu\text{g/g}$), vitamin E ($3.87 \pm 0.15 \mu\text{g/g}$) and vitamin A ($0.08 \pm 0.01 \text{IU}$).

3.2 Biochemical Studies

3.2.1 Acute Toxicity Profile of Ethyl-acetate Fraction of *Cola hispida* Leaves.

Table 6 shows the acute toxicity profile of ethyl-acetate fraction of *Cola hispida* leaves. The acute toxicity study showed that there was no mortality in all the groups ranging from 10, 100, 1000, 1600, 2900 and 5000 mg/kg b.w that received the *Cola hispida* leaves ethyl-acetate fraction.

3.2.2 Effect of Ethyl-acetate Fraction of *Cola hispida* Leaves on DPPH Radical Scavenging Activity

The ability of the fraction to scavenge DPPH radicals was investigated at various concentrations of the fraction. As shown in Table 7, the fraction possessed substantial dose-dependent antioxidant activity and the activity was compared to that of ascorbic acid, which was used as a control antioxidant. The percentage inhibition of ethyl-acetate fraction of *Cola hispida* leaves was concentration dependent with (IC_{50}) of 3.65 ± 0.25 compared to standard (Ascorbic acid) with IC_{50} of 3.21 ± 0.23 which implies that the standard was more effective in scavenging/inhibiting the DPPH radical than the fraction. The IC_{50} value represents the concentration of the fraction or standard that caused 50% inhibition of the radical.

Table 4: Mineral Composition of *Cola hispida* Leaves Methanol Extract

MINERAL CONSTITUENT	QUANTITY
Potassium (mmol/L)	0.85± 0.01
Iron (mg/100g)	0.49± 0.00
Zinc (mg/100g)	0.14 ± 0.00
Copper (mg/100g)	0.03± 0.02

Values are Mean±SD, (n= 3).

Table 5: Vitamin Contents of *Cola hispida* Leaves Methanol Extract

VITAMINS	QUANTITY
Vitamin C (mg/g)	26.10± 0.03
Vitamin E (µg/g)	3.87 ± 0.15
Vitamin A (IU)	0.08 ± 0.01

Values are Mean±SD, (n= 3).

Table 6: Acute Toxicity Profile of *Cola hispida* Leaves Ethyl-acetate Fraction.

PHASE 1	DOSAGE (mg/kg b.w)	MORTALITY
GROUP 1	10	0/3
GROUP 2	100	0/3
GROUP 3	1000	0/3
PHASE 2		
GROUP 1	1600	0/3
GROUP 2	2900	0/3
GROUP 3	5000	0/3

n= 3.

Table 7: Effect of Ethyl-acetate Fraction of *Cola hispida* Leaves on DPPH Radical Scavenging Activity

Concentration (µg/ml)	% Inhibition	% Inhibition STD
15.63	71.81 ± 1.16	62.89±1.14
31.25	74.76 ± 0.52	68.25±1.58
62.5	84.27 ±0.32	75.85±0.98
125	82.37 ± 0.86	86.01±1.07
250	74.68 ± 1.42	86.86±0.84
500	68.05 ±1.32	89.68±0.77
1000	43.25 ±1.38	93.26±0.58
IC ₅₀ (µg/ml)	3.67 ±0.25	3.21±0.23

Each value is expressed as mean ± standard deviation (n=3). IC₅₀ of the fraction (µg/ml) is 3.67±0.25 as compared to that of the standard, IC₅₀ of 3.21±0.23.

3.2.3 Effect of Ethyl-acetate Fraction of *Cola hispida* Leaves on Cardiac Biomarkers of Albino Rats.

Table 8 summarizes the serum level of cardiac biomarker enzymes in rats. Group that received DOX only showed significant ($p < 0.05$) increases in the serum level of creatine kinase (CK), lactate dehydrogenase (LDH) and aspartate aminotransferase (AST) when compared with the normal control group.

Administration of the graded doses of the fraction significantly decreased the concentration of these biomarker enzymes to near normal as the doses increased from 100 – 400 mg/kg b.w, ameliorating the toxic effect of Dox as compared to the rats treated with Dox only. In addition, significant ($p < 0.05$) decreases in the concentration of the enzymes were also observed in the group that received the standard drug (Vit E).

At the highest dose of 400 mg/kg b.w, *Cola hispida* leaves fraction significantly ($p < 0.05$) suppressed the toxic effect of Dox on (LDH and AST) more effectively than the standard drug.

Table 8: Effect of *Cola hispida* Ethyl-acetate Fraction on Cardiac Biomarkers of Albino Rats

GROUPS	CK (U/L)	LDH (U/L)	AST (IU/L)
Normal Control	6.87 ± 0.70 ^b	15.72 ± 1.71 ^b	105.09 ± 1.98 ^b
Dox only	9.36 ± 0.83 ^c	26.31 ± 1.17 ^d	117.30 ± 3.55 ^c
100mg/kg b.w vit E + Dox	5.01 ± 0.52 ^a	20.22 ± 2.09 ^c	108.90 ± 5.52 ^b
100 mg/kg b.w of <i>Cola hispida</i>	7.51 ± 0.88 ^b	17.83 ± 2.31 ^b	105.97 ± 3.76 ^b
200 mg/kg b.w of <i>Cola hispida</i>	7.47 ± 1.17 ^b	16.25 ± 2.07 ^b	105.8 ± 3.37 ^b
400mg/kg b. w of <i>Cola hispida</i>	7.39 ± 1.20 ^b	12.96 ± 1.13 ^a	94.25 ± 6.42 ^a

Values are expressed as mean±SD, n=4. Values in the same column having different superscripts differ significantly (P < 0.05). CK = Creatine kinase; LDH = Lactate dehydrogenase; AST = Aspartate aminotransferase.

