COMPARATIVE ANTI-ANEMIC EFFECTS OF METHANOL EXTRACTS OF *Dennettia tripetala* AND *Cola acuminata* LEAVES ON PHENYLHYDRAZINE-INDUCED HEMOLYTIC ANEMIA IN ALBINO WISTAR RATS

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BENIN CITY

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A THESIS WRITTEN IN THE DEPARTMENT OF BIOCHEMISTRY AND SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN BIOCHEMISTRY OF THE UNIVERSITY OF BENIN, BENIN-CITY.

OCTOBER, 2024

CERTIFICATION

We certify that this work was carried out by Mrs. Amenze Imuetinyan OKUNGBOWA in the Department of Biochemistry, Faculty of Life Sciences, University of Benin, Benin-City.

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CERTIFICATION OF THESIS

We the undersigned attest and declare that the thesis of Mrs. Amenze Imuetinyan OKUNGBOWA titled "Comparative Anti-anemic Effects of Methanol Extracts of *Dennettia tripetala* AND *Cola acuminata* Leaves on Phenylhydrazine-Induced Hemolytic Anemia in Albino Wistar Rats" has successfully passed the anti-plagiarism test and does not violate any copyright regulations.

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PROF. N.P. OKOLIE DATE
SUPERVISOR

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PROF. E.C. ONYENEKE DATE
HEAD OF DEPARTMENT

DEDICATION

I dedicate this work first to God Almighty who is the very essence of my existence and my inspiration all through this research.

Also, to my mother, Mrs. Patience EropkaidanmwenIkponmwen of blessed memory, who ensured that I got my first set of leaf samples for this research.

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LIST OF ABBREVIATIONS

Abbreviation	Meaning
PHZ	Phenylhydrazine
WHO	World Health Organization
G6PD	Glucose 6-phosphate Dehydrogenase
Dcytb	Duodenal cytochrome B
IREG1	Iron regulatory 1
DMT1-IRE	Divalent metal transporter 1
HO1	Heam oxygenase 1
TFR1	Transferrin receptor 1
JAK-STAT	Janus Kinase signal transducers and activators of transcription
LD_{50}	Lethal dose

ABSTRACT

Anemia is a medical condition in which the concentration of circulating red blood cells is less than 13g/dL for males and 12g/dL for female adults. The leaves of *Dennettia tripetala* (DT) and *Cola acuminata* (CA) have been used to manage anemia in adults and children by herbal practitioners. This study compared the anti-anemic effects of *Dennettia tripetala* and *Cola acuminata*methanol leaf extracts on phenylhydrazine (PHZ)-induced hemolytic anemia in albino Wistar rats.

The study was divided into four phases. In Phase I, nutritional and mineral composition, qualitative and quantitative phytochemical content, *In vitro* antioxidant capacity, High Performance Liquid Chromatography (HPLC) and Gas Chromatography Mass Spectrometry (GC-MS) were carried out in accordance with standard methods. In Phase II, acute and subacute toxicity of each extract were determined. In Phase III, the effect of each extract on biochemical parameters for kidney, liver and splenic functions were ascertained. Histology of bone marrow, kidney, liver and spleen and hematological parameters were also determined. In Phase IV, quantitative polymerase chain reaction (qPCR) for mRNA expression levels of IREG, HO-1, DMT-IRE and TFR1 genes in the liver and spleen was determined.

Proximate analysis reveal that both leaf extracts contain substantial amounts of proteins, lipids, carbohydrates, fibre and ash, with low moisture content. Minerals present are phosphorus, calcium, sodium, iron, magnesium, zinc, and potassium. Qualitative phytochemical and HPLC analysis reveal appreciable amounts of cardiac glycosides, saponins, alkaloids, terpenoids, coumarins, tannins, phenols, amino acids and reducing sugars. Steroids were sparingly present, while flavonoids were abundant. GC-MS analysis showed the presence of terpenoids, hydrocarbons and fatty acids. *In vitro* anti-oxidant analysis indicates that CA scavenged DPPH radical better than DT. However, DT had a higher total anti-oxidant capacity than CA. Acute toxicity studies show that both extracts had LD_{50} values >5,000 mg/kg body weight, with no mortality. Sub-acute toxicity revealed modulation of biochemical and hematological parameters. The effective dose of DT and CA against PHZ toxicity were 1,500 and 500mg/kg body weight, respectively. Administration of DT and CA resulted in significant ($p<0.05$) improvement of antioxidant status, biochemical indices and hematological parameters when compared with the negative control. These results were confirmed

by the histological changes in the kidney, liver and spleen. Prussian blue staining show that CA and DT restored bone marrow iron stores to levels comparable to both normal and positive controls. qPCR revealed that DT and CA downregulated mRNA expressions of HO-1 gene (in spleen) and IREG gene (in liver) similar to the positive control. Overall, CA, was more effective than DT in restoring liver function, hematological indices, organ morphology and bone marrow iron stores, while DT was more effective than CA in restoring kidney functions. Antioxidant defenses were restored in the kidney, liver and spleen by both extracts. The therapeutic effect of these extracts may be attributed to the presence of bioactive phytochemical compounds such as β-Longipinene, Cetene, Heptadecane, Neophytadiene, Eicosane and Squalene as well as rich iron contents of both extracts. These results provide scientific evidence for the medicinal use of *Dennettia tripetala* and *Cola acuminata* methanol leaf extract in the treatment of anemia.

CHAPTER ONE

INTRODUCTION

1.1. Background of the study

Natural remedies for various health conditions have become increasingly significant to scientists and also to medical practitioners. In many cultures, traditional medicine has a long history of using natural remedies obtained from plants,and other natural sources. The rising cost of health care and prescription medications have driven many individuals to seek natural remedies often available at lower cost, as alternative affordable options for improved health conditions including anemia and anemia related illnesses or diseases.

Anemia is a widespread issue worldwide, impacting about 500 million women within ages 15-49 and 269 million children between 6-59 months of age. As of 2019, about 30% of non-pregnant women (539 million) and 37% of pregnant women (32 million) in the 15-49 age group were affected by anemia according to WHO, (2023). This condition is a significant public health concern across developed and developing nations, with higher prevalence among children under five and pregnant women. Anemia is medically defined as having a lower concentration of circulating red blood cells than the normal thresholds of 13g/dL for males and 12g/dL for female adults (WHO, 2023). Symptoms typically manifest withlow levels of hemoglobin around 7.0 g/dL for most patients (Turner *et al*., 2023).

Anemia severity is categorized into levels depending on the hemoglobin concentrations in the bloodstream (Turner *et al*. 2023). Mild anemia is associated with hemoglobin concentrations of 10.0-10.9 g/dL in pregnant women and children below 5years of age, and 10.0-11.9 g/dL for non-pregnant women. Moderate anemia is synonymous with a level of 7.0-9.9 g/dL, while severe anemia corresponds to a level

less than 7.0 g/dl. Anemia may also be classified as hypoproliferative(i.e. corrected reticulocyte count <2%) or hyperproliferative (i.e., corrected reticulocyte count >2%) (Turner *et al*. 2023). Inhemolytic anemia the red blood cells are destroyed. This could be extravascular hemolysis or intravascular hemolysis. It is prominent in hemoglobinopathies (such as thalassemia and sickle cell anemia), enzyemopathies (G6PD deficiency, pyruvate kinase deficiency), membrane defects hereditary spherocytosis, hereditary elliptocytosis) cancer and some other drug induced anemia (Turner *et al*. 2023).

Anemia is not a diagnosis in itself, but may result from poor nutrition, excessive bleeding, deficient or defective erythropoiesis, or other underlying disease such as cancer or haemoglobinopathies. If not properly managed, anemia can lead to hypoxia in organs including the heart and the brain, resulting in organ dysfunction or outright organ failure, stroke, shortness of breath, or death. Therefore, any symptom of anemia is to be treated with utmost urgency to save life.

Management and treatment of the condition is usually determined by the cause. These may include improved diet with iron, folic acid and vitamin B12 supplementation, blood transfusion, discontinuation of offending medication if the cause is drug related, bone marrow stem cell transplant, surgical removal of the spleen (splenectomy) if the cause is due to spleen dysfunction.

In recent times, research has tilted towards the application of herbs and herbal products for the treatment and management of diseases. Numerous plants have been found to contain potent phytochemical constituents which are effective in curing certain infections and diseases. They have also been proven to improve health status by enhancing immune function and antioxidant concentrations in the blood. Several

plant leaves, barks, fruits and roots have been shown to combat anemia effectively such as *Telferia occidentalis* (flutted pumpkin), beetroot, etc.

Herbal medicine practitioners claim that certain herbs improve the heamoglobin levels, haematocrit and other blood parameters in anemic conditions. Such herbs include *Telferia occidentalis*, *Carica papaya*, *Dennettia tripetala* leaves, *Cola acuminata* leaves, beetroot, etc. *Dennettia tripetala* and *Cola acuminata* leaves, are renowned for their phytochemical composition of flavonoids, tannins, alkaloids, and other bioactive compounds known for their potential therapeutic effects.

Dennettia tripetala Baker (syn. *Uvariopsis tripetala* (Baker F.) G.E. Schatz) is a small woody shrub 12 - 18 m tall. It is popularly known as 'pepper fruit' in English, ‘ako’ in Edo, ‘mmimi’ in Igbo, ‘nkaika’ in Ibibio, ‘imako’ in Urhobo, ‘opipi’ in Idoma and ‘igberi’ in Yoruba languages of Nigeria (Egharevba and Idah, 2015). The fruit is enjoyed for its sweet spicy taste, medicinal purposes and as a food preservative (Okolie *et al.,* 2014; Iseghohi, 2015; Omage*etal*., 2021). In traditional medicine, the leaves and seeds are utilized to treat fever, cough, asthma, catarrh, toothache, diarrhea, and rheumatism. Additionally, they are employed to stimulate appetite, alleviate throat discomfort, reduce excessive salivation, soothe coated tongues, and alleviate nauseation (Okolie *et al*., 2014, Muhammed *et al*., 2021). The seeds are a significant element in the diets of women who put to birth, due to the notion that incorporating herbs and spices can help stimulate uterine contractions (Muhammed *et al.,* 2021). The plant is known for variedhealth benefits, such as antioxidant, antidiarrheal, anticonvulsant, antimicrobial, anti-trypanosomal, antimalarial, anti-inflammatory, antiparasitic, pain-relieving properties and anti-snake venom effects (Iseghohi, 2015,Muhammed *et al*., 2021).

Cola acuminata (P. Beauv.) Schott and Endler,isa medium sized evergreen tree having low grey or dark green branches, dark green leaves and white flowers (Facciola, 1998). Kola is commonly called ‘evbee’ in Edo. The nut occupies a unique place in West Africa where it is widely consumed. Cola contains five species of edible nuts, each having their own importance. These species include; *Cola nitida, Cola acuminata, Cola ballayi, Cola verticillata and Cola sphaerocarpa.* Of the five species, the most common species are the *Cola nitida* and *Cola acuminata*. Traditionally, people chewed kola nuts to serve as masticatory substance, helping to stimulate saliva flow.But now, they are eaten as snacks especially among elders in West and Central Africa (Adebayo and Oladele, 2012; Ajai *et al*., 2012). Kola nuts are frequently utilized for treating conditions like asthma and whooping cough due to their ability to widen the bronchial airways, a result of the caffeine they contain (Adebayo and Oladele, 2012; Kanoma*et al*., 2014). *Cola acuminata* nuts are known to boost alertness and physical energy, improve mood, curb appetite, and are even employed as an aphrodisiac (Adebayo and Oladele, 2012; Kanoma*et al*., 2014).

Phenylhydrazine (Hydrazino benzene) is a yellow to pale brown compound which was characterized by the scientist Hermann Emil Fischer in 1895 (Singh *et al.,* 2014). It was originally used for therapeutic purposes until it was proven that the red blood cells became dysfunctional when phenylhydrazine was administered.Several blood parameters were altered by the effect of this compound. Notably, PCV (Packed Cell Volume), hemoglobin levels (HBG), Red Blood Cell (RBC) count were decreased. On the other hand, MCHC (Mean Corpuscular Hemoglobin Concentration), MCH (Mean Cell Hemoglobin) and MCV (Mean Cell Volume) are elevated by phenylhydrazine. In the spleen and liver, extramedularhaematopoiesis occurs, while oxidative stress which leads to formation of methemoglobin and other hemichromesusually occurs in

response to PHZ toxicity (Singh *et al.,* 2014). Eventually, hemoglobin is precipitated to form of Heinz bodies (Singh *et al.,* 2014). Phenylhydrazine results in damage to proteins, peroxidation of lipids, cation imbalances,depletion of ATP, and reduced deformability of membrane. These symptoms show evidence of hemolytic anemia (Singh *et al.,* 2014). Phenylhydrazine-induced anemia increases iron absorption and expression of the iron transport genes (Dcytb, Ireg and DMT1-IRE)in the spleen, duodenum and liver. It also induces immunological activation (Singh *et al*., 2014),which triggers splenic and liver phagocytosis. Phenylhydrazine inhibits red blood cell maturation via the EPO receptors of JAK-STAT pathway.

Free radicals are atoms, molecules, or ions having unpaired electrons on outer shells, thus are very reactive and prone to participate in chemical reactions involving other molecules including proteins, lipids, and DNA, thereby disrupting their function. They are generated during endogenous cellular reaction or from exogenous sources (Gupta *et al*., 2010a). Oxidative stress occurs when there is an imbalance between the production of reactive oxygen species (ROS), such as free radicals, and the ability of cells to detoxify or repair them (Hamid *et al.,* 2010). The resulting effect of oxidative stress is the dysfunctionality of the cells and biomolecules which leads to disease formation and the aging process(Gupta *et al*., 2010).

Green leafy vegetables, fruits and nuts are rich sources of varied exogenous antioxidants in nature. The abundance of natural antioxidants and increased toxicity and/or mutagenicity of synthetic antioxidants has propelled researchers to focus their attention more on the natural than synthetic (Kumar and Sharma, 2006, Gupta and Sharma, 2010).

1.2. Justification of the Study

The practice of using herbs in the management of ailments is as old as man himself. Plants contain many vital nutrients and phytochemicals, which help to nourish the body, improve health status, fight infections, protect and build defensive mechanism against diseases. This method of treating sickness is not only limited to adults, but also to children, babies and toddlers. It suffices to say that even with the advent of and availability of orthodox drugs, many people still depend on herbs. This may be due to the high cost of living in places like Africa, India, Nigeria in particular. Hence the poor are not able to afford the high cost of drugs or medical care. Many drugs too have been found to have debilitating and adverse side effects, many of which become another illness altogether.

In recent times, herbal practitioners have found relevance in the field of medicine. Their knowledge and experience in the use of herbs to manage diseases, including terminal diseases like cancer, diabetes, arteriosclerosis, etc, have gained global recognition. However, many of these herbal extracts are used without specific doses, and ascertaining their toxicological effects. Among such herbs are the leaves of *Dennettia tripetala* and *Cola acuminata*.

Dennettia tripetala and *Cola acuminata* seeds are popular for their rich taste and ceremonial use. Their leaves are applied in the treatment of fever, anemia and splenomegaly conditions in children and adults. Although herbal practitioners claim its effectiveness in the management of these conditions, not much work has been done to give scientific backing for their use as anti-anemics in folk medicine. This study is thus designed to provide scientific evidence for the use of *Dennettia tripetala* and *Cola acuminata*leaves in anemia management.

1.3. Aim and Objectives of the Study

This study aims to compare anti-anemic effects of methanol extracts of *Dennettia tripetala* and *Cola acuminata* leaves on phenylhydrazine-induced hemolytic anemia in albino Wistar rats.

The specific objectives were to:

i. determine the LD_{50} of each leaf extract;
ii. ascertain the nutritional and mineral composition of each extract;
iii. evaluate the phytochemical content and *In vitro* antioxidant capacity of each extract;
iv. determine the effect of each extract on the kidney, liver and spleen enzymes and oxidative stress markers of rats given phenylhydrazine;
v. determine the bioactive compounds in the leaf extracts;
vi. determine the effect of each extract on hematological parameters of rats given phenylhydrazine;
vii. ascertain the effect of each extract on the expression of iron metabolism genes (Heam oxygenase 1(HO1), Iron regulatory 1(IREG1), Divalent metal transporter 1(DMT-IRE) and Transferrin receptor 1(TFR1)) in the liver and spleen of rats given phenylhydrazine and each extract;
viii. examine the effect of each extract on the histology of kidney, liver and spleen of rats given phenylhydrazine;
ix. examine the sequestration of iron in bone marrow of rats given phenylhydrazine and each extract.

CHAPTER TWO

LITERATURE REVIEW

2.1. Description of *Dennettia tripetala*plant

Dennettia tripetala Baker (syn. Uvariopsis tripetala (Baker F.) G.E. Schatz) is a small woody shrub 12 - 18 m tall with a fibrous bark and a potent distinctive smell. Cultivated in Nigeria, Ivory Coast and Cameroon, it is generally identified as 'pepper fruit' in English, ‘ako’ in Edo, ‘mmimi’ in Igbo, ‘nkaika’ in Ibibio, ‘imako’ in Urhobo, ‘opipi’ in Idoma and ‘igberi’ in Yoruba languages of Nigeria (Ejechi*et al*., 2005; Egharevba and Idah, 2015; Muhammed *et al*., 2021). It grows in Nigeria, around regions of the tropical rainforest and the Savana (Okwu *et al*., 2005). The plant blossoms at the beginning of the raining season, from April to June. Typically, fruiting is from March through May. Its flowers emerge in small clusters with a light brown outside and a red interior (Egharevba and Idah, 2015). When unripe, the fruit is green but red when mature. Between each seed is a finger-like carpel constriction.

Classification of *Dennettia tripetala*

Dennettia tripetala is classified as follows:

Kingdom: Plantae

Phylum: Magnoliophyta

Class: Magnolidae

Order: Magnoliales

Family: Annonanceae

Genus: Dennettia

Specie: tripetala

(Ihemeje, *et al*.,2013; Iseghohi, 2015)

Plate 2.1 *Dennettia tripetala* Leaves (Personal)

2.1.1. Domestic uses of *Dennettia tripetala*

*Dennettia tripetala*fruits, bark, and leaves are primarily used as seasonings or spices for meat, sausage, stew, soup, and vegetables(Ejechi and Akpomedaye, 2005; Iseghohi, 2015).