Group A: received distilled water (Normal control).

Group B: received only 20 mg/kg b.w DOX without treatment (Positive control).

Group C: received 100 mg/kg b.w vitamin E + 20 mg /kg b.w DOX (Standard control).

Group D: 100 mg/kg b.w of ethyl acetate fraction + 20 mg/kg b.w DOX.

Group E: 200 mg/kg b.w of ethyl acetate fraction + 20 mg /kg b.w DOX.

Group F: 400 mg/kg b.w of ethyl acetate fraction + 20 mg /kg b.w DOX.

3.2.4 Effect of Ethyl-acetate Fraction of *Cola hispida* Leaves on Serum Lipid Profile of Albino Rats.

Table 9 shows the serum lipid profile of the test animals. Intoxication with DOX caused an increase in serum lipid parameters amongst the group that received DOX only from 8.63 ± 0.50 mmol (TC), 1.66 ± 0.37 mmol (TAG), 6.03 ± 0.54 mmol (LDL-C) in the normal group to 9.35 ± 1.08 mmol (TC), 2.16 ± 0.25 mmol (TAG) and 7.08 ± 0.61 mmol (LDL-C) respectively. However, the serum level of HDL-C decreased from 1.90 ± 0.19 mmol in the normal control to 1.56 ± 0.11 mmol in the group that received DOX only.

Relative to the group that received DOX only, pretreatment with *Cola hispida* leaves ethyl-acetate fraction significantly ($p < 0.05$) reversed and ameliorated the effect of DOX intoxication towards normal; thus, significantly ($p < 0.05$) decreasing the serum lipid concentrations of total cholesterol (TC), triacylglycerol (TAG), and low density lipoprotein (LDL-C) while high density lipoprotein (HDL-C) increased. The effect of *Cola hispida* leaves fraction was comparable with that of the standard drug, vitamin E.

Table 9: Effect of Ethyl-acetate Fraction of *Cola hispida* Leaves on Serum Lipid Profile of

GROUPS	TC (mmol/L)	TAG (mmol/L)	LDL (mmol/L)	HDL (mmol/L)	AI
Normal Control	8.63 ± 0.50 ^c	1.66 ± 0.37 ^a	6.03 ± 0.54 ^{bc}	1.90 ± 0.19 ^a	0.01
Dox only	9.35 ± 1.08 ^c	2.16 ± 0.25 ^b	7.08 ± 0.61 ^c	1.56 ± 0.11 ^c	0.14
100mg/kg b.w vit E + Dox	5.98 ± 0.71 ^b	1.76 ± 0.15 ^a	3.80 ± 0.66 ^a	1.72 ± 0.16 ^{abc}	-0.01
100mg/kg b.w of <i>Cola hispida</i>	6.07 ± 0.67 ^b	1.70 ± 0.16 ^a	4.08 ± 0.54 ^a	1.66 ± 0.17 ^a	0.01
200mg/kg b.w of <i>Cola hispida</i>	5.32 ± 0.38 ^{ab}	1.72 ± 0.02 ^a	4.03 ± 0.76 ^a	1.78 ± 0.18 ^c	-0.01
400mg/kg b.w of <i>Cola hispida</i>	4.72 ± 0.58 ^a	1.59 ± 0.23 ^a	3.48 ± 0.45 ^a	1.83 ± 0.13 ^b	-0.06

Values are expressed as mean±SD, n=4. Values in the same column having different superscripts differ significantly (P< 0.05). TAG = Triacylglycerol; TC = Total cholesterol; LDL = Low density lipoprotein; HDL = High density lipoprotein; Atherogenic index (AI) = Log (TAG/HDL).

Group A: received distilled water (Normal control).

Group B: received only 20 mg/kg b.w DOX without treatment (Positive control).

Group C: received 100 mg/kg b.w vitamin E + 20 mg /kg b.w DOX (Standard control).

Group D: 100 mg/kg b.w of ethyl acetate fraction + 20 mg/kg b.w DOX.

Group E: 200 mg/kg b.w of ethyl acetate fraction + 20 mg /kg b.w DOX.

Group F: 400 mg/kg b.w of ethyl acetate fraction + 20 mg /kg b.w DOX.

3.2.5 Effect of Ethyl-acetate Fraction of *Cola hispida* Leaves on Cardiac Enzyme Antioxidant Activity

The results of the effect of different treatments on catalase, superoxide dismutase (SOD) and glutathione reductase (GPx) activities are shown in Table 10. The activity of catalase in the heart significantly ($p < 0.05$) varied after DOX treatment, decreasing from 3.71 ± 0.19 U/L in the baseline (Normal) group to 3.09 ± 1.13 U/L in the group that received Dox only.

Furthermore, the activities of SOD and GPx also reduced significantly ($p < 0.05$) from 11.38 ± 0.03 and 227.48 ± 21.67 to 10.87 ± 0.54 and 174.90 ± 16.98 U/L respectively. Table 9 also showed that pretreatment of the animals with different doses of the leaves fraction for (27) days, significantly ($p < 0.05$) inhibited the effect of the DOX on the cardiac enzymatic antioxidants in a dose dependent manner. The activities of all the tested cardiac enzymatic antioxidants were significantly increased by the leaves fraction at the different doses. This increase in the activities of CAT, SOD and GPx observed were higher (though not significant) in rats administered with 400 mg/kg b.w fraction compared to that recorded for vitamin E.

Table 10: Effect of Ethyl-acetate Fraction of *Cola hispida* Leaves on Cardiac Enzyme Antioxidant Activity

GROUPS	CAT (U/mg)	SOD (U/mg)	GPx (U/L)
Normal Control	3.71 ± 0.19 ^{ab}	11.38 ± 0.03 ^b	227.48 ± 21.67 ^a
Dox only	3.09 ± 1.13 ^a	10.87 ± 0.54 ^a	174.90 ± 16.98 ^c
100mg/kg b.w vit E + Dox	4.57 ± 0.34 ^c	11.39 ± 0.04 ^b	195.75 ± 10.41 ^b
100mg/kg b.w of <i>Cola hispida</i>	3.70 ± 0.59 ^{ab}	11.36 ± 0.16 ^b	174.95 ± 3.93 ^a
200mg/kg b.w of <i>Cola hispida</i>	4.39 ± 0.43 ^{bc}	11.39 ± 0.05 ^b	181.57 ± 7.01 ^{ab}
400mg/kg b.w of <i>Cola hispida</i>	4.83 ± 0.19 ^c	11.40 ± 0.18 ^b	196.67 ± 6.12 ^b

Values are expressed as mean±SD, n=4. Values in the same column having different superscripts differ significantly (P<0.05). CAT = Catalase; SOD = Superoxide dismutase; GPx = Glutathione peroxidase.

Group A: received distilled water (Normal control).

Group B: received only 20 mg/kg b.w DOX without treatment (Positive control).

Group C: received 100 mg/kg b.w vitamin E + 20 mg /kg b.w DOX (Standard control).

Group D: 100 mg/kg b.w of ethyl acetate fraction + 20 mg/kg b.w DOX.

Group E: 200 mg/kg b.w of ethyl acetate fraction + 20 mg /kg b.w DOX.

Group F: 400 mg/kg b.w of ethyl acetate fraction + 20 mg /kg b.w DOX.

3.2.6 Effect of Ethyl-acetate Fraction of *Cola hispida* Leaves on Lipid Peroxidation

Fig. 3 shows that treatment of animals with DOX induced an elevated amount ($p < 0.05$) of malondialdehyde (MDA) from 2.35 mg/ml in the normal control (baseline) to 3.89 mg/ml in the group intoxicated with Dox only.

Administration of the plant fraction significantly ($p < 0.05$) inhibited the generation of MDA in a dose dependent manner. The highest tested dose of Ethyl-acetate fraction of *Cola hispida* leaves (400 mg/kg b.w) inhibited the generation of a stable product of lipid peroxidation (MDA) from 2.35 ± 0.32 mg/ml in the normal control to 2.62 ± 1.10 mg/ml. Also, the 400 mg/kg b.w fraction showed a significant ($p < 0.05$) inhibition of lipid peroxidation compared to that recorded for Vit E (2.85 ± 0.51 mg/ml).

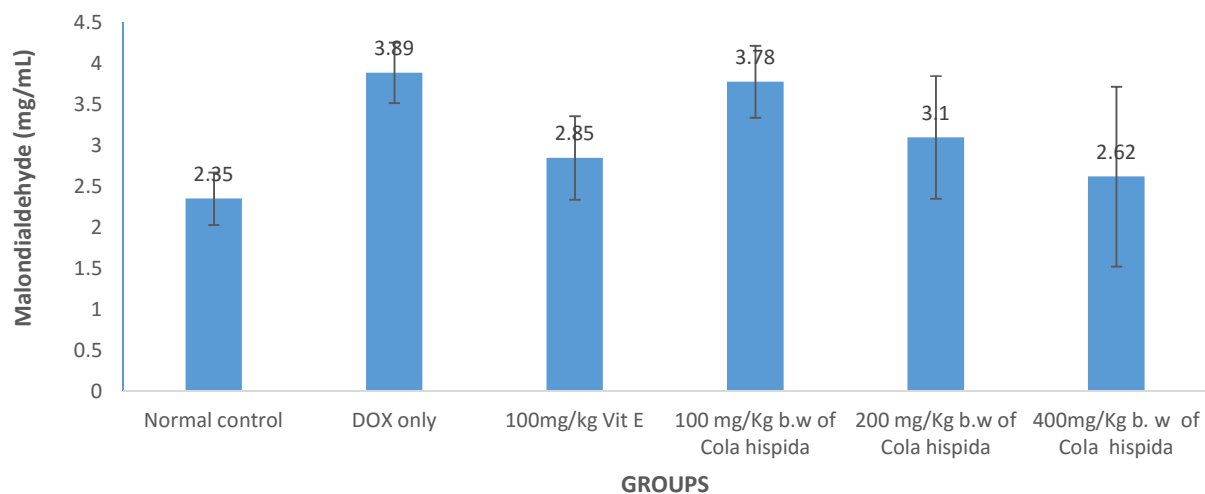


Fig.3: Effect of Ethyl-acetate Fraction of *Cola hispida* Leaves on Malondialdehyde Levels

3.3 Histological Examination of Heart Tissue

The micrograph of the heart tissue was characterized by normal striations of the cardiac muscle fibre consisted of adequate regular branching / anastomosing pattern as shown in plate A. The blood vessels observed consisted of three major tunics (intima, media and adventitia). The cardiac muscles are joined end to end by specialized junction region called the intercalated disc which provides anchorage from myofibrils and allow rapid spread of contractile stimuli between cells. Haemorrhagic conditions associated with rupture of blood vessels were observed in plate B also called hyperemic condition. It also has nucleus located at the mid tissues region. However, longitudinal muscle fibre of cardiac muscle with the presence of mixed cellular infiltrates, was observed in plate C. Minor degeneration of the cardiac muscle evident by loss of myofibrillar slender threads of a muscle fibre was observed in plate D. Plate E showed myxomatous degeneration characterized by thickened ventricular surface of the mitral valves while inflammatory lesions not associated with cardiomyopathy but composed of more mononuclear cells is observed in (plate F).