2.1.2. Medicinal uses of *Dennettia tripetala*

The leaves and seeds of *Dennettia tripetala* play a role in traditional medicine for addressing feverish conditions, asthmatic effects, dental defects, diarrhea, and rheumatism (Oyemitan*et al*., 2006). *Dennettia tripetala*is effective in stimulating appetite, alleviating throat discomfort, controlling excessive salivation, alleviating coated tongues, and mitigatingnauseation (Ejechi and Akpomedaye (2005), Ukeh*et al.,* 2012). Its seeds are considered a crucial dietary element for women post-childbirth due to the belief that spices and herbs contribute to uterine contractions. Additionally, they are commonly used in combination with mango leaves for the treatment of mild ailments (Nwinuka,*etal*., 2008).Human intraocular pressure (IOP) has been demonstrated to decrease in response to *Dennettia tripetala* seeds. According to Timothy and Okere (2008), oral administration of 0.75g of *Dennettia tripetala* lowers intraocular pressure for around half an hour following treatment. There was a 17.30% decrease in IOP (12.90 mmHg) relative to the 15.60 mmHg mean baseline. Flavonoids complement the effects of vitamin C and help stabilize collagen, as reported by Head, (2001). They are believed to influence intra-ocular pressure by reducing the blood-aqueous membrane permeability in the eye increased glutathione in glaucoma patients' lacrimal fluid and red blood cells has been demonstrated to be caused by lipoic acid (fatty acid), which lowers intraocular pressure (Head, 2001).

2.1.3. Biochemical effects of *Dennettia tripetala*

Oyemitan*et al*. (2006) demonstrated that β-Phenylnitroethane and essential oil derived from *Dennettia tripetala* exhibit significant memory-enhancing effects in the Y-Maze test and *In vitro* anticholinesterase activity. Furthermore, the seed oil of *Dennettia tripetala* has been noted for its antibacterial and broad-spectrum antimicrobial properties. Similarly, the methanol extract of *Dennettia tripetala* seeds was found to reduce serum levels of low-density lipoprotein (LDL), very low-density lipoprotein (VLDL), high-density lipoprotein (HDL), total cholesterol, and triacylglycerol in a diabetic rat model, leading to an improvement in high-density lipoprotein (Aniok*et al*., 2017). Previous research has also indicated that leaf extracts possess antidiabetic, antilipidemic, and antibacterial properties (Abonyi *et al*., 2020; Nnenna *et al*., 2015). The methanol seed extract has been shown to reduce sleep latency and enhance total sleeping time in pentobarbital-induced mice, with reported anxiolytic and antiepileptic effects (Uruaka and Georgwill, 2020). These findings are consistent with studies carried out by other researchers who reported the anticonvulsant, hypnotic and anxiolytic effects of 1-nitro-2-phenylethane and other essential oil components present in the seeds (Oyemitan*et al*., 2013). The n-hexane seed extract revealed the identification of twenty-five compounds, such as linoleic acid ethyl ester, caryophyllene, 3-carene, phenyl ethyl alcohol, and cubebene (Elekwa, 2017).

2.1.4. Phytochemical Constituents of *Dennettia tripetala*

Research indicates that *Dennettia tripetala* contains a variety of phytochemicals, and the composition varies depending on the plant part. Adedayo *et al*. (2010) found that the fruits contain alkaloids, cardiac glycosides, flavonoids, tannins, saponins, terpenoids, and steroids. This slightly contrasts with the findings of Egharevba and Idah (2015), who identified tannins, alkaloids, steroids, terpenes, flavonoids, balsams

(resin), and phenol in the fruits. They noted a similar phytochemical profile in the leaves, except for the absence of balsams (resin) and steroids. Elekwa*et al*. (2017) reported the presence of alkaloids, tannins, saponins, flavonoids, terpenoids, steroids, and cardiac glycosides in the seeds.

2.1.5. Nutritional Composition of *Dennettia tripetala*

Dennettia tripetala fruits have been acknowledged for their nutritional significance, with variations in nutrient proportions between ripe and unripe fruits. Ihemeje*et al*. (2013) reported the proximate composition of *Dennettia tripetala* fruits (both unripe and ripe) revealing that moisture, fat, and carbohydrate contents increase with ripeness, while crude fiber and protein contents decrease. Notably, the fruits exhibit high carbohydrate content ranging from 53% to 68% (Okwu and Morah, 2005; Dike, 2010; Ihemeje*et al*., 2013). These fruits have inherent laxative properties and serve as dietary fibre by adding bulk to the diet. They also contain sugars such as glucose, sucrose, and fructose, as well as hemicellulose and pectin (Muhammed *et al*., 2021). According to Okwu and Morah (2005), the fruits possess caloric value of 480.24g cal·100 g–1. Furthermore, *Dennettia tripetala* fruits are reported to be rich in minerals and water-soluble vitamins, including ascorbic acid, thiamine, riboflavin and niacin (Okwu and Morah, 2005). Vitamins A and C seemto increase significantly with ripening (Ihemeje*et al*., 2013). These vitamins contribute to the fruit's traditional uses in treating common colds and managing conditions such as prostate cancer (Muhammed *et al*., 2021). *Dennettia tripetala* fruits are notably high in potassium (2.48%) and calcium (1.80%) while exhibiting low concentrations of sodium, zinc, copper, manganese, cobalt, nickel, and cadmium, (Okaka and Okaka, 2001). The fruits contain a substantial percentage of iron (17.75%), a vital constituent of heme moieties in hemoglobin (Okwu and Morah, 2005).

2.1.6. Anti-nociceptive and Anti-inflammatory Effect of *Dennettia tripetala*

According to Oyemitan*et al*. (2006), *Dennettia tripetala* exhibits strong and anti-inflammatory anti-nociceptive properties. It has been discovered that the pain relieving impact of the essential oils from *Dennettia tripetala* fruits is comparable to that produced by aspirin, indomethacin, and the potent opioid morphine. Both the early and late stages of inflammation were demonstrated to benefit from the anti-inflammatory activity (Oyemitan*et al*., 2008). Research hasdemonstrated that essential oil of *Dennettia tripetala* reduces inflammation in edematous rodents to a degree comparable to dexamethasone (Oyemetan*et al*., 2008).

2.1.7. Effect on Oxidative Stress and Antioxidant Activities

Reactive oxygen species (ROS) are typically produced throughout a living organism's metabolism, and antioxidants mitigate the impacts of these species. A portion of these antioxidants are created by the body on an endogenous level, while others are found in plants as antioxidant nutrients (Ofem *et al*., 2014). According to a number of research, *Dennettia tripetala* is used to treat infections and illnesses linked to oxidative stress (Aderogba*et al*., 2011). In accordance with a 2010 study conducted at the Federal University of Technology, Akure, unripe *Dennettia tripetala* fruits have higher antioxidant activity than ripe ones. The study assessed the changes in the antioxidant contents and potentials of the fruit with ripening (Aiyeloja and Bello, 2006). These physiological alterations which coincide with fruit ripening, raise total phenol content and lower ascorbic acid concentration, cause the decline in antioxidant activities. As a result, the ripe fruit's antioxidant properties are diminished (Aiyeloja and Bello, 2006). Aderogba*et al*. (2011)stated that, these flavonoids efficiently scavenge free radicals because they were seen to quickly degrade 1, 1-diphenyl-2-picrylhydrazine (DPPH) purple colouration.

An additional investigation examined the potential of the ethanolic root extract of *Dennettia tripetala* to hinder lipid peroxidation in frozen heart muscle slices. The results indicated a significantly ($p < 0.05$) greater antioxidant effect of the extract in comparison to ascorbic acid, proving that the antioxidant-rich root extract of *Dennettia tripetala*improves frozen meat preservation (Okolie *et al*., 2014). Research conducted by Iseghohi and Orhue (Iseghohi, 2015; Iseghohi and Orhue, 2017) demonstrated that by preventing the oxidative stress linked to tetrachloromethane (CCl4) metabolism, the administration of fruit extracts, both aqueous and ethanolic, prevented elevation of plasma γ-glutamyl transaminase (GGT) activity, alanine transaminase (ALT), and alanine phosphatase (ALP) enzymes.

2.1.8. Antimicrobial Effect of *Dennettia tripetala*

The antibacterial properties of *Dennettia tripetala* have been linked to several phytochemicals, including flavonoids, fatty-acids, linoleic acid, palmitate, eicosanoic acid, and ethyl ester in its content (Anyaele and Amusan, 2003; Nnenna *et al*., 2015; Kumar *et al*., 2010).According to another study (Ejechi and Akpomedaye, 2005), essential oils and phenolic acids in the seed extract prevented the growth of food-borne bacteria. Additionally, Nwachukwu and Osuji (2008) discovered that *Dennettia tripetala* leaves effectively suppress the proliferation of *Sclerotiumrolfsii* which causes cocoyam spoilage, both *in vitro* and *in vivo.*

There have been reports of insecticidal (Ukeh*et al*., 2012) and antifungal (Nwachukwu and Osuji, 2008) capabilities in *Dennettia tripetala* extracts. Anyaele and Amusan (2003) reported on the fruit's potential to protect cowpea from storage insect pests. They found that the fruit's hexane extract has larvicidal activity against Aedesaegypti mosquito larvae. According to Ukeh*et al*. (2012), pepper fruit's activity against insects rises with concentration and exposure duration. Moreover, *Dennettia*

tripetala fully regulated the development adults and eradication of the larva of corn weevils (Sitophilluszeamis) and cowpea weevils. Research by Asawalam*et al*., (2007), proves that 2-nitroethyl-benzene and beta-phenyl nitroethaneconstituent of *Dennettia tripetala* confer its insecticidal activity.

2.1.9. Anti-hyperglycaemic Effect of *Dennettia tripetala*

Dennettia tripetala extracts in chloroform, ethyl acetate, and methanolic form have been shown by Anaga and Asuzu (2010) to bring hyperglycemic rats' plasma glucose levels down to values comparable with those with normal glucose levels. In order to explore a potential mechanism for *Dennettia tripetala*'s antihyperglycemic effect, researchers have employed 3T3-L1 adipocytes brefeldin. They found that part of the mechanism involves the mobilization of glucose transporters from the cytosol to the plasma membrane (Muhammed*et al*., 2021).

2.1.10. Effects of *Dennettia tripetala* on the Nervous System

Fruits, leaves, and seeds of *Dennettia tripetala*are known to contain an essential oil (1-nitro-2-phenyl ethane), which is thought to be the source of the oil's observed neuropharmacological effects. In mice, this substance possesses hypnotic, anticonvulsant, and anxiolytic properties (Oyemitan, 2008). The ethanolic extract of the seeds inhibit cell proliferation and enhance cytotoxicity in prostate cancer cell lines PC3 and LNCaP, *In vitro* (Muhammed *et al*., 2021).

2.2. Description of *Cola acuminata*

Cola acuminata (P.Beauv.) Schott and Endler, tree isan evergreen, medium sized tree, having low grey or dark green branches, very green leaves and whitish flowers (Facciola, 1998). It is popularly known as kola nut but commonly called ‘evbee’ in Edo, ‘Oji’ in Igbo, ‘obi’ in Yoruba and ‘goro’ in Hausa (Ugwuowo*et al*., 2021).

Plate 2.2: Leaves of the *Cola acuminata* plant and the capsule shaped fruits (Lowe *et al.,* 2014).

Cola acuminata is classified as follows:

Kingdom: Plantae

Order: *Malvales*

family: *Sterculiaceae*

Genus: Cola

Specie: acuminata

The genus *Cola acuminata* comprises approximately 125 native species found in Africa's tropical rain forests. It was then moved into the Sterculiaceae family after being previously classified in the Malvaceae family and subfamily, Sterculioidea, according to phylogenetic data (Kanoma*et al.,* 2014).

Cola contains five species of edible nuts, each having their own importance. They include; *Cola nitida*, *Cola acuminata, Cola ballayi*, *Cola verticillata* and *Cola sphaerocarpa. Cola nitida* and *Cola acuminata* are more widely utilized than other species. Apart from these two species, *Garcinia kola* belonging to the *Clusiceae*family is also important and the seeds are added to numerous herbal formulations and have potential therapeutic benefits. Nigeria accounts for about 70% of kola nut produced worldwide. However, about 90% is consumed while the remaining 10% is exported (Adebayo and Oladele 2012; Dah-Nouvlessounon*et al*., 2015; Kanoma*et al.,* 2014).

2.2.1. Traditional use of *Cola acuminata*

Cola acuminata and the *Cola nitida*are used during weddings, naming ceremonies, Chieftaincy ceremonies, funerals and other rituals peculiar to Africa (Adebayo and Oladele, 2012; Ajai *et al.*, 2012). Traditionally, kola nuts are consumed, to enhance saliva secretions but now serve as snack most commonly by African elders in west and central regions of Africa (Leakey, 2001). In traditional medicine, dehydrated cola nuts are ground to powder and mixed with honey to treat cough (Adebayo and

Oladele, 2012). Mboto (2009) reported proof of improvedrecovery in a combination regimen of*Vernonia amygdalina,*honey and *Garcinia kola* in treatment of chronic ulcers, fresh injuries and male circumcision related wounds.

2.2.2. Medicinal use of *Cola acuminata*

Cola acuminata has various medicinal uses. They contain large caffeine and theobromine amounts and are therefore used as stimulants. The caffeine in their nuts expand the bronchia, for this reason they are mostlyappliedas remedy for asthma and whooping cough. Bitter kola (*Garcinia kola*) is suspected to detoxify the eosophagus and intestines, without any adverse reaction when consumed excessively. *Cola acuminata* also improves alertness and physical energy, elevates mood, reduces appetite and it is also used as asex enhancer. The nut extracts of *Cola acuminata* are antiparasitic especially against trichomoniasis infection in women (Adebayo and Oladele, 2012; Kanoma*et al*., 2014).

2.2.3. Toxicity of *Cola acuminata*

Although *Cola acuminata* has positive effects, there are also negative effects due to high consumption of *Cola acuminata*, effects like insomnia or ulcer in pregnant women and high blood pressure (Ajai *et al.*, 2012).

2.2.4. Antioxidant and Anticholesterase Properties of *Cola acuminata*

A study showed that the seed extracts of *Cola acuminata* inhibited the activities the neurotransmitters, acetylcholinesterase and butyrylcholinesterase in a dose dependent fashion. Acetylcholinesterase and butyrylcholinesterase inhibition have been the basic treatment regime for mild Alzheimer's disease. The extracts of *Cola acuminata* also prevented oxidative stress induced neurodegeneration due to its high radical scavenging and Fe^{2+} chelating activities. This inhibition could be as a result of phytochemicals such as caffeine and flavonoids (Oboh *et al*., 2014).

2.2.5. Anticancer properties of *Cola acuminata*

Lowe *et al*. (2014)reported that hexane nut extract killed 100% of the breast and prostate (DU-145 and PC3) cancer cell lines. Another research showed the cytotoxicity of *Cola acuminata* in cell lines of breast cancer (MCF-7 and DA-MB 468) and prostate cancer (LNCaP). The reduction of viability of these cell lines is via the mechanism of apoptosis (Edrini*et al*., 2011; Fortenort*et al*., 2007).

2.2.6. Phytochemical composition of *Cola acuminata*

Phytochemicals are plant chemicals having protective or disease preventive capacity. Research by Victoria *et al*. (2014) on both qualitative and quantitative phytochemical analysis of *Cola acuminata* leaves established its secondary metabolites constituents usually responsible for the pharmacological potential of medicinal plants. Some phytochemicals identified were phenols, alkaloids, saponins, flavonoids and carotenoids.

Quantitative analysis was carried for each of the phytochemicals to reveal high concentrations of alkaloids and phenolic acids. The quantitative analysis of the phenolic acid content of the leaves shows high content of caffeic and quinic acidsand their ester, chlorogenic acid and low contents of cedar acid (syringic acid), protocatechinic acid, ellargic acid, p-coumaric acid and rosmarinio acid. This proves that the leaves of *Cola acuminata* can be used against the development of dementia or Alzheimer's disease because phenolics act against oxidative stress induced diseases. Also, intake of diets/supplements rich in phenolic acid may have similar effects *In vivo*(Victoria *et al*., 2014).

Alkaloids concentration in the leaves of *Cola acuminata* are very high. Alkaloids are said to possess cytotoxic activities, anti-viral properties, anti-bacterial properties and carcinogenic or mutagenic properties (Victoria *et al*., 2014; Bribi, 2018). Quantitative

analysis of the alkaloid content in the leaves revealed the presence of caffeine, theobromine, theophylline, nitidine, akuammidine, trigonelline, vocangine and echitammidine (Victoria *et al*., 2014; Bribi, 2018).

The quantitative analysis of flavonoid content confirms catechin content, epicatechin, isoquercetin, isohamnetin, apigenin, kaemferol, luteolin and quercetin in small concentrations (Victoria *et al*., 2014). Flavonoids are naturally occurring phenols which are potent antioxidants. They scavenge free radicals thereby preventing oxidative cell damage and cancer. Quantitative analysis of carotenoid content revealed the presence of lutein, viola-xanthin, beta-carotene, neo-xanthin, malvidin and beta-cryptoxanthin in small concentrations (in comparison to that of concentration of alkaloids and phenolic acids (Victoria *et al*., 2014). Carotenoids have antioxidant and anti-cancer properties. Lutein acts directly to absorb damaging ultraviolet light in order to protect the retina (Victoria *et al*., 2014).

2.3. Anemia

Anemia is described as a condition in which the concentration of hemoglobin, hematocrit or red blood cell count in the blood is reduced below the normal range (Sharmanov, 1998; Turner *et al*., 2023). According to WHO, Anemia is a significant public health concern, mostly affecting children, pregnant and postpartum women, and menstrual adolescent girls and women.It is estimated that 30% of women aged 15 to 49 worldwide, 37% of pregnant women, and 40% of all newborns aged 6 to 59 months suffer from anemia.