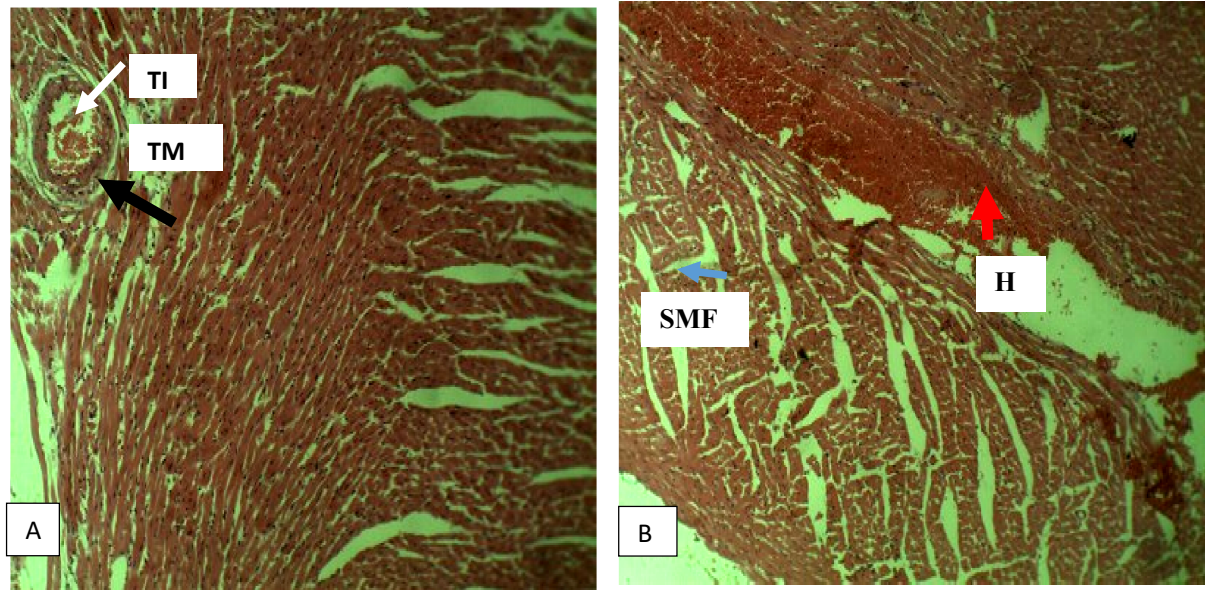


Plate A (Normal control): Photomicrograph of heart showed (1). Passive occlusion (OCC) of the blood vessels (red arrow) (artery). The three tunics (intima, media and adventitia) were observed in the artery which consists of tunica intima (TI) (blue arrow) and media (TM) (white arrow) (2). The cardiac muscle joined end to end by specialized junction's region.

Plate B (Positive control): Photomicrograph of heart showed normal striations of the muscle fibre (SMF) (red arrow). Severe hyperemic condition was observed. (star). H&E. mag. 100X.

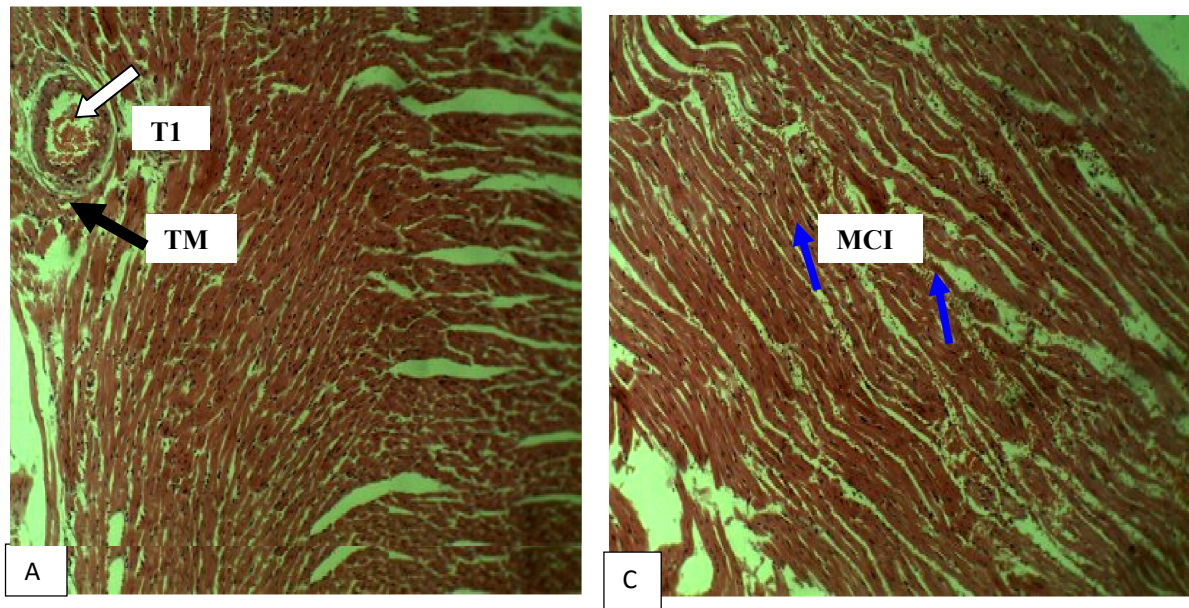


Plate A(Normal control): Photomicrograph of heart showed (1). Passive occlusion (OCC) of the blood vessels (red arrow) (artery). The three tunics (intima, media and adventitia) were observed in the artery which consists of tunica intima (TI) (blue arrow) and media (TM) (white arrow) (2).

Plate C (Standard control): Photomicrograph of heart showed longitudinal muscle fibre of cardiac muscle with presence of mixed cellular infiltrates (MCI) (blue arrow) H&E. mag. 100X.

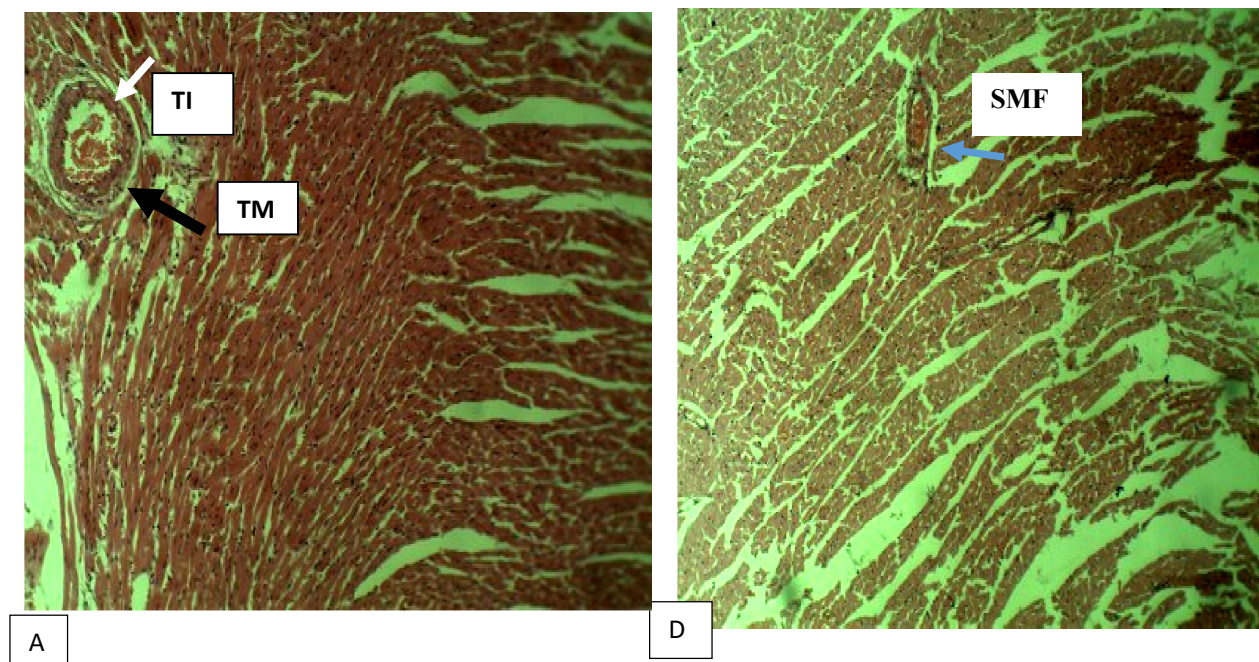


Plate A (Normal control): Photomicrograph of heart showed (1). Passive occlusion (OCC) of the blood vessels (red arrow) (artery). The three tunics (intima, media and adventitia) were observed in the artery which consists of tunica intima (TI) (blue arrow) and media (TM) (white arrow) (2).

Plate D (100mg/kg b.w Treated Group): Photomicrograph of heart showed minor degeneration of the cardiac muscle evident by loss of myofibrils, slender threads of a muscle fibre (STMF) (black arrow) H&E. mag. 100X.