Although anemia is not a disease, it is often an indication of another medical issue. Typically, drops in hemoglobin concentration less than 7.0g/dL, a patient experiences the symptoms of anemia, such as fatigue, dizziness, yellowing of the eyes, low blood pressure, etc (Figure 2.1). The normal hemoglobin ranges are as follows: 13.5 to 18.0

g/dL in men, 12.0 to 15.0 g/dL in women, 11.0 to 16.0 g/dL in children and for pregnant women the values differ based on trimester, but in general more than 10.0 g/dL (Turner *et al*., 2023).

When hemoglobin levels are insufficient, the body's ability to transport oxygen to tissues and organs is compromised, resulting in various symptoms experienced by anemic individuals. These symptoms include weakness, fatigue, and heightened susceptibility to illnesses. Anemia poses significant risks, especially for expectant mothers, potentially resulting in pre-term birth and lowered infant birth weight. Additionally, anemia can hinder the mental and physical development of children, elevating the chances of illness and death among those affected (Sharmanov, 1998).

Erythropoietin (EPO), a hormone produced in the kidney, is involved in stimulating the production of red blood cells (RBCs). The primary trigger for EPO generation is hypoxia state of tissues, and typically, EPO levels are inversely correlated with hemoglobin concentration. Patients with anemia and renal failure often exhibit unusually low EPO levels. In another instance, patients with anemia associated with chronic diseases (AOCD), have marked increases in EPO levels although below the extent anticipated, emphasizing insufficiency of EPO (Turner *et al*., 2023).

2.3.1.Classification of Anemia

Anemia is categorized into macrocytic, microcytic, or normocytic. Anemic patients usually show non-specific symptoms like, lethargy, weakness or tiredness. While syncope, reduced exercise tolerance and shortness of breath are manifestations of severe anemia (Turner *et al*., 2023). Anemia can be divided into several categories depending on the aeitiology. Anemia may arise from a variety of RBC abnormalities, in addition to those affecting RBC maturation (megaloblastic), production (aplastic

anemia), synthesis of haemoglobin (anemia due to deficiency of iron), genetic defects in maturation (thalassaemia), or hemorrhage (hemolytic anemias).

Anemia results from either continuous loss of blood or reduced rate of red blood cells production. There are varied types categories of anemia some of which are;

i. Blood Loss Anemia

In the case of excessive bleeding, the body is able to replace the fluid portion of the plasma within three days, while red blood cells concentration normalizes within three to six weeks as long as subsequent hemorrhage is avoided. In chronic situations, iron absorption from the intestine is not as adequate as to accommodate the frequency of blood loss. Therefore, smaller red blood cells having very small hemoglobin are produced (Turner *et al*., 2023). This gives rise to microcytic, hypochromic anemia.

ii. Aplastic Anemia

Aplastic anemia refers to a condition in which the bone marrow has lost its function as it relates to red blood cell formation. The condition is caused by factors such as exposure to gamma radiation, excessive x-ray treatment, some industrial chemicals and drugs (Turner *et al*., 2023).

iii. Megaloblastic Anemia

Red blood cells, which grow too large, and having odd shapes are called megaloblasts(Figure 2.2). They are formed as a result of poor absorption or lack of vitamin B12, folic acid or other intrinsic factors from the stomach mucosa. Megaloblasts are mostly oversized and typically with fragile membranes (Turner *et al*., 2023). They rupture easily, leading to reduced number of red blood cells.

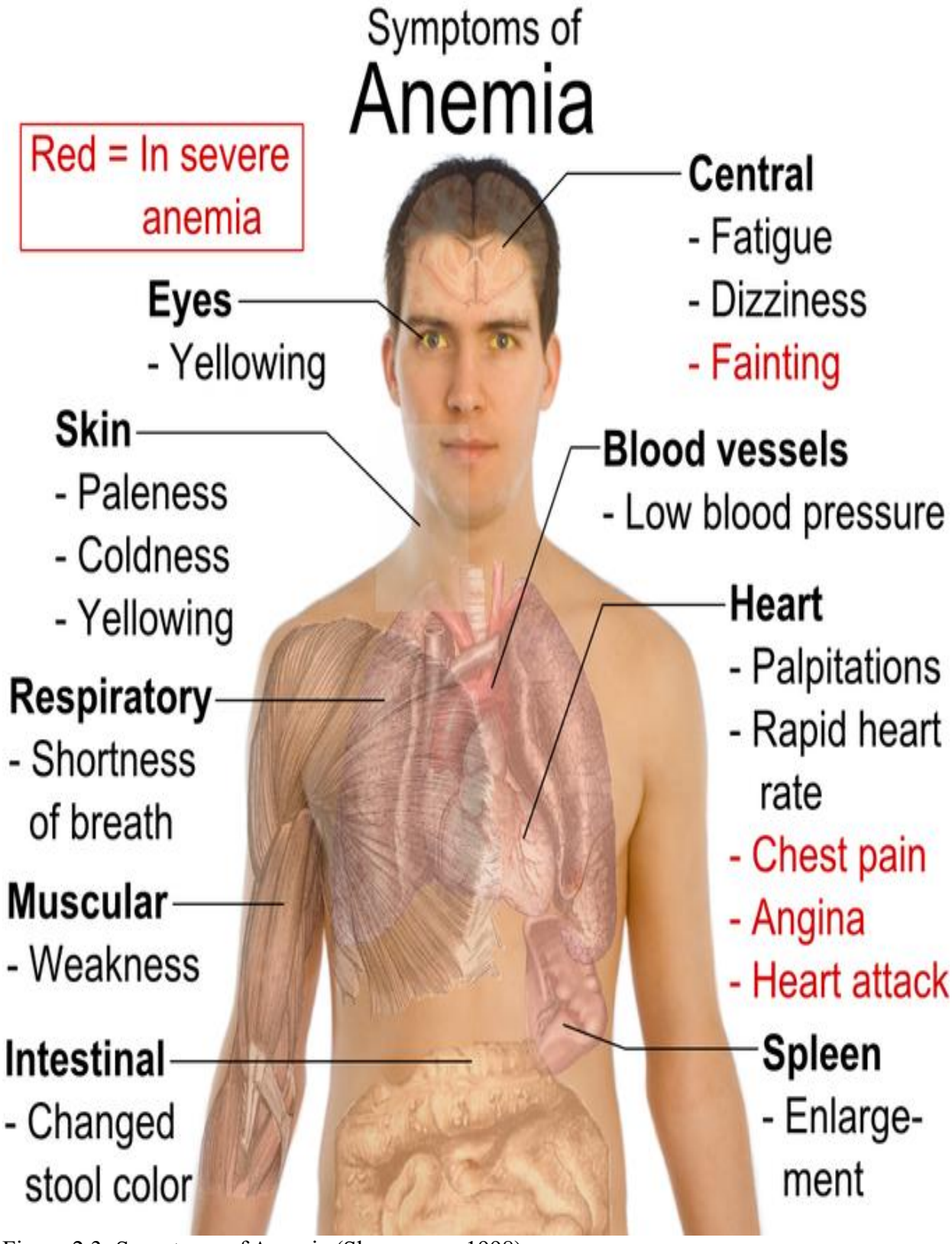


Figure 2.3: Symptoms of Anemia (Sharmanov, 1998).

iv. Hemolytic Anemia

Hemolytic anemia, in which the red blood cells are destroyed are in different forms. These abnormalities of the red blood cells, many of which are hereditarily, make the cells fragile, so that they rupture easily as they go through the capillaries, especially through the spleen (Turner *et al*., 2023). Even though the number of red blood cells formed may be normal, or even much greater than normal in some hemolytic diseases, the life span of the fragile red cell is so short that the cells are destroyed faster than they can be formed, and serious anemia results. Some of these types of anemia are the following:

a. Hereditary Spherocytosis

Hereditary spherocytosis, is characterized by red blood cells that are very small and sphericalinstead of the normal biconcave discs. Due to the difference in features, the cells are not able to withstand compression forces as they lack the normal loose, baglike cell membrane structure of the biconcave discs (Turner *et al*., 2023). They are easily ruptured by even slight compression when passing through the splenic pulp and some other tight vascular beds.

b. Sickle Cell Anemia

Sickle cell anemia, a genetic disorder ismarked by red blood cells which have the abnormal hemoglobin ‘S’. It iscaused by abnormal composition of beta chains in the hemoglobin molecule. At reducedoxygen levels in the blood, hemoglobin ‘S’precipitatesforming long crystals which elongate the cell making it sickle in shape rather than biconcave (Turner *et al*., 2023). This precipitationmakes the cell membrane highly fragile, leading to red blood cell destruction and subsequently, serious anemia called a sickle cell disease.

c. Erythroblastosis Fetalis

Erythroblastosis fetalis is a blood condition where by a fetus with a Rh-positive red blood cells areattacked by the antibodies ofits Rh-negative mother. Theantibodies make the Rh-positive cells of the fetus fragile, leading to quick rupture, allowing the child to be born with severe anemia. The condition triggers extremely rapid formation of red blood cells to compensate for the destroyed ones, thereby resulting in a large number of early blast forms of red cells being released from the bone marrow into the blood. Red blood cell hemolysis also occasionally results from transfusion reactions, health conditions like malaria, certain drugs, and in some autoimmune processes. (Kamil*et al*., 2022).

2.4. Free Radicals

A free radical is any molecular species which contains an unpaired electron in an atomic orbital, captures electrons from other substances in order to neutralize themselves and is capable of existing independently (Halliwell and Gutteridge, 1999). Free radicals allow enormous chain chemical reactions in the body due of their ability to easily react with other molecules. These reactions are known as oxidation. They could be of benefit or may be harmful (Kumar, 2011).

Most radicals have a very short half-life (10^{-6}seconds or less) in biological systems due to their high reactivity. Several radicals are very reactive as they easily donate an electron to or extract an electron from other molecules (Halliwell and Gutteridge, 1989). However, some reactive species can survive much longer than their normal half-life. When free radicals donate/accept electrons from other molecules, they become stabilized and start producing other radicals in the process which leads to chain

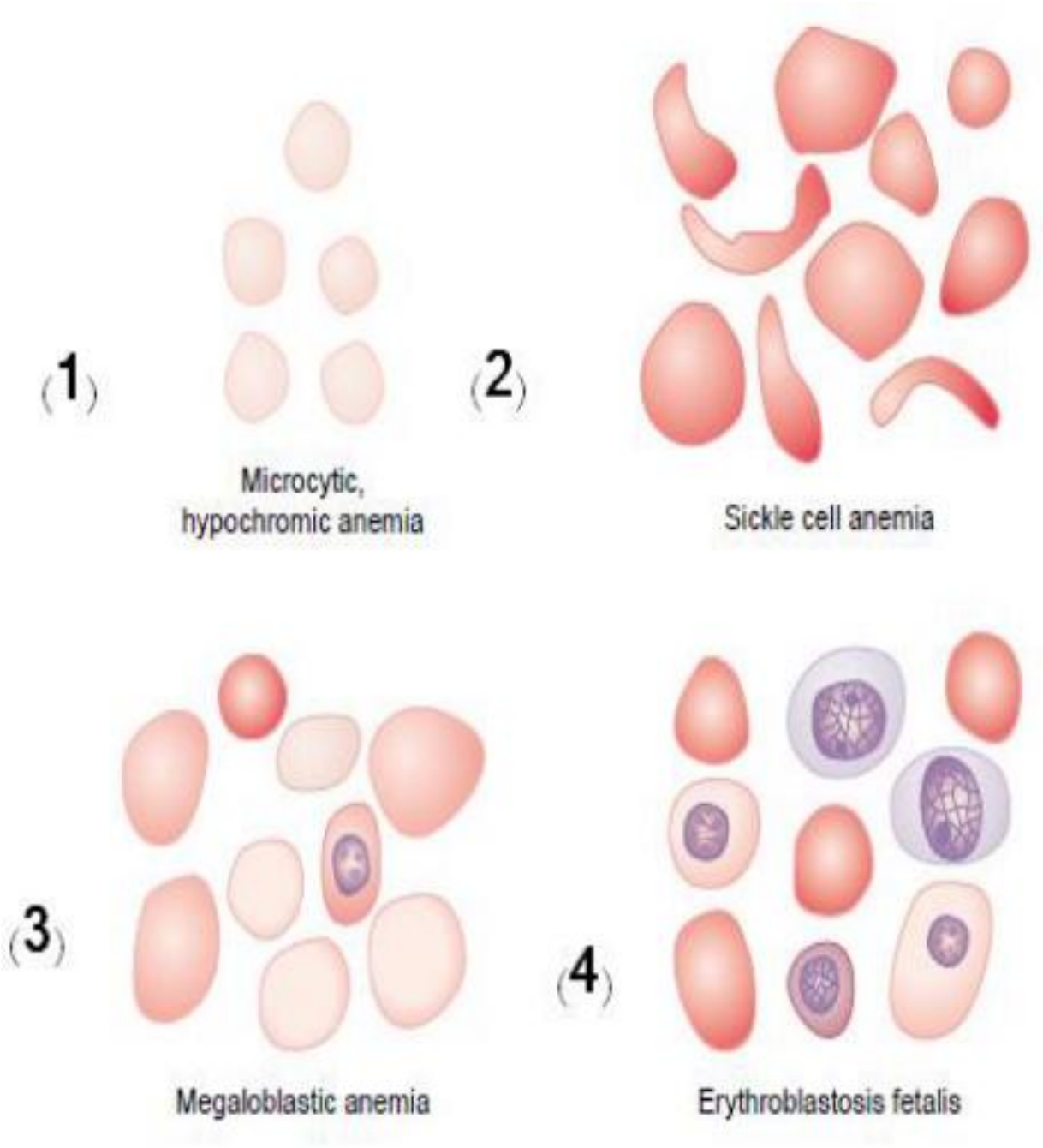


Plate 2.3: Shapes of red blood cells in the different types of anemia. Source: Kamil*et al*., (2022)

reactions and series of several free radical reactions take place within few seconds of the primary reaction (Cheeseman and Slater, 1993; Kumar, 2011).

Free radicals can be formed by three ways

a. Homolytic cleavage of covalent bond of normal molecule, witheach fragment retaining one of paired electrons.

$$X{:}Y \longrightarrow X^{*} + Y^{*}$$

b. Loss of single electron from normal molecule

$$X{:}YX^{-} + Y^{-} \longrightarrow$$

c. Addition of single electron to normal molecule

$$X + e^{-}X^{-} \longrightarrow$$

Figure 1. Mechanism of free radicalsformation (Kumar, 2011)

Free radicals are harmful and capable of causing damage to important biological molecules such as DNA, proteins, lipids and carbohydrates. Super oxides and hydroxyl radicals are the most significant free radicals in many disease states (Young and Woodside, 2001)

2.4.1. Free Radicals and their Chemical Reactions

Free radical formation in the body areas a result of enzymatic and non-enzymatic reactions. Enzymatic reactions usually include reactions involved in the respiratory chain, phagocytosis, prostaglandin synthesis, and in the cytochrome P-450 system (Liu *et.al*., 1999).

These free radicals attack the body's healthy cells and cause severe disorders, diseases or damages such as cancers, heart diseases, brain function decline and immune system (Harman 1992).

i.Super oxides (O_2^-): These radicals are produced by the addition of one electron to oxygen. Molecules like thiol compounds, flavin nucleotides, adrenaline,glucose, and

several other molecules can be oxidized to produce super oxides. These reactions are accelerated greatly by transition metals most especially copper and iron. For Instance, Hydrogen peroxide reacts with iron II (or copper I) generating the hydroxyl radical, a reaction first described by Fenton in 1894:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\cdot} + OH^{-}$$

Enzymatic reactions in the body may lead to the formation of free radicals. The activity of liver enzymes involved in the production of adrenal hormones and cytochrome P_{450} oxidase can also result in the leakage of few electrons into the cytoplasm forming super oxides. Super oxides are responsible for lipid peroxidation and reduction of activity of enzymatic antioxidant defense system like catalase (CAT) and glutathione peroxide (Halliwell and Gutteridge, 1992; Lloyd *et al*., 1997; Barbacanne*et al*., 1999; Valko *et al*., 2007).

ii. Hydrogen Peroxide (H_2O_2): Hydrogen peroxide, althoughby itself not a free radical, is categorized as a reactive oxygen species (ROS). Hydrogen peroxide is a weak oxidizing agent capable of directly disrupting structural architecture of proteins and enzymes (which have reactive thiol groups). Hydrogen peroxide is highly reactive with the propensityof freely crossing the cell membrane and damaging tissues. This is a vital property and ability of hydrogen peroxide because super oxides do not have the ability to do so (Valko *et al*., 2007).

iii. Hydroxyl Radical (OH^-): Hydroxyl radicals are the most potent cytotoxic agents and most reactive species which can attack and cause damages to molecules found in living tissues, such as lipids, sugars, nucleotides and amino acids.Super oxides and hydrogen peroxides exert most of their pathological effects by inducing the formation of hydroxyl radicals (Stohs and Bagchi, 1995).

2.5. Oxidative Stress

The term“oxidative stress” was invented by Helmut Sies in 1985; the term “stress” emphasizes the disruptionswhichtake place in the existing cellular prooxidant-antioxidant balance (Galaris*et al*., 2019). Oxidative stress refers to an imbalance in antioxidants and oxidants within the cells as a result of increased reactive nitrogen species (RNS), reactive oxygen species (ROS), and Reactive Sulfur species (RSS) production or an antioxidant deficiency, these may lead to potential cellular damage (Aziz *et al*., 2016; Law *et al*., 2017).

Categories of reactive oxygen species (ROS) are; Hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-), superoxide anion radical (O_2^-), perhydroxyl radical (HO_2^-), hypochlorous acid (HClO), nitric oxide radical (NO^-), hypochlorite radical (OCl^-), peroxynitrite (ONOO), and various lipid peroxides.

Reactive nitrogen species (RNS) are produced when nitric oxide reacts either exogenously or endogenously with reactive oxygen species such as superoxide anion radical (O_2^-) and hydrogen peroxide (H_2O_2), while Reactive Sulphur species (RSS) are produced when thiols react with reactive oxygen species (ROS) (Krishnamurthy and Wadhwani, 2012; Lu *et al*., 2009). Generally, cells protect themselves against ROS damage through metal chelating actions, free radical scavenging actions and intracellular enzymatic reactions, these actions retain the ROS homeostasis at a low level. Recall, oxidative stress may be due to increased ROS within the body, therefore if the ROS levels are low, the cells protect themselves against oxidative stress (Lu *et al*., 2009; Krishnamurthy and Wadhwani, 2012).

2.5.1. Oxidative Damage to Proteins

Oxidative damage in proteins may result in a variety of specific damage products due to modifications of amino acids such as increased electrical charge, aggregation of

cross-linked products and fragmentation of peptide chains. When proteins have been oxidized, they are more likely to undergo proteolysis and increased number of oxidized proteins (free radical damage to proteins) may be implicated in cataract and aging (Kumar, 2011; Law *et al*., 2017).

2.5.2. Oxidative Damage to Lipids

Lipids perform very vital roles in the structure and function of cell membranes. After apoptosis, lipids in membranes become easily oxidized via peroxidation processes. Polyunsaturated fatty acids are specific targets for ROS attack. Hydroxyl radicalinitiates ROS chain reactions and lipid peroxidation of polyunsaturated fatty acids (Kurutas, 2016). Several compounds produced because of lipid peroxidation are used as markers in lipid peroxidation assays. Such compounds include alkanes, isoprostanes and malondialdehyde. These compounds have been confirmed in various diseases including neurogenerative diseases, heart disease, and diabetes (Bagchi and Puri, 1998; Law *et al*., 2017).

Lipid Peroxidation refers to oxidative damage of polyunsaturated fatty acids (PUFA). This damage is destructive because it proceeds as a self-perpetuating chain reaction. This process is explained in the reaction below

$$LH + R^{\cdot} \rightarrow L^{\cdot} + RH \qquad (1)$$

$$L + O_2 \rightarrow LOO^{\cdot} \qquad (2)$$

$$LOO^{\cdot} + LH \rightarrow LOOH + L^{\cdot} \qquad (3)$$

From the equations above, LH represents, target polyunsaturated fatty acid (PUFA) while $R^{\cdot}$ represents, initiating oxidizing radical. In equation 1, LH is oxidized to form a fatty acid radical $L^{\cdot}$. On addition of oxygen, $L^{\cdot}$ forms a fatty acid peroxy radical $LOO^{\cdot}$ as seen in the second equation. In the third equation, the fatty acid peroxy radical ($LOO^{\cdot}$) can further oxidize polyunsaturated fatty acid molecules (LH) and

initiate new chain reactions, generating lipid hydro peroxides (LOOH) which can further generate more reactive radical molecules (Balasaheband Pal, 2015).

2.5.3. Oxidative Damage to DNA

Activated oxygen and agents that produce oxygen-free radicals have the potential to causeDNA damage, for example, ionizing radiations like x-rays, promote damage in DNA that leads to deletion, mutations, and other negative genetic effects. The damage also makes the sugar and base moieties in the DNA structure prone to oxidation, leading to base degradation, single strand breakage and cross linkage to proteins. Free radical damage to DNA underlies the development of cancer and accelerated aging (Pandey and Rizvi, 2010; Law *et al.*, 2017).

2.6. Antioxidants

Antioxidants are compounds which have the capacity to inhibit oxidation, even at small concentrations.

They act as free radical scavengers by reacting with reactive radicals and rendering them less active, and less dangerous substances than the neutralized radicals. Antioxidants neutralize free radicals by accepting or donating electron(s) thus removing the unpaired status of the radical (Lu *et al*., 2009).

Antioxidants tend to reduce the cellular level of free radicals by inhibiting the expression of free radical generating enzymes like xanthine oxidase and NAD(P)H oxidase or by promoting the activity and expression of antioxidant enzymes like superoxide dismutase (SOD) and catalase (CAT) (Shebis*et al*., 2013; Shih *et al*., 2007). Cells and organs in the body can be protected by antioxidants from the deleterious effect of oxidative stress through a variety of defensive mechanisms, including enzymatic and non-enzymatic reactions that cooperate with each other.

Endogenous antioxidants are produced by the body to neutralize the free radicals in the body and shield the body

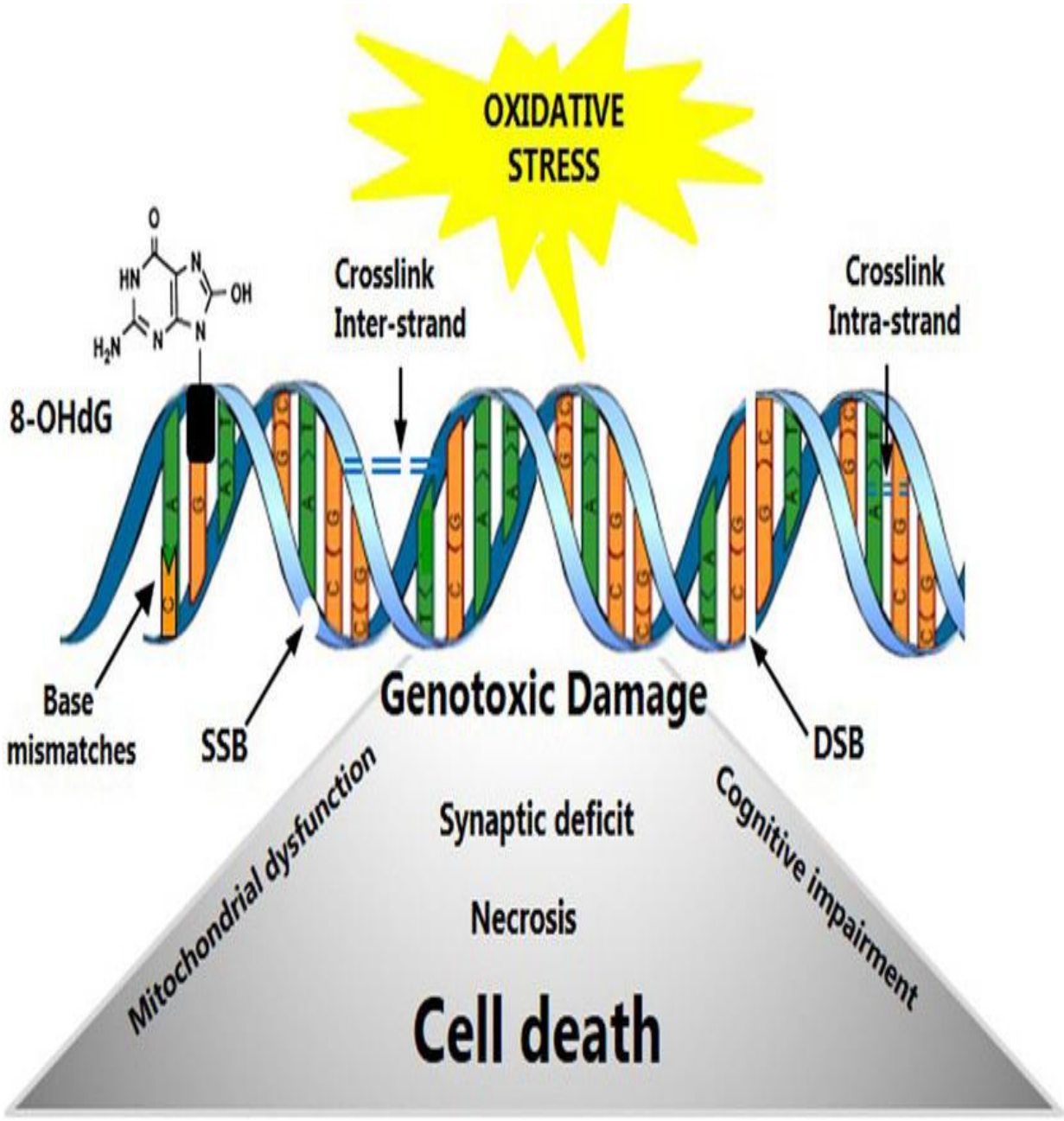


Figure 2.2: DNA damage types due to oxidative stress (Castillo and Aristizabal-pachon, 2017)

from different diseases. Exogenous antioxidants are supplied to the body via food intake.

2.6.1 Classification of Antioxidants

Generally, antioxidants are classified asenzymatic and non-enzymatic antioxidants but can also be classified on various basis. The various classifications include;

a. Based on their activity, antioxidants are classified as enzymatic and non-enzymatic antioxidants. Enzymatic antioxidants break down and get rid of free radicals by converting dangerous oxidative products to H_2O_2 and water with the aid of cofactors like copper (Cu), manganese (Mn), zinc (Zn), selenium (Se), and iron (Fe) (Shahidi and Zhong, 2010,Nimse and Pal, 2015).

Non-enzymatic antioxidants act by interfering with free radicals chain reactions. Examples include vitamin C, vitamin E, plant polyphenol and carotenoids.

b. On the basisof solubility, antioxidants are classified as water-soluble or lipid-soluble antioxidants. Vitamin C is a water-soluble vitamin present in cellular fluids such as cytosol or cytoplasmic matrix (Shahidi and Zhong, 2010,Nimse and Pal, 2015).

c. According to size, antioxidants are classified as small molecule or large-molecule antioxidants. The small molecule antioxidants neutralize ROS in a process named radicals scavenging. The major small molecule antioxidants are Vitamin E, glutathione (GSH), Vitamin C and carotenoids while large molecule antioxidants include superoxide dismutase (SOD), Catalase (CAT), glutathione peroxidase (GPX) and albumin. These group of antioxidants react

with ROS,inhibiting them from destroying other proteins(Shahidi and Zhong, 2010;Nimse and Pal, 2015).

d. Based on occurrence**,** antioxidants are classified as natural or synthetic antioxidants. Natural antioxidants function by breaking chains, reacting with free radicals to transform them into more stable substances. They are all non-enzymatic and generally phenolic in structure. They are further categorized, they include;

i. Antioxidant minerals:These refer to cofactors like selenium, copper, iron, zinc, and manganese, which are essential components of antioxidant enzymes. (Shahidi and Zhong, 2010, Nimse and Pal, 2015).

ii. Antioxidant vitamins: they are important and are necessary for most metabolism functions in the body. They include vitamin C, E, and B (Shahidi and Zhong, 2010,Nimse and Pal, 2015).

iii. Phytochemicals: They are derivatives of phenolic compounds which are neither minerals nor vitamins. For example,carotenoids, flavonoids, catechins, lycopene, carotene, herbs and spices such as diterpene, thyme and rosmari quinone (Shahidi and Zhong, 2010, Nimse and Pal, 2015).

Synthetic antioxidant compoundsinclude butylated hydroxyl toluene (BHT), propyl gallate (PG), and many other compounds (Shahidi and Zhong, 2010,Nimse and Pal, 2015).

2.6.1.1. Antioxidant Enzymes

Enzymatic antioxidants disintegrate and eliminate free radicals by transforming dangerous oxidative products to H_2O_2 and water with the aid of cofactors. They basically catalyze reactions that neutralize free radicals and ROS. Glutathione peroxidase (GPX), Catalase (CAT), and Superoxide dismutase (SOD) are the best-

known enzymes of the antioxidant protection system (Shahidi and Zhong, 2010,Nimse and Pal, 2015).

i. Catalase

This was the first enzyme to be characterized. It is responsible for the degradation of hydrogen peroxide (H_2O_2) generated by oxidases involved in respiration, catabolism of purines and β-oxidation of fatty acids. Catalase hasfour subunits, each of which containsone heme molecule plusan NADPH specie. Catalases catalyze the conversion of hydrogen peroxide to water and oxygen occurs in two steps/stages

i. catalase–Fe(III) + $H_2O_2 \rightarrow$ compound I

ii. compound I + $H_2O_2 \rightarrow$ catalase–Fe(III) + $2H_2O + O_2$

Catalase is used in the food industry during cheese production to remove H_2O_2 from milk, used in textile industries to ensure the materials are peroxide free by removing H_2O_2 , esthetic industries now use this enzyme in facial masks because the combination of CAT with hydrogen peroxide can be used to increase cellular oxygenation in the epidermis upper layer (Krishnamurthy and Wadhwani, 2012).

ii. Glutathione Peroxidases

Glutathione peroxidases catalyze the oxidation of glutathione using hydrogen peroxide as substrate or other peroxides like lipid hydroperoxide.

$$ROOH + 2GSH \rightarrow GSSG + H_2O + ROH$$

Glutathione peroxidases require seleniumat the active site, and the presence of reduced glutathione (GSH). Within cells, high levels of glutathione are predominant in the cytosol and mitochondria but also in the liver. This does not dispute the fact that glutathione peroxidase is found in almost all tissues. The ratio of reduced glutathione to oxidized glutathione is maintained at high levels by the enzyme glutathione reductase, the pentose phosphate supply is responsible for the supply of the NADPH

required by glutathione reductase in maintaining the ratio of reduced to oxidizedglutathione or replenishing the supply of reduced glutathione (Gibson *et al*., 1985; Holben and smith, 1999).

$$GSSG + NADPH + H^{+} \rightarrow 2GSH + NADP^{+}$$

Glutathione peroxidase is an important antioxidant enzyme in the body. The functionality of the enzyme relies on the continual presence of reduced glutathione (GSH). GSH functions as an immune system booster of the body. It protects the white blood cells which are responsible for the immune system, ensures red blood cellsareunchanged and increase the protection of the body from free radicals by combining with other antioxidants like Vitamin C, Vitamin E and glutathione peroxidase (Krishnamurthy and Wadhwani, 2012).

iii. Superoxide Dismutase (SOD)

Superoxide dismutase catalyzes the dismutation reaction of superoxide to hydrogen peroxide, which are removed by either a catalase or a glutathione peroxidase.

$$O_2^{-} + O_2^{-} + 2H^{+} \rightarrow H_2O_2 + O_2$$

Three forms of SOD exist in mammalian tissues, all categorized by their metal cofactors, each form is produced by distinct genes but they all catalyze the same reaction. They include copper zinc superoxide dismutase (CuZn-SOD), manganese superoxide dismutase (MnSOD) and extracellular superoxide dismutase (ECSOD).

(a) **Copper zinc superoxide dismutase (CuZn-SOD):** CuZn-SOD occurs in the cytoplasm and organelles of most mammalian cells, having a molecular mass of 32,000kDa, two protein subunits with each consisting of a copper and zinc atom (both catalytically active) (Young and Woodside,2001).

(b**) Manganese superoxide dismutase (MnSOD):** Unlike CuZn-SOD, this is present in the mitochondria of nearly all mammalian cells. It has a molecular mass of 96,000

kDa, four protein subunits with each subunit containing a single manganese atom. In a mixture of the two enzymes (MnSOD and CuZn-SOD), the activity of MnSOD can be easily distinguished from the other enzymes on the basis of its amino acid sequence which is totally different from that of CuZn-SOD and on the basis of its inhibition (MnSOD is not inhibited by cyanide, but CuZnSOD is inhibited by cyanide) (Young and Woodside, 2001).

(c) **Extracellular superoxide dismutase (ECSOD):** It is a secretory copper and zinc containing SOD distinct from CuZnSOD synthesized by very few cell types like fibroblasts and endothelial cells, this enzyme is bound to heparin sulphates and expressed on cell surfaces (Young and Woodside,2001).

2.6.1.2 Non-Enzymatic Antioxidants

In our diet, antioxidant constituents improve health status via prevention of oxidative stress and certain diseases. For example, plant derived compounds (phytochemicals) performvitalfunction in body functions. Due to the presence of hydroxyl groups in the structure of natural compounds in food materials, they are usually said to possess antioxidant properties (Shui and Leong, 2004).

Synthetic and natural antioxidants scavenge free radicals formed in different biochemical pathways, preventing oxidative damage to the essential macromolecules (in humans) like proteins,nucleic acids and lipids (Shui and Leong, 2004). These antioxidants include Vitamin C, Vitamin E and β-Carotene, flavonoids, tannins, coumarins, phenolics and terpenoids. Aside phytochemical antioxidants, other antioxidants like polyphenols, lutein and lycopene protect the body from oxidative damage also (Moon and Shibamoto, 2009; Perumalla and Hettiarachchy, 2011).

2.6.1.3. Low Molecular Weight Antioxidants

Low molecular weight antioxidants are subdivided into lipid soluble antioxidants like carotenoids, tocopherol, quinones, bilirubin and water-soluble antioxidants like ascorbic acid (Vitamin C), uric acid and other polyphenols.

i. Vitamin E

Lipid phase chain-breaking antioxidants serve to eliminate radicals that are present in membranes and lipoprotein particles, thereby preventing lipid peroxidation. They basically prevent lipid peroxidation (Arredondo, 2016). Vitamin E stands out as the principal lipid phase antioxidant in this context. It manifests in eight variations, encompassing both tocopherols (α, β, γ, and δ) and tocotrienols($\alpha, \beta, \gamma \; and \; \delta$). The key distinguishing feature between tocopherols and tocotrienols lies in the unsaturated tail structure of tocotrienols (Horwitt, 1991).

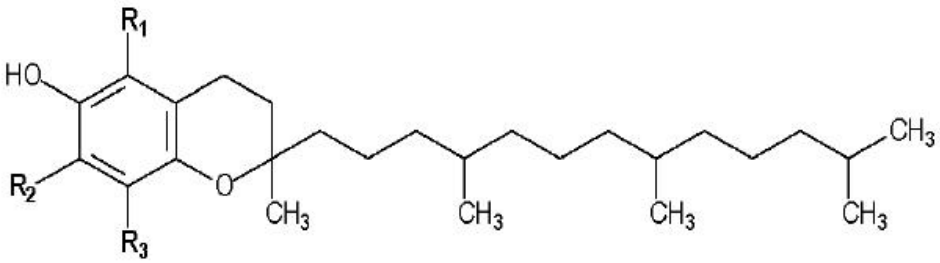
α-tocopherol is the most powerful of the tocopherols and the primary form of Vitamin E possessing antioxidant and immune function. It has been proven to be a very efficient inhibitor of lipid peroxidation induced by peroxy-nitrate, and an inhibitor of inflammatory reactions. α -tocopherols can be regenerated by reaction with an aqueous phase chain breaking antioxidant like ascorbate, urate or glutathione (McCormick and Parker, 2004; Walter and Marchesan, 2011). In vitro, tocotrienols have amazing and excellent antioxidant activity and are also said to restrain ROS more than tocopherols.

ii. Carotenoids

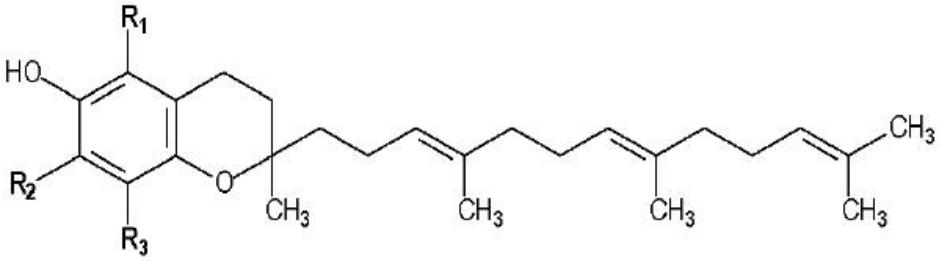
Carotenoids, a group of lipid soluble antioxidants are natural pigments (functionally and structurally different) present in numerous fruits and vegetables. The antioxidant characteristics of carotenoids include scavenging single oxygen, trapping peroxyl

radicals at low oxygen pressure with great efficiency as that of α-tocopherol, sulfonyl, sulfur and NO_2 radicals and finally protection of lipids from hydroxyl and superoxide radical attack (Chaudiere and Ferrari-Iliou, 1999; Rahman, 2007).