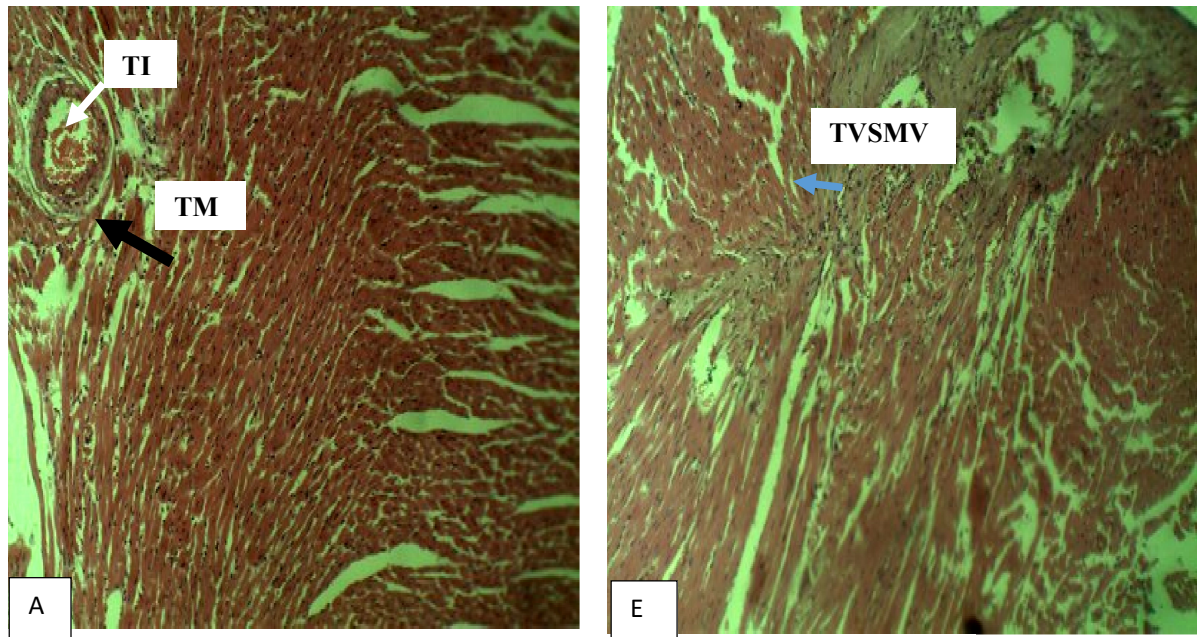


Plate A (Normal control): Photomicrograph of heart showed (1). Passive occlusion (OCC) of the blood vessels (red arrow) (artery). The three tunics (intima, media and adventitia) were observed in the artery which consists of tunica intima (TI) (blue arrow) and media (TM) (white arrow) (2).

Plate E (200mg/kg b.w Treated Group): Photomicrograph of heart showed a myxomatous degeneration accompanied by focal thickened ventricular surface of the mitral valves (TVSMV) (black arrow) H&E. mag. 100X.

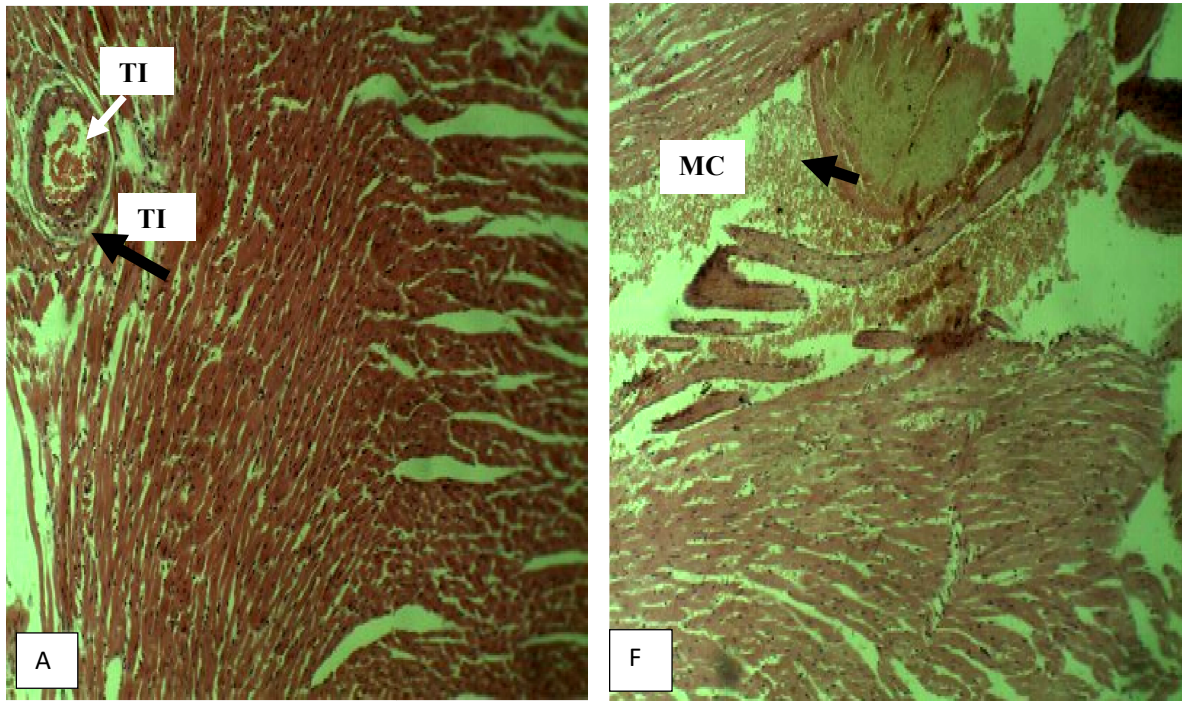


Plate A (Normal control): Photomicrograph of heart showed (1). Passive occlusion (OCC) of the blood vessels (red arrow) (artery). The three tunics (intima, media and adventitia) were observed in the artery which consists of tunica intima (TI) (blue arrow) and media (TM) (white arrow) (2).

Plate F (400mg/kg b.w Treated Group): Photomicrograph of heart showed inflammatory lesions not associated with cardiomyopathy but composed of more homogenous population of mononuclear cells (MC) (black arrow) H&E. mag. 100X.

CHAPTER FOUR

DISCUSSION

This study investigates the cardio-protective effect of ethyl acetate fraction of *Cola hispida* leaves on doxorubicin-induced cardiotoxicity in wistar albino rats.

The phytochemicals constituents of *cola hispida* leaves are non-nutritive plant chemicals that have protective or diseases preventive properties. Qualitative analysis showed that the leaves of *Cola hispida* contains bioactive components; it showed high concentration of terpenoids and saponins while flavonoids, alkaloids and tannins are in smaller concentration when compared with terpenoids and saponins while cardiac glycosides are in minute quantity in *Cola hispida* extract as shown in Tables 1 and 2 hence gives credence to the report of (Akintehinse *et al.*, 2016) on the phytochemical composition of *Cola acuminata* leaves. The presence of this phytochemicals proves that the leaves of *Cola hispida* can be used in the treatment of various diseases. They possess free scavenging potentials, also saponins are hypoglycemic, antifungal and antihyperlipideamic agents in animals. The scavenging of free radical by various constituents of plant may be due to phenolic acids and flavonoids which play a protective role against oxidative damage (Boumerfeg *et al.*, 2009). Terpenoids, another large family of plant secondary metabolites extracted from aromatic plants antioxidant activity act as regulators of metabolism and play a protective role as antioxidants (Cinzia *et al.*, 2019) the same as tannins. Iron and zinc serve as cofactors required for synthesis and proper functioning of catalase (CAT) and superoxide dismutase (SOD) (Ying *et al.*, 2018).