Metabolites derived from carotenoids, as well as carotenoids themselves, serve a protective function against disorders associated with reactive oxygen species (ROS), such as cancer, cardiovascular diseases, and myocardial infarction, especially among smokers. Intake of foods or supplements rich in carotenoids can decrease the morbidity in non-smokers and risk of prostate cancer (Skowyra, 2014).



Tocopherol structure



Tocotrienol structure

Figure 2.3: Structure of Tocopherol and Tocotrienol (Young and Woodside, 2001)

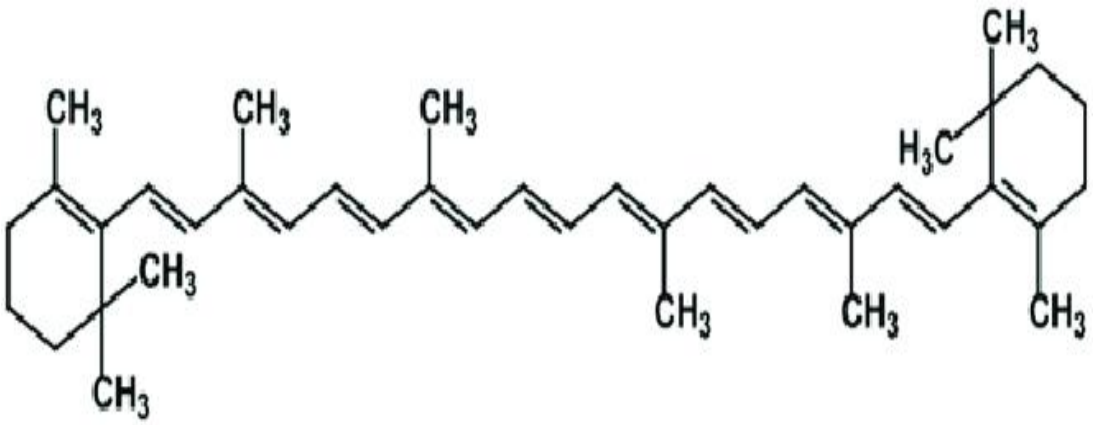


Figure 2.4: Chemical structure of Beta Carotene (Shankaranarayanan*et al.,* 2018)

iii. Vitamin A

Vitamin A is a fat-soluble vitamin possessing free radicals scavenging properties, which allows it to function as a natural antioxidant within the body, thereby helping to prevent chronic illnesses such as cancer and cardiovascular diseases.Trans retinol, the primary dietary variant of vitamin A, is naturally found in the form of fatty acid esters like retinyl palmitate, whereas retinal and retinoic acid are less common natural dietary forms of Vitamin A (Arredondo, 2016).Although Vitamin A cannot be synthesized in body and is gotten by food supply, it is essential for life in mammals due to its antioxidant role. It functionsas a unique preventive nutrition regimen against neurodegenerative diseases (Sauvant*et al*., 2012).

iv. Uric Acid

Uric acid, is a potent free radical scavenger with an estimate of about 60% radical scavenging activity in the plasma. It acts as a potent antioxidant by inhibiting the generation of reactive oxygen species (ROS) when xanthine oxidase (XO) is active. This enzyme produces free radicals during the breakdown of xanthine and hypoxanthine (Ames *et al.*, 1981; Skowyra, 2014).

v. Vitamin C

Vitamin C is a potent antioxidant found in the aqueous phase of cells. It is a water-soluble vitamin that protects biomembranes from lipid peroxidation through the elimination of peroxyl radicals in the aqueous phase before peroxidation can even be initiated. In humans, ascorbate is as an important cofactor in enzymes that catalyze hydroxylation reactions. For instance, in the synthesis of collagen, ascorbate acts as a cofactor for the enzymes,prolyl and lysyl oxidases (Arredondo, 2016; Thomas *et al*., 1996). Vitamin C loses electrons to stabilize reactive species. Ascorbate undergoes a twoelectron reduction where it is reduced to semidehydroascorbyl radical and

subsequently to dehydroascorbate. The semidehydroascorbyl radical exhibits relatively stable characteristics, whereas dehydroascorbate, in contrast, is comparatively unstable and readily undergoes hydrolysis to form diketogulonic acid. Subsequently, diketogulonic acid breaks down into oxalic acid.Dehydroascorbate can be converted back to ascorbate through two pathways;

i. one pathway involves mediation by a selenoenzyme, thioredoxin reductase and;
ii. another pathway utilizes a non-enzymatic reaction mechanism that (Pietri *et al*., 1994).

Ascorbate functions as a scavenger of superoxide, hydrogen peroxides, hypochlorous acids, aqueous peroxyl radicals, hydroxyl radicals and singlet oxygen (Skowyra, 2014).

2.6.2. Plant Derived Antioxidants

Phenolics (large, heterogenous groups of secondary plant metabolites) are said to possess high antioxidant properties, they act as chain breaking antioxidants by competing for chain carrying peroxyl radicals with the substrate. Phenolic acids that possess antioxidant properties include; vanilic, caffeic, sinapic and coumaric acids (Taruscio*et al*., 2004). Many berries, including strawberries, raspberries, cloudberries, and blueberries, are rich in cinnamic acids, ellagic acid, ferulic acid, and numerous other derivatives (Zadernowski*et al*., 2005).

i. Flavonoids

Flavonoids, the major class of phenolic compounds, form a significant group of polyphenolic antioxidants found in plants, i.e fruits, vegetables, and beverages like tea and wine.

Polyphenols are compounds with several or many phenolic hydroxyl substituents (Young and Woodside, 2001). Flavonoids, which have a low molecular weight, play vital roles as antioxidants by acting as reducing agents, singlet oxygen scavengers, and hydrogen donors due to their high redox potential. They are categorized into different groups based on their chemical structures, such as flavonols (e.g., quercetin and kaempferol), flavanols (like the catechins), flavones (e.g., apigenin), and isoflavones (such as genistein) (Walter and Marchesan, 2011; Young and Woodside, 2001).

Flavonoids possess metal chelating potential.When individuals consume fruits or vegetables abundant in flavonoids, there is evidence suggesting a correlation between flavonoid intake and a loweredsusceptibility to diseases including breast and prostate cancer (Jaganathan *et al*., 2014; Sharmila *et al*., 2014; Yiannakopoulou, 2014).

iii. Tannins

Tannins unlike flavonoids are large molecular weight compounds constituting the third indespensable group of phenolics. Tannins are categorized into hydrolysable tannins (these heterozygous polymers contain phenolic acids especially, gallic acid/ 3, 4, 5- trihydroxyl benzoic acid including simple sugar) and condensed tannins (these are usually produced by the polymerization of flavonoid units. The focus of research primarily centers on condensable tannins, which predominantly derive from flavan-3-ols like (-) epicatechin and (+) –catechin (Walter and Marchesan, 2011; Skowyra, 2014).

Tannins have various effects on biological systems due to their metal ion chelating properties, their antioxidant properties and due to the fact that they are protein-precipitating agents. Although they are effective in biological systems, their structural variations and diversity in biological roles has made it difficult to modify models that

would allow a precise prediction of their effects in any biological system. This implies that the modification of tannin structure and activity relationship of tannins are important in the protection of their biological effects (Skowyra, 2014).

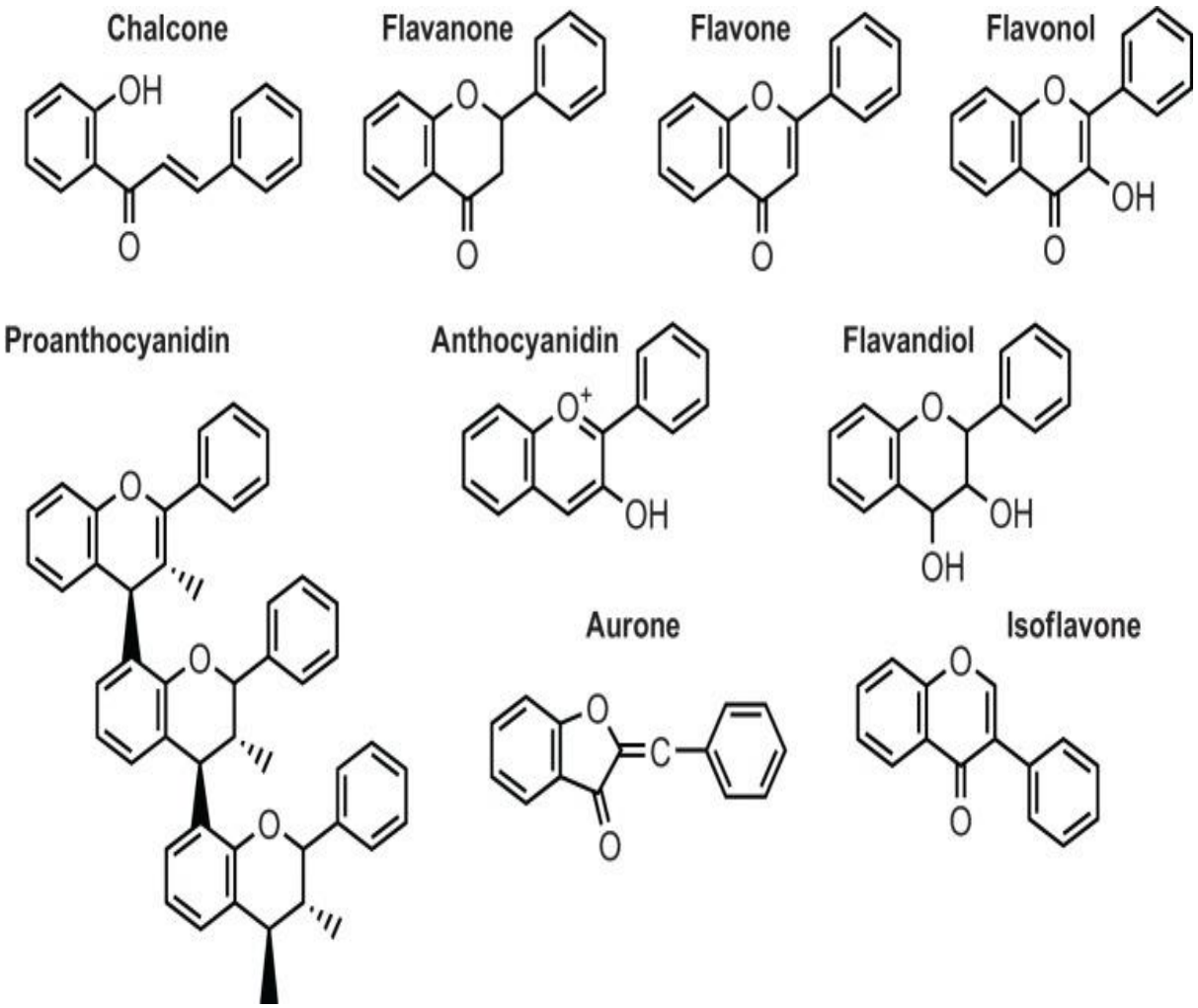


Figure 2.5: Structures of main classes of flavonoids (Ferreyra *et al*., 2012)

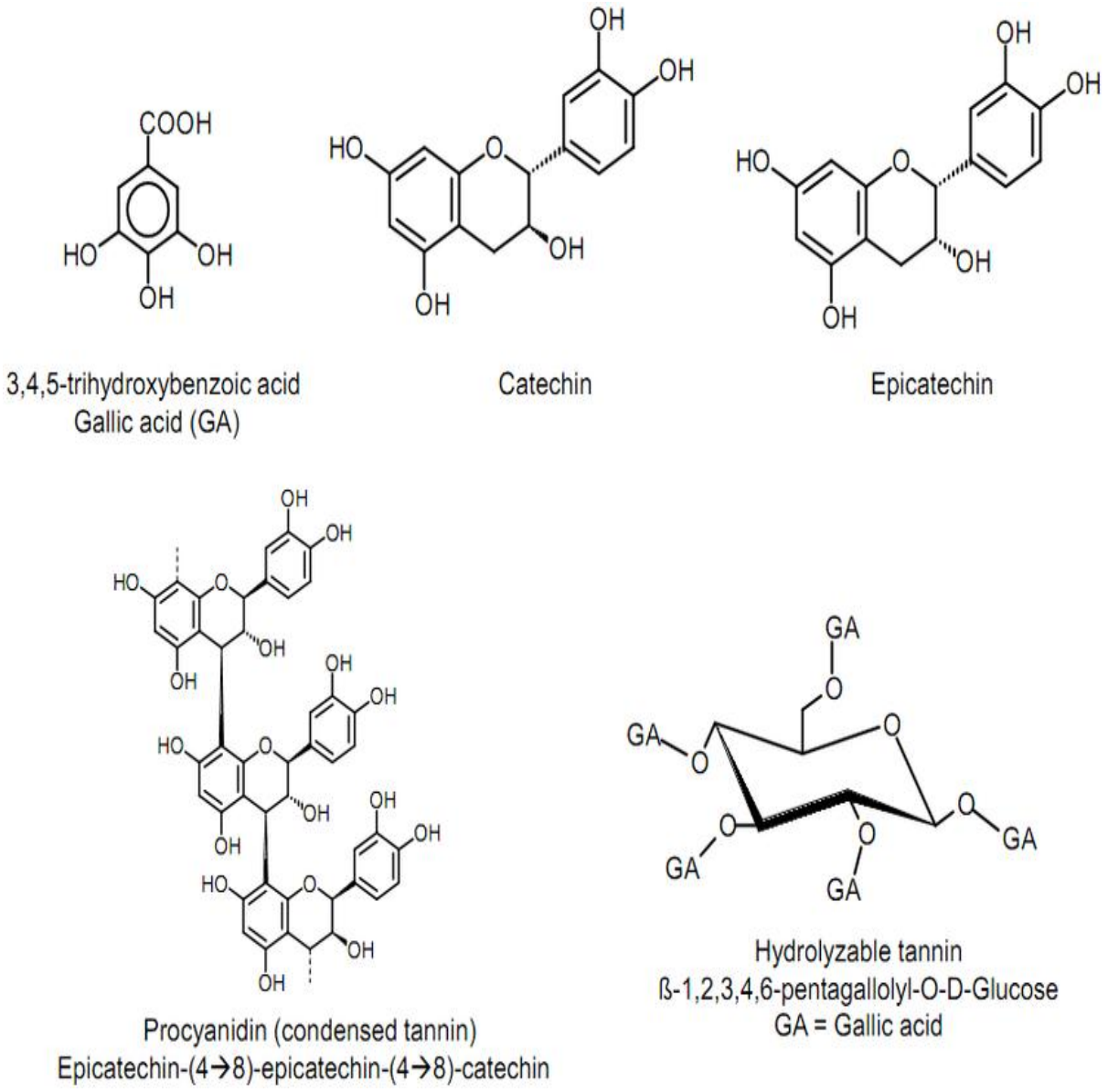


Figure 2.6: Typical structures of hydrolysable and condensed tannins (Lamy *et al.,* 2011)

2.7. Phenylhydrazine

Phenylhydrazine (Hydrazino benzene) (PHZ)was first characterized by Hermann Emil Fischer in 1895 (Singh *et al.,* 2014). It is a yellow to pale brown oily liquid. It has the emperical formula, $C_6H_8N_2$, density of 1.10 g/cm³, molecular mass of 108.14g/mol, with boiling point of 243.5 °C and melting point of 19.5 °C(Singh *et al.,* 2014).

It is generally used as a chemical intermediate in the agrochemical, pharmaceuticaland chemical industries.Derivatives of phenylhydrazine were primarily used as antipyretics until their toxic effects on the red blood cells was reported. Thereafter, PHZbecame the first choice for chemical induction of experimental anemia in animal models. It is an effectiveagentin treating polycythemia Vera2 a disordersynonymous with increased number of erythrocytes (Singh *et al.,* 2014). Phenylhydrazine reduces hemoglobin levels, RBC (Red Blood Cell) count and PCV (Packed Cell Volume) while increasing the MCV (Mean Cell Volume), MCH (Mean Cell Hemoglobin), MCHC (Mean Corpuscular Hemoglobin Concentration) and extramedular hematopoiesis in the liver

andspleen (Singh *et al.,* 2014).

2.7.1. Mechanism of Phenylhydrazine Induced Hemolytic anemia

Phenylhydrazine actively induces hemolytic anemia. Exposure to the compound is via inhalation, oral and skin contacts. Metabolism of the compound generates benzene, nitrogen, hydrogen peroxide, superoxide anion and phenyl radicals which induce oxidative stress within erythrocytes. As a result, hemoglobin is oxidized thereby leading to methemoglobin formation and subsequently irreversible hemichromes. This reaction causes hemoglobin precipitation as ‘heinz bodies’ are formed (Singh *et al.,* 2014). PHZ also damages skeletal proteins and lipids, resulting in ATP reduction,

cation alterations, and reduced deformation of membranes. The overall effect of these reactions is destruction of red blood cells and hemolytic anemia (Singh *et al.,* 2014).

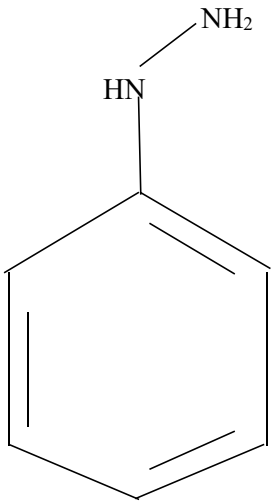


Figure 2.7: Chemical structure of Phenylhydrazine

Phenylhydrazine-induced anemia increases iron absorption (Latunde-dada *et al*.,2006; Singh *et al.,* 2014; Shwetha, *et al.,*2019),whichpromotes gene expression in the duodenum forDcytb, DMT1-IRE and Ireg genes, involved in iron transport. In the spleen and liver, Dcytb and Ireg1 genes are also involved in iron metabolism (McKie *et al*., 2004).

In the immune system, PHZinduces immune activation (Singh *et al.,* 2014). It crosses red blood cells membrane and binds with free autologous antibodies. The macrophage receptors recognize the antigen-antibody complex, initiating phagocytosis of the red blood cells in the spleen and in the liver. Consequently, the spleen removes damaged cells intact. In addition to storing blood and supporting immune function, the spleen serves as the primary organ for engulfing red blood cells in rodents and rabbits experiencing PHZ-induced hemolytic anemia (Singh *et al.,* 2014).

Phenylhydrazine impacts the EPO receptors of JAK-STAT pathway, crucial in red blood cells maturation. Following phenylhydrazine-induced anemia, EpoRHM mice showed inadequate response to splenic stress erythropoiesis (Singh et al., 2014). The erythropoietin receptor belonging to the cytokine receptor family, activates JAK2 tyrosine kinase upon binding to erythropoietin thereby triggering various intracellular pathways, including Ras/MAP kinase, phosphatidylinositol 3-kinase and STAT transcription factors. The activated erythropoietin receptor plays a role in promoting the survival of erythroid cells. Malfunctions of the erythropoietin receptor may lead to conditions such as erythroleukemia and familial erythrocytosis. Dysregulation of this cytokine may impact on tumor size.

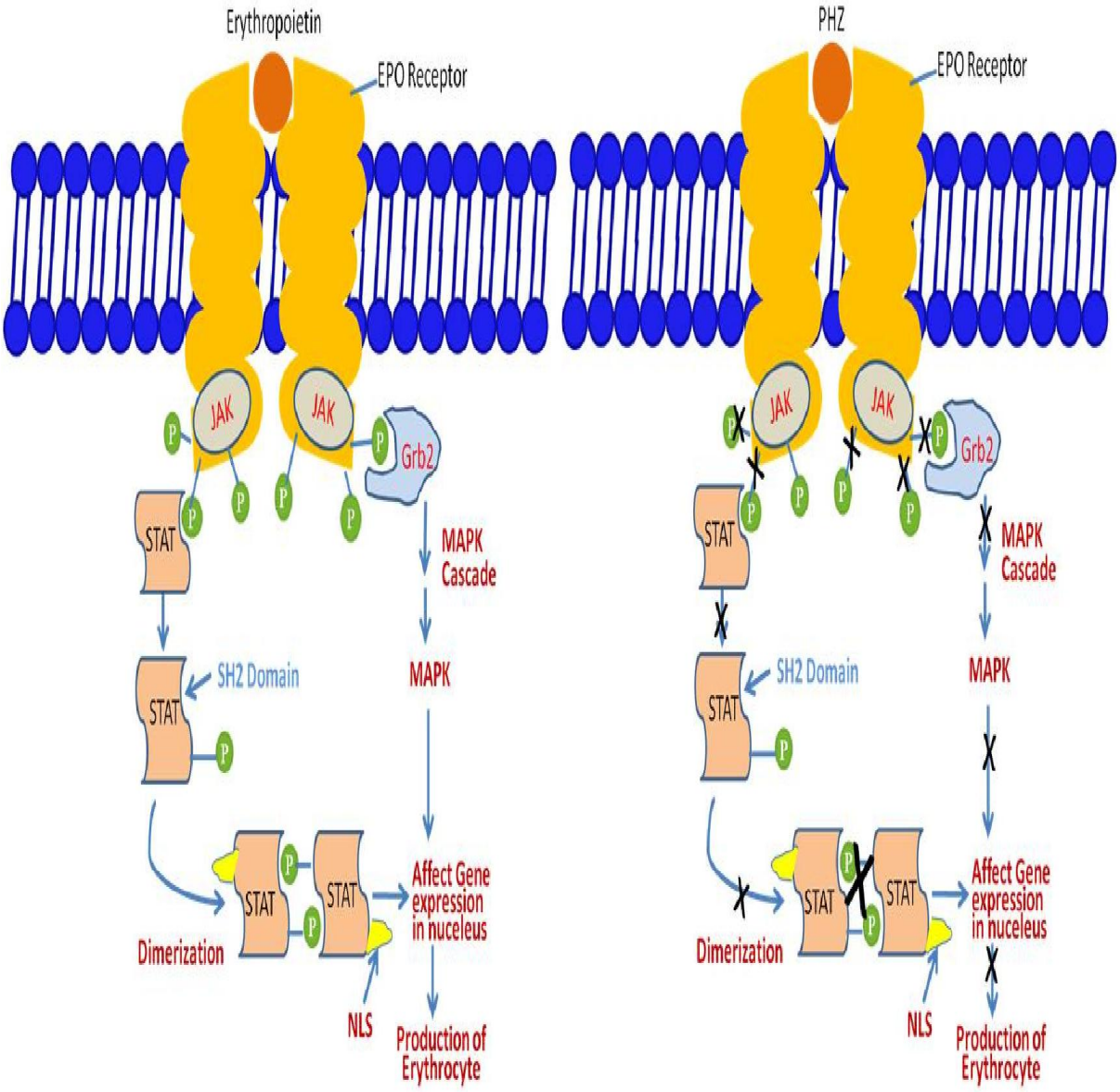


Figure 2.8: (a). Normal functioning ofFigure 2.8(b). Abnormal functioning JAK STAT Pathway. of JAK STATPathway.

(Singh *et al*., 2014)

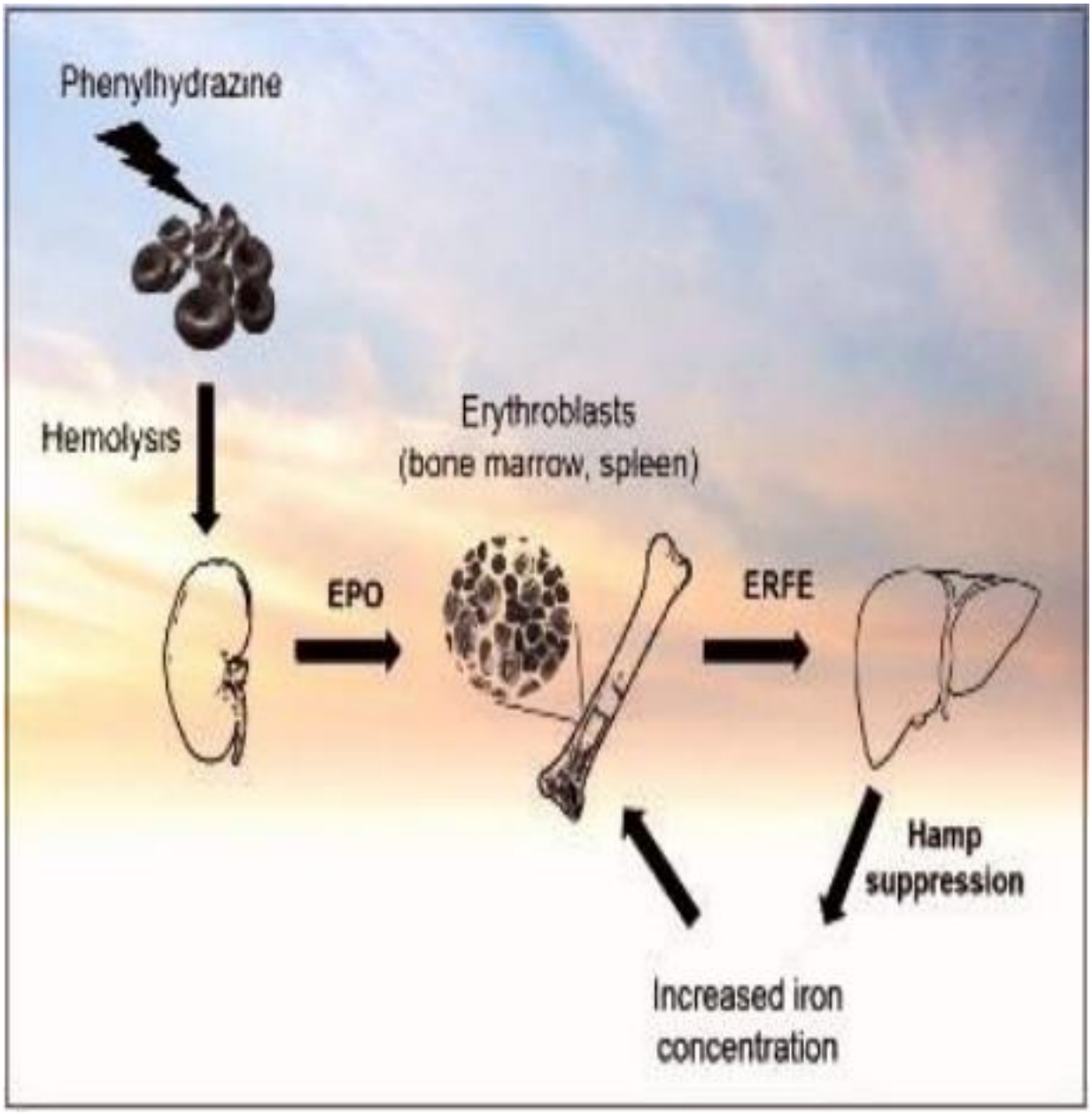


Figure 2.9: Mechanism of phenylhydrazine-induced anemia(EPO - erythropoietin, ERFE- erythroferrone, Hamp- Hepcidin antimicrobial peptide)
(Shwetha *et. al.,*2019)

2.8. Iron Metabolism and Anemia

Iron, classified as a transition metal, is a vital component found in nearly all multicellular and single celled organisms. Itsfunction in various biochemical processesis pivotal as a constituent of numerous metalloproteins. These processes include the transportation of oxygen within tissues, electron transfer reactions during mitochondrial respiration, DNA synthesis and repair, and the metabolism of xenobiotics (Galaris*et al*., 2019). Additionally, iron is highly abundant on Earth, being the most prevalent element overall and the fourth most abundant metal in the Earth's crust (Frey and Reed, 2012).Its electron configuration enables the formation of stable ferrous (Fe+2) or ferric (Fe+3) ions, and it readily forms complexes with organic molecules, which holds significant biological significance.Therefore, heme, iron-sulfur clusters or iron-oxo centers, which are iron cofactors are utilized by proteins for numerous electron transfer reactions within a broadelectrochemical potential (Eo from −500 to +500 mV) (Williams, 2012).

Iron is indispensable for various physiological functions, such as oxygen transport facilitated by hemoglobin, muscle oxygenation by myoglobin, cellular respiration through proteins within the mitochondrial electron transport chain, and DNA synthesis mediated by ribonucleotide reductase (Gkouvatsos*et al*., 2012).Furthermore, iron is involved in numerous enzymatic reactions across cytosolic and cellular compartments. In the adult human body, iron content typically ranges from approximately 3 to 5 grams, with a significant portion, about 70%, utilized in the erythron, while any excess is stored primarily in the liver (Williams, 2012).

Transferrin, a carrier protein, transports approximately 25–30 mg/day of iron from the plasma to developing erythroid cells in the bone marrow to support heme biosynthesis during hemoglobinization. It also supplies smaller amounts of iron, up to around 5

mg/day, to other tissues. Despite containing only around 3 mg of iron at steady-state, transferrin undergoes turnover approximately 10 times daily to meet physiological demands. The body replenishes its iron primarily through iron provided by tissue macrophages following the clearance of senescent red blood cells. During erythrophagocytosis, heme undergoes enzymatic degradation via heme oxygenase 1 (HO-1), and inorganic iron is subsequently released from macrophages to plasma through the transmembrane ferrous exporter ferroportin for re-utilization (Lesjak *et al*., 2014).

CHAPTER THREE

MATERIALS AND METHODS

3.1 MATERIALS

3.1.1. Experimental Animals

Male albino rats Wistar strain (100-120 g), bred in the animal houses of the departments of Biochemistry and Anatomy, University of Benin, Benin City, Edo State, were used for this research. A total of one hundred and ninety-four (194) rats were used for the study.

Experiments were performed according to the guidelines for the care and use of laboratory animals and was approved by the Life Sciences Ethics Committee for Animal Research with approval number, FLSRE-2023-008.

3.1.2. Collection of Plant Materials

Fresh leaves of *Dennettia tripetala*and *Cola acuminata* were obtained from a farm in Benin City, Edo State, Nigeria. The leaves were identified and authenticated by Prof. H.A. Akinnibosun in the Department of Plant Biology and Biotechnology, University of Benin, and herbarium specimens were assigned voucher numbers UBH-D488 and UBH-C317 respectively.

3.1.3. Chemicals and Reagents

Phosphotungstate reagent, concentrated sulphuric acid, L-ascorbic acid, Oxalic acid, xylene, Batophenanthroline, Anhydrous iron chloride, crystalline orthophosphoric acid, α-tocopherol, Anhydrous ethanol, Total antioxidant capacity reagent solution (0.6M sulphuric acid, 28mM sodium phosphate, 4mMammonium molybdate), Phosphate buffer, distilled water,$FeCl_3$ solution, Sodium hydroxide, glacial acetic acid, potassium mercuric iodine (mayer's reagent), Hager's reagent, Potassium ferricyanide, Trichloroacetic acid.

3.1.4. Equipment and Apparatus

Rotary evaporator (Surgifield, model SM-52CS-1), blender, water bath (HH-W21-Cr 4211, Techmel and Techmel Texas, USA), oven, UV Visible spectrophotometer (Genesys 10S UV-VIS Thermo scientific), ISE 4000 SFRI auto analyzer, table top centrifuge (Surgifield model SM 80-2, England), digital weighing balance (Scout Pro Spuo 401, OHAUS Corporation, USA), sensitive weighing balance (OHAUS Corporation, USA, PAG 21), Teflon homogenizer, test tubes and test tube racks, beakers, measuring cylinder, micro pipette, foil paper, conical flask, spatula, filter paper, dropper, muslin cloth, volumetric flask.

3.2 Methods

3.2.1. Preparation of Plant Extracts

Fresh leaves of *Dennettia tripetala* and*Cola acuminata* were air-dried and ground to powder using a blender. Five grams of each powdered sample was macerated in 2,500mL methanol separately for 72 hours at room temperature with continuous stirring at 24 hours interval. A muslin cloth was then used to obtain each filtrate. The resulting crude extract was concentrated with a rotary evaporator at 45°C.

3.2.2. Experimental Design

The study was divided into four phases.

Phase I:Nutritional and mineral composition, qualitative and quantitative phytochemical content, *In vitro* antioxidant capacity, High Performance Liquid Chromatograph (HPLC) and Gas Chromatography Mass Spectrometry (GC-MS) were carried out in accordance with standard methods.

Phase II: Acute and subacute toxicity of each plant extract were determined. This was done in accordance with Lorke**,** (1983), for acute toxicity testing. Nine animals per extract were used for the first phase of Lorke's method. They were separated into

three groups of three animals each. Each group received different doses (10,100 and 1000mg/kg) of each extract. The animals were placed under observation for 24hours to monitor their behaviour as well as if mortality occurred. In the second phase of Lorke's method, three animals were used per extract. They were distributed into three groups of one animal each. The animals were given higher doses of the extract (1600, 2900, and 5000mg/kg). They were observed for 24hours for behaviour as well as mortality.

For sub-acute toxicity studies, twenty-eight male albino Wistar rats were grouped into seven groups of four rats each, per extract. Each extract was administered separately with graded doses as follows: 10,100, 1000, 1600, 2900, and 5000mg/kg for groups 2-7 respectively, while group 1 received normal saline. Thereafter, animals were sacrificed and blood was collected, liver, kidney and spleen were harvested for biochemical and hematological analysis.

Phase III, Experiment 1:The effective dose of each extract against phenylhydrazine-induced hemolytic anemia was studied. Forty-two male albino Wistar rats were grouped into seven groups of six rats each, per extract. Group 1 received normal saline (normal control), group 2 received only phenylhydrazine (negative control, 60mg/kg body weight) for two consecutive days, while groups 3-7 received PHZ + 500, 1,500, 2,500, 3,500, and 4,500mg/kg body weight of each extract respectively. At the end of fourteen days treatment, animals were sacrificed and blood, kidney, liver and spleen were collected for biochemical, and hematological analysis.

Phase III, Experiment 2:The anti-anemic effect of each extract on PHZ-induced hemolytic anemia was compared. Thirty male albino Wistar rats were grouped into five groups of six rats each. Group 1 received normal saline (normal control), group 2 received 60mg/kg body weight phenylhydrazine (negative control) for two

consecutive days, group 3 received PHZ + 1,500mg/kg body weight *Dennettia tripetala* methanol leaf extract, group 4 received PHZ + 500mg/kg body weight *Cola acuminata*methanol leaf extract and group 5 received PHZ + 100mg/kg body weight vitamin C (positive control). Treatment lasted for fourteen days after which animals were sacrificed after an overnight fast. Blood was collected, kidney, liver and spleen were harvested for biochemical, hematological and histological analysis. Bone marrow histology was also studied.,

Phase IV:Quantitative polymerase chain reaction (qPCR) was carried out to determine the levels of mRNA expression of the IREG, HO1, DMT-IRE and TFR1 genes in the liver and spleen of phenylhydrazine-induced anemic rats treated with *Dennettia tripetala* and*Cola acuminata* separately.