Proximate analysis of the leaves extract of *Cola hispida* showed some disparity with some earlier research on the leaves of *Cola acuminata* (Dewole *et al.*, 2013). The proximate analysis revealed a higher moisture content (16.71 ± 0.05) and carbohydrate content (68.45 ± 0.02) when compared with the results obtained from Dewole *et al.* (2013) and Akintehinse *et al.* (2016) while the values obtained by Dewole *et al.* (2013) and Akintehinse *et al.* (2016) for protein (19.14 %), crude fibre (7.30 %), fat (3.03 %) and ash (2.27 %) were higher. The proximate composition of kola nut differs relatively from what has been reported by Arogba (1999) that dried kola nut had 69% carbohydrate, 18% crude fat and 3.1% ash dried weight. The varying composition as reported by various workers may be due to different environment where the plants were planted, seasons and climatic condition.

Table 4 shows the mineral contents of *Cola hispida* leaves extract; Potassium (K), iron (Fe), zinc (Zn) and copper (Cu) were found to be relatively present in the leaves extract. The concentrations of K, Fe, Zn, and Cu in *Cola hispida* leaves extract were found to be $0.85 \pm$

0.01 $\mu\text{g/g}$, $0.49 \pm 0.11 \mu\text{g/g}$, $0.14 \pm 0.05 \%$ and $0.03 \pm 0.01 \%$ which are low respectively. Iron is well established as an essential element and is a component of haemoglobin and is present in several human enzymes. Iron is present in high levels in red blood cells and muscle tissue. High doses of iron can cause hepatotoxicity. Acute liver failure has been reported after an overdose of ferrous sulphate in a young adult. Iron deficiency leads to anemia, which is the most well-known nutritional deficiency disorder (Upasana *et al.*, 2018). In addition, according to Mussie *et al.*, (2016), zinc is an essential element and Cellular zinc promotes homeostatic control to avoid accumulation of surplus zinc. Exposure to excessive zinc results in copper deficiency and cell apoptosis also, zinc deficiency has been linked to a suppressed immune response. Zinc plays a crucial role in the storage and secretion of insulin, which subsequently increases the uptake of glucose (Mussie *et al.*, 2016). Zinc and copper are considered important for metabolizing glucose and lowering cholesterol nutritional deficiency disorder.

Copper is found in various human transcription factors and enzymes and is considered as an important essential trace element. However, excessive intake of copper can lead to liver damage and Wilson's disease. Wilson's disease results from accumulation of free copper in the liver, brain, and kidney (Elzbieta *et al.*, 2017). Deficiency of copper can have adverse health effects, including aneurysms, blood vessel damage, hernias, and nosebleeds and can also affect the movement of nutrients through cell walls.

The vitamins present in the extract as shown in Table 5 includes vitamin C, E and A. Vitamin C also known as ascorbic acid (AA) plays an important role in many physiological processes in humans. It is needed for the repair of tissues in all parts of the body. The important functions of vitamin C include the formation of protein used to make skin, tendons, ligaments, and blood vessels for healing wounds and forming scar tissue, for repairing and maintaining cartilage, bones, and teeth and aid in the absorption of iron. It can also act as a reducing and capping agent for metal nanoparticles (Juliet *et al.*, 2017). Vitamin E is known to be the most effective chain breaking antioxidant located in the cell membrane which protects membrane fatty acids from lipid peroxidation. Dietary antioxidants enzymes such as vitamin C has been cited as being capable of regenerating vitamin E, also Beta-carotene and other carotenoids are also believed to provide antioxidant protection to lipid-rich tissues. This may justify the claim of Kurutas (2015) who suggested that beta-carotene work synergistically with vitamin E. The result in Table 6, showed no mortality in the acute toxicity of ethyl-acetate fraction on the experimental animals up to the highest dose. This may be attributed to little or no toxicity of the plant fraction at 5000 mg/kg b.w.

Table 7 showed that the percentage inhibition (DPPH) of *Cola hispida* leaves ethylacetate fraction was concentration dependent with (IC_{50}) of 3.65 ± 0.25 compared to the standard (ascorbic acid) with IC_{50} of 3.21 ± 0.23 which implies that the standard was more effective in scavenging/inhibiting the DPPH radical than the fraction.

The results in Table 8, showed a decrease in the activities of CK, AST and LDH in heart. This may be attributed to damage of myocardial cells according to the report of Balakrishna *et al.* (2015). The leakage of these enzymes into the extracellular fluid can be prevented by maintaining the membrane integrity of myocardium as reported by Saravanan *et al.*, (2013). Doxorubicin treated groups showed a significantly ($p < 0.05$) increased level of serum cardiac marker enzymes of (CK, AST and LDH) when it was compared to the normal group. This indicates damage or rupture of the myocardial cells containing these marker enzymes, consequently leading to the leakage of these enzymes into the plasma. Ability of the fraction to significantly lower the concentrations of these marker enzymes in the extracellular fluid suggests its cardio-protective effect on membrane integrity of myocardium, hence, preventing the leakage of these enzymes into the plasma. Furthermore, previous study by Swamy *et al.* (2012) has reported that the oxidative damage to tissues and their cellular components caused by the administration of Doxorubicin associated with an antioxidant deficit can be prevented by certain antioxidant metabolites present in the plants hence significantly protects the myocardium by reducing the elevated level of biomarker enzymes such as LDH and CPK and biochemical parameters such as AST, ALT and ALP back to normal (Swamy *et al.*, 2012). This could be part of properties of the plant that justifies its use in the management of disease conditions.