3.2.3. Sacrifice and sample collection

At the end of treatment period of fourteen days (phase 2-4) animals were fasted overnight and sacrificed by light anesthesia. Blood was collected by cardiac puncture into EDTA and lithium heparin bottles for hematology and biochemical analysis respectively. Liver, kidney and spleen were harvested and preserved in buffered solution for other biochemical analysis. Portions of the liver, kidney and spleen were preserved in formol-saline for histological studies. Bone marrow was collected for histology analysis. Small portions of the liver and spleen were also reserved in normal saline for molecular studies.

3.2.4. Preparation of Tissue Homogenate

Weighed portions of the tissues (liver, spleen and kidney) were homogenized with a Teflon homogenizer in physiological saline (0.9 %) to obtain tissue homogenates. The resulting homogenate was centrifuged at 5,000 g for 10 min and the supernatant

obtained was stored at -4ºC in a freezer, until when needed for subsequent biochemical analysis.

3.2.5. Proximate Analysis of Leaves

The proximate analysis of the plant leaves was carried out in accordance with the Association of Official Analytical Chemists (AOAC, 2000) methods.

3.2.5.1. Determination of Percentage (%) Moisture Content

Principle: Moisture content of any sample is principled upon weight loss of mass that occurs as the sample is heated. The sample weight is taken prior to heating and again after reaching a steady-state mass subsequent to drying.

Procedure

Moisture was determined according to the standard method (AOAC, 2010). Sample (10 g) was transferred to a porcelain dish and dried for 24hours at 105 ^{0}C in an oven. The sample was cooled by placing in desiccators to cool to a constant weight before weighing again to obtain the final weight after drying.

The percentage moisture content was calculated as:

$$\% M.C = \frac{Weight\ of\ sample\ before\ drying - Weight\ of\ sample\ after\ drying}{Weight\ of\ sample} \times 100$$

3.2.5.2. Determination of % Ash Content

Principle: The principle of ashing is to burn off the organic matter and to determine the inorganic matter remained. Heating is carried out in two stages: firstly, to remove the water present and to char the sample thoroughly; and finally, ashing at 550°C in a muffle furnace.

Procedure

Three grams (3g) of dried powered leaf sample was weighed into a porcelain dish that had previously been weighed. This was dried at 100ºC for three hours in an oven. The

dish with content was transferred to a muffle furnace and ignited for six hours at 500 ºC until free from carbon (residue appears grayish-white). This was removed from the oven and the ash moisture with a few drops of water (to expose bits of unashed carbon). The ash was re-dried in the oven at 100 ºC for three hours and re-ash in in the furnace at 500 ºC for another one hour. This was removed from the muffle furnace in desiccators until it cooled and was then weighed. The percentage ash was calculated as follows:

$$\% A.C = \frac{Weight\ of\ ash}{Weight\ of\ sample} \times 100$$

3.2.5.3. Determination of Crude Lipids

Principle: A dried, ground sample is extracted with diethyl ether which dissolves fats, oils, pigments and other fatsoluble substances. The ether is then evaporated from the fat solution. The resulting residue is weighed and referred to as ether extract or crude fat.

Procedure

A 250 mL extraction (round bottom) flask was dried in an oven at 105ºC.The flask was allowed to cool in a desiccator and the empty weight was taken. One gram (1 g) of the sample was weighed into a porous thimble. The porous thimble was tied. Then about 250 mL of N-hexane was measured into the round bottom flask, attached to the Soxhlet extractor and heated in a heating mantle. The sample was extracted for 6 hr. The flask was thereafter attached to a rotary evaporator and the hexane evaporated. The flask was cooled in a desiccator and weighed. Crude lipid content was calculated as follows:

$$\% C.L = \frac{Weight\ of\ ash}{Weight\ of\ sample} \times 100$$

3.2.5.4. Determination of % Crude fibre content

Principle: Crude fiber is determined gravimetrically after chemical digestion and solubilization of other materials present. The fiber residue weight is then corrected for ash content after ignition.

Procedure

Five (5 g) of sample was treated successively with boiling solution of 0.26NH_2SO_4 and 0.28N KOH. The residue was then filtered, cleaned, transferred to a crucible, and placed in an oven at 65^{o}C for 24 hr. The sample and crucible were weighed and placed in a muffle furnace at 500^{o}C for 24 hr. The sample was cooled in a desiccator and re-weighed. The ash content of the sample was calculated as follows:

$$\% \text{ C. F} = \frac{Dryweightofresiduebeforeashing - Weightofresidueafterashing}{Weightofsample} \times 100$$

3.2.5.5. Determination of % Crude protein content

Principle: Protein in the sample was determined by Kjeldahl method. The samples were digested by heating with concentrated sulphuric acid (H_2SO_4) in the presence of digestion mixture. The mixture was then made alkaline. Ammonium sulphate thus formed, released ammonia which was collected in 2 % boric acid solution and titrated against standard HCl. Total protein was calculated by multiplying the amount of nitrogen with appropriate factor (6.25) and the amount of protein was calculated.

Procedure

The crude protein content was determined using the method described by AOAC (2010). A 100 mL round bottom flask was attached to a Soxhlet apparatus reflux condenser and 0.5g of the dried powdered sample was placed into different filter papers and folded. Crude protein for each was determined by placing each of the

folded filter papers containing each sample into a Kjeldahl round bottom flask. The sample was digested with 10ml concentrated Sulphuric acid for about 2hoursusing selenium powder as catalyst. The apparatus was assembled and the solution was digested until the solution was clear. It was distilled by adding 100 mL of distilled water and 10 mL of a 40 % of sodium hydroxide (NaOH) and sodium thiosulphate to avoid loss of ammonia in the process. Afterwards, 10mL of boric acid and 3 drops of methyl red were then added to a 100 mL conical flask and placed at the receiving end. The apparatus was set until it got to the 100 mL point inside the conical flask. The solution was titrated with 0.1M sodium hydroxide (NaOH) to a yellow red end point and the titre value was recorded and calculated as:

$$\% \, C.P = \frac{0.001410 \times 6.25 \times 25 \, T}{W}$$

Where; W= weight of sample; 6.25 = protein conversion Factor T = titre value

3.2.5.6. Determination of Carbohydrate

The total amount of carbohydrate in the sample was obtained by using the weight difference percentage. This was done by subtracting the percentage sum of the food nutrients (% crude protein, % crude fat, % crude fibre, % moisture content and % ash) from 100% dry weight. Percentage carbohydrate was calculated, using the formula below;

Carbohydrate (%) = 100 – (%Protein + %Fat + %Fibre + %Ash + %Moisture)

3.2.6. Determination of Minerals

The sample was preserved according to recommended practices as contained in United States Environmental Protection Agency (USEPA); America Standard for Testing and Materials (ASTM). Laboratory analysis was conducted using approved standard test method.

Procedure

A 10 mL aliquot ofdried *C. acuminata* leaf was measured into a beaker and then digested with a combination of 0.04 mL of concentrated HNO_3 and0.25 mL of concentrated HCl. The mixture was then covered with a ribbed watch glass and heated on a steam bath at 95ºC until the volume was reduced to 2 mL. The beaker was removed and allowed to cool after which the sample was filtered.The filtrate was made up to 100 mL with newly prepared distilled water and the solution was used for the determination of Ca, P, Mg, K, Fe, Se, and Na using an atomic absorption spectrophotometer at 660 nm.

3.2.7. Qualitative Phytochemical Screening of Leaf Extracts

Phytochemical screening was conducted to qualitatively determine the presence or absence of the following secondary metabolites, Alkaloids, Saponins, Cardiac glycosides, Tannins, Flavonoids using the method outlined by (Enabulele and Ehiagbonare, 2011).

3.2.7.1. Alkaloids

The presence of alkaloids in the leaf extracts were determined by the method according to Enabulele and Ehiagbonare, (2011). Three drops of Hager's reagent were added to 1ml of plant extracts. The formation of a yellow coloured precipitate indicated the presence of alkaloids. Three drops of mayer's reagent were added to 1ml of plant extract. The formation of a cream coloured precipitate indicated the presence of alkaloids.

3.2.7.2. Saponins

The presence of Saponins in the leaf extracts was determined the method of Enabulele and Ehiagbonare, (2011). Five (5mL) of distilled water was added to 1 mL of the leaf

extracts in a test tube and shaken vigorously. The formation of foams or stable frothing following the shaking indicated the presence of saponins.

3.2.7.3. Cardiac glycosides

The presence of Cardiac glycosides in the plant extracts were determined according to Enabulele and Ehiagbonare, (2011). One ml of the plant extract was mixed with 2ml of glacial acetic acid and one drop of 15% $FeCl_3$ solution and 1mL of concentrated H_2SO_4 was added to the mixture obtained, to form a layer. The presence of a brown ring at the interface indicated de-oxy sugar characteristic of cardiac glycosides.

3.2.7.4. Tannins

The presence of Tannins in the leaf extracts were determined by the method of Enabulele and Ehiagbonare, (2011). One ml of extract was heated for 5 minutes and few drops of 15% $FeCl_3$ solution were added to the extracts in a test tube followed by shaking. A dirty green or dark blue coloration confirmed the presence of tannins.

3.2.7.5. Flavonoids

The presence of Flavonoids in the leaf extracts was determined according to Enabulele and Ehiagbonare, (2011). One ml of dilute ammonia and 1ml of concentrated sulphuric acid were added to 1ml of the plant extracts. The presence of a yellow colour confirmed the presence of Flavonoids.

3.2.8. Quantitative Phytochemical Screening of Leaf Extracts

3.2.8.1 Determination of Total Flavonoid Content

Principle

The principle of total flavonoid content is determined by the Aluminum Chloride colorimetric method. The aluminum chloride colorimetric method's main principle is that aluminum chloride forms a stable complex with C-4 keto group and either with

the C-3 or C-5 hydroxyl group of flavonoids. The $AlCl_3$ also forms labile complex with ortho-dihydroxyl groups present in A or B ring of flavonoids. The complex imparts yellow color and is detected by the absorbance at about 510 nm.

Procedure

Total flavonoids were quantified as per the method of Ebrahimazaden*et* al., (2008). In this method, 0.5mL of the extract (1 mg/ mL in methanol) was mixed 1.5mL of methanol and 0.1 mL of 10% aluminum chloride and 2.5mL of distilled water. Leave the mixture at room temperature for 30 minutes. A spectrophotometer was used to measure absorbance at 415nm. The results extrapolated from the standard curve were expressed as milligrams quercetin equivalent (QE) per gram of extract (mgQE/g extract). The standard curve was calibrated with quercetin using graded concentration of 0.02, 0.04, 0.06, 0.08, 0.10 mg/ml.

3.2.8.2. Determination of Total Phenolic Content

Principle

The principle of total phenolic content is a method used to measure the amount of phenolic compounds in a sample. It involves the use of calorimetric assays, such as the Folin- Ciocalteaumethod, which relies on the reduction of a reagent by phenolic compounds resulting in the formation of a blue-coloured complex a peak absorbance at 760 nm occurs concurrently with the process. The TPC, describe as the Gallic acid equivalent, is directly proportional to the absorbance.

Procedure

The method of Folin and Ciocalteau(2007) was used to determine total phenolics. In this method, 0.5mL of the extract (1 mg/ mLin methanol) was mixed 2.5mL of Folin-Ciocalteau reagent (0.1 %v/v) and 2 mL of 7.5% Sodium Carbonate in this method. Leave mixture at room temperature for 30 minutes. A spectrophotometer was used to

measure absorbance at 760nm. The results extrapolated from the standard curve were expressed as milligrams gallic acid equivalent (GAE) per gram of extract (mgQE/g extract). The standard curve was calibrated using gallic acid at graded concentration of 0.02, 0.04, 0.06, 0.08, 0.10 mg/ml.

3.2.9. Gas Chromatography-Mass Spectrometry (GC-MS) And High Performance Liquid Chromatography (HPLC) Analysis

The GC-MS analysis of the leaves was performed on an Agilent 6890 gas chromatograph (GC) which was interfaced to an Agilent 5973N Mass Spectrometer (MS) and fitted with a META X5 coated fused capillary column Length: 30m, Diameter: 0.25mm with a film thickness of 0.25µm; and a maximum temperature, 325 °C. The HPLC analysis of the extracts was performed using a RESTEK 15METER MXT-1 HPLC system with methanol AT 5PSI as carrier.

3.2.10. *In Vitro* Antioxidant Activity Determination

3.2.10.1. Total Antioxidant Capacity (TAC) assay:

The Total Antioxidant Capacity (TAC) of *Dennettia tripetala* and*Cola acuminata* leaf extracts was determined by the phosphomolybdate method according to Prieto *et al*. (1999). 1 ml of aliquot (0.1, 0.2, 0.4, 0.6, 0.8, 1.0 mg/mL) of the leaf extracts were mixed with 3mL of the reagent solution (0.6M sulphuric acid, 28mM sodium phosphate, 4mM ammonium molybdate) in test tubes. The tubes were capped with aluminum foil and incubated in boiling water at 95°C for 90 min. The reaction mixture was allowed to cool at room temperature and the absorbance of the solution was measured at 695nm against a blank. The blank contained 3ml of the reagent solution and the appropriate volume of the dissolving solvents. This was incubated

under the same conditions as the test samples. Ascorbic acid was used as the standard reference compounds to compare the activities of the extracts. The test was done in triplicates.

3.2.10.2. Reducing Power Assay

The reducing power of *Dennettia tripetala* and *Cola acuminata* methanol leaf extracts was determined according to the method described by Anandjiwala*et al.* (2007).

Principle

In this assay the yellow color of the test solution changes to green or blue depending on the reducing power of antioxidant sample.

Procedure

Different concentrations (0.1, 0.2, 0.4, 0.6, 0.8, 1.0mg/ml) of each extract were prepared and 1 mL of each concentration was mixed with 2.5 mL of phosphate buffer (0.2 M, pH 6.8) and 2.5 mL of potassium ferricyanide. The mixture was incubated at 50^{o}C for 20 minutes. To this mixture, 2.5 mL of trichloroacetic acid (10%) was added to stop the reaction then centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5 mL) was mixed with 2.5 mL of distilled water and 0.5 mL of 0.1% . The absorbance of the mixture was measured at 700nm. Higher absorbance values indicated higher reducing power. Vitamin C served as the reference control.

3.2.10.3. DPPH Radical Scavenging Activity

2,2-diphenyl-1-picrylhydrazyl (DPPH) was determined according to the method described by the method of Brand-Williams *et al*. (1995).

Principle

The assay is based on the ability of an antioxidant compound to reduce 2,2-diphenyl-1-picrylhydrazyl (DPPH) by donation hydrogen resulting color change from deep purple violent to golden yellow.

Procedure

About 0.1 mL of the extract and fractions ((100 mg/ mL) was added by 3.9 mL of 0.15mM DPPH in ethanol. The solution then was mixed vigorously and allowed to stand in the dark for 30 mins at room temperature. All tests were carried out in triplicate and ascorbic acid was used as a standard control at concentrations comparable to the test samples, a blank solution containing 0.1 mL ethanol with 3.9 mL DPPH was used as a control. Absorbance was measured at 517 nm using a spectrophotometer. The DPPH scavenging activity was calculated according to the equation (Jamous *et al*., 2015)

$$\%\text{Radical Scavenging Activity}=\frac{Absorbance\ of\ control-Absorbance\ of\ test}{Absorbance\ of\ control}x100$$

3.2.11. Evaluation of Kidney Function Parameters

3.2.11.1 Estimation of Plasma Electrolytes

Principle

The Electrolyte analyzer is a highly automated machine which applies the Ion Selective Electrode (ISE) technology in its working. The Ion Selective Electrode is a type of electrochemical sensor which converts ion activity to the electric potential of the electrode. The relation is in line with the NERNST equation, in which the Logarithm of the ion activity has a linear relationship with the electrode potential. Thus, the contents of potassium (K^+), sodium (Na^+), chloride (Cl^-)and bicarbonate(HCO_3^-)in plasma were measured (ISE series operator manual version 2.0).

Procedure

A small amount of sample (plasma), 150µL was required. The reagents for analysis of the various electrolytes were pre-loaded into the analyzer. On application of the run button, the sample was aspirated via the sample probe then the analysis was run automatically and the results displayed on the screen or printed out.

3.2.11.2. Estimation of Plasma Urea Concentration

Principle

Urea in plasma is hydrolyzed to ammonia in the presence of urease. The ammonia was then measured photometrically by Berthelot's reaction (Fawcett and Scott, 1960)

$$\text{urea} + H_2O_2 \xrightarrow[\text{urease}]{} 2NH_3 + CO_2$$

$$NH_3 + \text{hypochlorite} + \text{phenol} \longrightarrow \text{indophenol (blue compound)}$$

Procedure

	Blank	Standard	Sample
Sample	-	-	5µL
Standard	-	5µL	-
Distilled H_2O	5µL	-	-
Reagent 1	50µL	50µL	50µL
Mix and incubate at 37ºC for 10minutes			
Reagent 2	1.25mL	1.25mL	1.25mL
Reagent 3	1.25mL	1.25mL	1.25mL

Mix and incubate for 15minutes at 37ºC. Read absorbance of sample and standard against blank at 546nm.

3.2.11.3. Estimation of Plasma Creatinine

Principle

Plasma creatinine levels are a useful indicator of renal function. Creatinine is derived from creatine and creatine phosphate in muscle tissues and may be defined as a nitrogenous waste product. Creatinine is not reutilized but is excreted from the body in the urine via the kidney. Creatinine is regarded as the most useful endogenous marker in the diagnosis and treatment of kidney diseases (Allen *et al.,* 1982).

Procedure

	Reagent BlankSO	**Standard SI**	**Sample**
ddH_2O	25µL		
Standard		25µL	
Sample			25µL
Working Reagent	250µL	250µL	250µL

3.2.12. Evaluation of Liver Function Parameters

3.2.12.1. Estimation of Plasma Protein

Protein concentration in plasma and tissue samples was determined by the Biuret method, using Randox kit (Gornall *et al.,* 1949).

Principle

Proteins form a complex with cupric ions in an alkaline solution as exemplified by the biuret reagent, which contains copper sulphate ($CuSO_4$), potassium Iodide (KI) and sodium potassium tartarate. The protein and biuret reagent form a complex with maximum absorbance at 546m. The concentration of protein in the sample is obtained by calculation.