Table 9 provides information on predisposition of the serum lipid profile of the animals to atherosclerosis and coronary heart disease. Increase in serum lipid profile of the animals administered with Dox alone predisposes them to cardiovascular risk. Reduction in the levels of TC, TAG, LDL-C and increase in HDL-C observed in the groups treated with vitamin E and graded doses of the fraction suggested reduced susceptibility to cardiovascular risk. This was supported by the reduction in the computed atherogenic index, a useful indicator of cardiovascular diseases. The ethyl-acetate fraction of *Cola hispida* plants could be described as possessing anti-atherogenic capacity.

The inhibition of free radical generation is important in providing protection against myocardial infarction (Renu *et al.*, 2017). The activities of antioxidant enzymes (CAT, SOD, GPx) in the heart tissues of rats as shown in Table 10; catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (Gpx), were significantly decreased in the group of animals

treated with Dox only when compared to the normal group. This may be due to overwhelming of the antioxidant defense system by Dox metabolites thus predisposing the cardiac tissue to increased free radical damage. The significant increase ($p < 0.05$) in activities of endogenous antioxidant enzymes caused by administration of vitamin E and fraction suggests protective action on antioxidant defense system which may be attributed to antioxidant activity of *Cola hispida* leaves fraction. Furthermore, the research works of Patrice *et al.*, (2016) on dietary antioxidants: vitamins C, E and A detected in the extract complement the activities of these enzymatic antioxidants in protecting the animals from excessive oxidative stress caused by free radicals. The presence of cofactors such as Fe, Cu, Zn and K in the extract, required for the synthesis and proper functioning of SOD and CAT might have contributed in providing protection against myocardial infarction (Mussie *et al.*, 2016). The decrease in the levels of antioxidant enzymes (SOD, CAT and Gpx) after the administration of Dox predisposes the cardiac tissue to increased free radical damage although was significantly augmented by the administration of vitamin E and the fraction, hence free radicals were prevented from overpowering the antioxidant defense system. The ability of the fraction to scavenge the reactive oxygen species such as (superoxide ion, hydroxyl radical and H_2O_2) further implies that it could be used in counteracting the deleterious effects of reactive oxygen species produced in vivo during chemical assault. In addition, consumption of the fraction may complement the *in vivo* free radical scavenging activities of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx), when the internal enzymatic mechanism tends to be inefficient.

Fig 3 represents the increase in MDA content of heart tissues of DOX-induced animals indicating that peroxidation of polyunsaturated fatty acids in the cardiac cell membrane has occurred. This led to the disruption of the structural integrity of the cardiac cell membrane. The significant ($p < 0.05$) decrease in the cardiac MDA content following the administration of vitamin E with the graded doses of the fraction showed that treatment with the fraction led to a protective effect against the DOX-induced cardiac lipid peroxidation. The protective effect of *Cola hispida* leaves fraction on lipid peroxidation may be attributed to some of its antioxidant components thus Ethyl-acetate fraction of *Cola hispida* leaves could be used to restore altered biochemical profiles induced by DOX exposure and promote membrane stabilization. Glutathione peroxidase plays an important role in protecting the heart against oxidative damage, it reduces lipid peroxides to their corresponding alcohol and reduces free hydrogen peroxide to water (Nachiappan *et al.*, 2010). The animals that were treated with vitamin E and graded doses of *Cola hispida* leaves fraction showed improved glutathione peroxidase activity.

This treatment promotes the regeneration of reduced glutathione for normal cell metabolism. The presence of phytochemicals in the extract such as flavonoids, alkaloids and phenols might also be responsible for the antioxidant activity of the fraction. This possibly may justify the effect of anti-lipid peroxidation property of *Cola hispida* leaves fraction.

Histological perturbation shown in the group that received Dox only could be justified with the decrease in oxygen supply with paramount rise in cardiac wall-stress. The histopathological examination of heart tissues shown in Plate B revealed that rats exposed to Dox only showed hydropic, degeneration of cardiac muscle by loss of myofibril and leucocytic cellular infiltration when compared to the Plate A (normal control). Heart tissues of animals treated with different doses of extract showed dose dependent protection of the heart from myocardial necrosis hence the histo-architectural structure of the heart of the heart was maintained.

4.2 CONCLUSION

This present study has shown that Doxorubicin (DOX) induction causes severe damage in the test animals as shown by changes in serum levels of cardiac biomarkers. Pre-treatment of the test animals (rats) with ethyl acetate fraction of *Cola hispida* leaves showed protective effects against Dox-induced cardiotoxicity. This is attributed to the free radical scavenging ability associated with the antioxidant phyto-constituents in the fraction. Consequently, ethyl-acetate fraction of *Cola hispida* leaves could be a beneficial therapeutic agent in treatment and management of cardiac dysfunction.

4.3 Suggestions for Further Studies

Much work has not been done on this plant species as shown from the literature review conducted. There is need to further purify this plant extract and carry out structural elucidation of the components of the extract in order to determine the bioactive compounds and their molecular mechanisms of action.

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