Procedure

	Reagent blank	standard	Sample
Distilled water	0.02ml	-	-
Standard	-	0.02ml	-
Plasma	-	-	0.02ml
R1	1ml	1ml	1ml

Mix and incubate for 30minutes at 20-25ºC. Read absorbance at 546nm.

Calculation:

$$\text{Total protein concentration} = \frac{\text{Absorbance of Sample} \times \text{Standard Concentration}}{\text{Absorbance of standard}}$$

3.2.12.2. Estimation of Plasma Albumin

Principle

Albumin is the most abundant plasma protein representing 55-65% of the total protein. It is synthesized in the liver and has a half-life of 2-3weeks. The main biological functions of albumin are to maintain the water balance in serum and plasma transport and store a wide variety of ligands e.g fatty acids, calcium, bilirubin and hormones such as thyroxine. Albumin also provides an endogenous source of amino acids (Grant,1987)

Procedure

	Reagent	**Standard**	**Sample**
Distilled H_2O	0.01mL		
Standard (cal)		0.01mL	
Plasma			0.01Ml
BCG reagent(R1)	3.00mL	3.00mL	3.00Ml

3.2.12.3. Estimation of Plasma Aspartate Aminotransferase (AST)

Principle

AST is measured by monitoring the concentration of oxaloacetate hydrazone formed with 2,4-dinitrophenylhydrazine (Reitman and Frankel,1957).

Procedure

	Reagent blank	**Sample**
Sample		0.1Ml
Reagent 1	0.5mL	0.5mL
Distilled Water	0.1mL	
Mix and incubate for exactly 30 minutes at 37˚c		
Reagent 2	0.5mL	0.5mL
Mix and allow to stand for exactly 20min at 25˚c		
Sodium Hydroxide	5.0mL	5.0mL
Mix, read the absorbance of the sample against the sample blank after 5 minutes		

3.2.12.4. Estimation of Alkaline Phosphatase (ALP)Rec.GSCC,1972

Principle

p-nitrophenylphosphate + H_2O→ phosphate + p-nitrophenol ().

Procedure

Sample	0.01mL
Reagent	0.5mL
Mix and read 405nm	

3.2.12.5. Estimation of Alanine Aminotransferase (ALT)

Principle

Alanine aminotranseferase is measured by monitoring the concentration of pyruvate hydrazone formed with 2,4-dinitrophenylhydrazine (Reitman and Frankel,1957).

Procedure

	Reagent Blank	Sample
Sample		0.1mL
Solution R1	0.5mL	0.5mL
Distilled water	0.1mL	
Mix, incubate for exactly 30min at 37˚C		
Solution R2	0.5mL	0.5mL
Mix, allow to stand for exactly 20mins, at 25˚C		
Sodium hydroxide	5.0mL	5.0mL

Mix, read the absorbance of sample against the reagent blank after 5mins.

3.2.13. Evaluation of *In Vivo* Oxidative Stress Indices in Kidney, Liver, and Spleen

3.2.13.1. Determination of Lipid Peroxidation

Lipid peroxidation was assessed as malondialdehyde (MDA) content of the kidney according to the method of Varshney and Kale (1990).

Principle

Under acidic condition, MDA produced from the peroxidation of membrane fatty acid and food products reacts with the chromogenic reagent 2-thiobarbituric acid (TBA), to yield a pink coloured complex with maximum absorbance at 532nm and fluorescence at 533nm. The pink chromophore is readily extractable into organic solvents such as butanol.

Procedure

An aliquot of 0.4ml of tissue post-mitochondria fraction was mixed with1.6ml of tris-KCL buffer to which 0.5ml of 30% TCA was added. Then 0.5ml of 0.75%TBA was added and placed in a water bath for 45mins at 80ºC. This was cooled in ice and centrifuged at 3,000g for 10minutes. The clear supernatant was collected and absorbance measured against a reference blank of distilled water at 532nm. The MDA level was calculated according to the method of Adam-Vizi and Seregi (1982). Lipid peroxidation in µmole/ml was computed with a molar extinction coefficient of $1.56x10^5$ $M^{-1}cm^{-1}$.

3.2.13.2. Determination of Catalase Activity

Catalase activity was assayed according to the method of Cohen *et al*., (1970).

Procedure

Zero point five (0.5) ml aliquot of the homogenate (kidney, liver and spleen) samples were added into ice-cold test tubes, while the blank tube contained 0.5ml of distilled water. The reaction was initiated by the sequential addition of 5ml cold 20mM H_2O_2 at fixed intervals. It was then mixed thoroughly by inversion. After 3 minutes, the reaction was stopped sequentially by the rapid addition of 1ml of 6.0M H_2SO_4 solution at fixed intervals. 7ml of 0.01M $KMnO_4$ was added to the test samples and the blank test tubes. The absorbance was read at 480nm after one minute interval.

3.2.13.3. Determination of Reduced Glutathione (GSH) Levels

Reduced glutathione level was determined by the method of Beutler *et al*., (1963).

Principle

Reduced glutathione consists mainly of cellular non-protein sulfhydryl groups. This method is based on the development of a relatively stable yellow colour when 5/,5/ -(2-nitrobenzoic acid) (DTNB) or Ellman's reagent is added to sulfhydryl compounds.

The chromophoric product resulting from the reaction of Ellman's reagent with the reduced glutathione 2-nitro-5-thiobenzoic acid possesses a molar absorption at 412nm.

Procedure

Zero point two milliliters (0.2ml) of sample was added to 1.8ml of distilled water and 3ml of the precipitating solution was mixed with kidney homogenate. The mixture was then allowed to stand for approximately 5 minutes and then filtered. At the end of the fifth minute, 1ml of filtrate was added to 4ml of 0.1M phosphate buffer. Finally, 0.5ml of the Ellman's reagent was added. A blank was prepared with 4ml of the 0.1M phosphate buffer, 1ml of diluted precipitating solution (3 parts to 2 parts of distilled water) and 0.5ml of the Ellman's reagent. The optical density was measured at 412nm. GSH was proportional to the absorbance at this wavelength and the estimate was obtained from the GSH standard curve.

3.2.13.4. Determination of Glutathione-S-Transferase (GST) Activity

Glutathion-s-transferase activity was measured by the method of Habig *et al*., (1981).

Principle

The principle of this assay is based on the fact that all known glutathione-s-transferase activity utilizes 1-chloro 2,4-dinitrobenzene as substrate. When this substance is conjugated with reduced glutathione, its absorption maximum shifts to a longer wavelength. The absorption increase at the new wavelength of 340nm provides a direct measurement of the enzymatic reaction.

Procedure

The medium for the estimation was prepared as shown in the table below and reaction allowed to run for 60 seconds each time before the absorption is read against the blank at 340nm. The temperature was maintained at 31ºC and the results were tabulated appropriately after the absorbance was read.

Reagent	Blank	Test
Reduced glutathione (0.1M)	30µl	30µl
CDNB (20mM)	150 µl	150 µl
0.1M Phosphate buffer. pH 6.5	2.82ml	2.79ml
Post mitochondria fraction	-	30 µl

3.2.14. Hematological analysis

Hematological analysis of the rat blood was carried out using a hemocytometer. A sample of blood was diluted with an isotonic solution (2% acetic acid), and the number of red blood cells in a fraction of this diluted blood was counted, and then multiply by a correction factor.

3.2.15. Histopathological examination of tissues

Histology analysis of kidney, liver and spleen tissues was carried out according to the method of Bancroft and Gamble, (2008). The excised organs were fixed in 10 percent formal saline. Fixed tissues were completely dehydrated in ascending concentrations of alcohol (70, 90, 96 and 100 percent). The tissues were placed in xylene to remove the alcohol, impregnated and embedded with molten paraffin wax. They were allowed to solidify before sectioning into 4um using a microtome (Leica RM 2235, UK) the 4um sections were placed on slides and stained with hematoxylin -eosin dye (Bancroft and Gamble, 2006). Stained slides were viewed using an optical photomicroscope (Olympus 230 V 50/60 He, China) and camera (Eakins 12Mega pixels, UK) at x40, x100 and x400 magnification.

3.2.16. Bone marrow histochemistry

Perl's Prussian blue staining of bone marrow tissues was done by Mallory's method (Luna, 1968; Sheehan, and Barbara (1980))

Principle

The reaction occurs with the treatment of sections of the bone marrow in acid solutions of ferrocyanides. The ferric ions (Fe^{3+}) in the tissue reacts with ferrocyanide to form a bright blue pigment known as 'Prussian blue".

Procedure

Prestaining was carried out by heat drying tissue section/slides in oven and cleansing glass wears to avoid residual iron staining. Staining was done by deparaffinizing sections thoroughly in three changes of xylene, 3 minutes each. Hydration was done through two changes each of 100% and 95% ethyl alcohols, 10 dips each. They were washed well with distilled water.

Fresh ferrocyanide working solution was prepared directly before use. A mixture of 20ml 20% hydrochloric acid and 20ml 10%, potassium ferrocyanide aqueous was prepared and placed in fresh ferrocyanide working solution for 20minutes.It was rinsed in three changes of tap water/ distilled water. Nuclear Fast Red Stain, Kernechtrotwas added and allowed to stand for 5 minutes. Thereafter, was rinsed again in distilled water.This was dehydrated in two changes each of 95% and 100% ethyl alcohol, then cleared in three changes of xylene, 10 dips each. A coverslip was placed over using a compatible mounting medium.

3.2.17. RNA Extraction and Quantitative Polymerase Chain Reaction (qPCR) Analysis

RNA was extracted from the liver and spleen of rats using the Quick-RNA Miniprep Kit (Zymo Research, Catalogue No. R1054). qPCR was carried out with Biorad CFX Mange instrument and CFX Manager SoftwareVersion 3.1.3086.0516.

3.2.18. Data Analysis

All values were expressed as mean ± standard deviation. One-way Analysis of Variance (ANOVA) was carried out and differences between means were determined by Fisher's multiple range post – hoc tests using MINITAB software. p-values ≤ 0.05 were be considered as significant.

CHAPTER FOUR

RESULTS

4.1. Proximate content of *Dennettia tripetala* and *Cola acuminata* Leaves

Tabe 4.1 shows nutritional composition of the leaves of *Dennettia tripetala* and *Cola acuminata.* The resultsreveal that both leaves contain proteins, lipids, carbohydrates and minerals. They also contain appreciable quantity of fibre and low moisture content. However, *Dennettia tripetala* had a higher percentage of protein while *Cola acuminata* had a higher percentage of lipids, fibre and carbohydrates. Moisture content of *Dennettia tripetala* was lower than that of *Cola acuminata.*

4.2. Qualitative Phytochemical Analysis of *Dennettia tripetala* and *Cola acuminata* Leaves

The results for the qualitative phytochemical analysis of *Dennettia tripetala* and *Cola acuminata* leaves are shown in table 4.2. Both leaves contain flavonoids, cardiac glycosides, saponins, terpenoids, alkaloids, coumarins and tannins. Steroids were absent in both leaves. Flavonoids were strongly present, cardiac glycosides, saponins and coumarins were moderately present in both *Dennettia tripetala* and *Cola acuminata* leaves. Tannins and alkaloids were strongly present in *Cola acuminata* but moderately present in *Dennettia tripetala* leaves.

4.3. Mineral Content of *Dennettia tripetala* and *Cola acuminata* Leaves

The mineral content of *Dennettia tripetala* and *Cola acuminata* leaves are shown in Table 4.3. Both leaves contain the essential minerals phosphorus, sodium, potassium, calcium, magnesium, zinc and iron. However, *Dennettia tripetala* contained higher amounts of phosphorus, calcium, zinc and iron, while *Cola acuminata* had higher amounts of sodium, potassium, and magnesium.

Table 4.1: Proximate composition of *Dennettia tripetala* and *Cola acuminata* Leaves

Nutrient	*Dennettia tripetala* (%)	*Cola acuminata* (%)
Ash	7.56 ± 0.10	6.40 ± 0.12
Protein	6.50 ± 2.65	6.12 ± 0.01
Lipids	0.12 ± 0.03	0.13 ± 0.05
Fibre content	0.56 ± 0.01	1.21 ± 0.02
Moisture content	1.99 ± 0.02	2.05 ± 0.03
Carbohydrate	83.27	84.09

Values are mean ± standard deviation (n = 3)

Table 4.2. Qualitative Phytochemical Analysis of *Dennettia tripetala* and *Cola acuminata* leaves

Phytochemicals	Inference	
	Dennettia tripetala	*Cola acuminata*
Flavonoids	+++	+++
Cardiac glycosides	++	++
Saponins	++	++
Steroids	-	-
Terpenoids	++	+++
Alkaloids	++	+++
Coumarins	++	++
Tannins	++	+++
Reducing sugars	+++	+++

Key: ++ =Strongly present ++ = Moderately present + = Slightly present - = Absent

Table 4.3. Mineral content of *Dennettia tripetala* and *Cola acuminata* leaves

Minerals	Plant sample	
	Dennettia tripetala (mg/100g)	*Cola acuminata* (mg/100g)
Phosphorous	100.5 ± 0.63	60.68 ± 1.26
Sodium	19.04 ± 0.57	26.66 ± 0.51
Potassium	24.3 ± 0.25	33.47 ± 1.34
Calcium	24.5 ± 0.34	8.63 ± 0.11
Magnesium	9.22 ± 0.08	22.34 ± 0.34
Zinc	1.97 ± 0.04	1.63 ± 0.02
Iron	5.3 ± 0.12	4.50 ± 0.03

Values are mean ± standard deviation (n = 3)

4.4. Quantitative Phytochemical Screening of Leaf Extracts

The results for the quantitative phytochemical analysis of the methanol leaf extract of *Dennettia tripetala* and *Colaacuminata* are presented in Table 4.4. *Cola acuminata* had higher amount of total flavonoids and less of total phenolics than *Dennettia tripetala*.

4.5. Gas Chromatography-Mass Spectrometry (GC-MS) of *Dennettia tripetala* and *Cola* acuminata leaves

Tables 4.5a and 4.5b, give the molecular weight, percentage composition and structure of some of the bioactive organic compounds found in the leaves of *Dennettia tripetala* and *Cola* acuminata respectively

4.6. High performance liquid chromatography analysis (HPLC) of *Dennettia tripetala* and *Colaacuminata* leaves

HPLC results for *Dennettia tripetala* and *Cola acuminata* leaves are presented inTables 4.6a and 4.6b.The results reveal that*Cola acuminata* had higher concentrations of Tannins, flavones, flavanones, catechins, steroids, narigenin and cyanogen glycosides, while *Dennettia tripetala*contains higher concentrations of proanthocyanidins, anthocyanidine and spartein.

4.7. *In Vitro* Antioxidant Capacity of *Dennettia tripetala* and *Cola acuminata* Leaves

Results for *In vitro* antioxidant capacity of *Dennettia tripetala* and *Cola acuminata* leaves are presented in Figures 4.1a and 4.1b.

Figure 4.1a reveals that *Dennettia tripetala*possess a higher total antioxidant capacity than *Cola acuminata* when compared with vitamin C, the standard drug.

Figure 4.1b reveal that *Cola acuminata* scavenged DPPH radical better than *Dennettia tripetala* when compared with vitamin C.

Table 4.4: Quantitative phytochemical determination of total flavonoid and phenolic contents of *Dennettia tripetala* leaf extract and*Cola* acuminata leaf extract

Phytochemical	*Dennettia tripetala*	*Colaacuminata*
Total flavonoids (mg QE/gram extract)	0.62±0.03	1.73 ± 0.09
Total phenolics (mg GAE/gram extract)	0.59±0.02	0.014 ± 0.001

*Values are expressed as mean ± SE (n=3), GAE = Gallic Acid Equivalent, QE = Quercetin Equivalent.

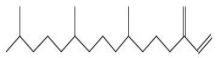
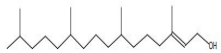
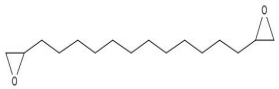
Table 4.5a. GC-MS analysis of *Dennettia tripetala*Leaves

PEAKNO.	RETENTION TIME (MIN)	% COMPOSITION	NAME OF COMPOUND	STRUCTURE	MOLECULAR WEIGHT	BIOLOGICAL ACTIVITY	REFERENCE
1	4.055	4.967	Pentadecane	$C_{15}H_{32}$ 	212	Alkane, hydrocarbon/antimicrobial	Konovalova *et al*, 2013
2	4.124	0.218	Tetradecane	$C_{14}H_{30}$ 	198	Alkane, hydrocarbon/ Antimicrobial, Cytotoxicity, Antipyretic, Anthelmentic, Tumour, Bronchitis, Asthma, Tuberculosis, Dyspepsia, Constipation, Anemia, Throat diseases, Elephantiasis, Antidiabatic, Anti-inflammatory, Antidiarrhoeal	Banakar and Jayaraj, 2017

Table 4.5a. GC-MS analysis of *Dennettia tripetala*Leaves

PEAK NO.	RETENTION TIME (MIN)	% COMPOSITION	NAME OF COMPOUND	STRUCTURE	MOLECULAR WEIGHT	BIOLOGICAL ACTIVITY	REFERENCE
3	6.521	0.538	Hexadecane	$C_{16}H_{34}$ 	226	Alkane, hydrocarbon/ Alkane, hydrocarbon/Cytotoxicity , Antimicrobial, Antioxidant , Antipyretic, Anthelmentic, Tumour, Bronchitis, Asthma, Tuberculosis, Dyspepsia, Constipation, Anemia, Throat diseases, Elephantiasis, Antidiabetic, Anti-inflammatory, Antidiarrhoeal	Banakar and Jayaraj, 2018.
4	6.544	1.479	Cetene	$C_{16}H_{32}$ 	224	Alkene, hydrocarbon/antibacterial, antioxidant	Yogeswari*et al*.,2012; Elgorban*et al*., 2019.
5	6.619	1.449	Heptadecane	$C_{17}H_{36}$ 	240	Alkane, hydrocarbon / anti-inflammatory	Kim *et al*., 2013; Pratama*et al*., 2019.
6	7.082	2.514	Octadecane	$C_{18}H_{38}$ 	254	Alkane, hydrocarbon/ Antioxidant, Anti-inflammatory, Cough, lung diseases, Cold and fever detoxification , Anticorrosion agent , Antisepsis	Banakar and Jayaraj, 2018

Table 4.5a. GC-MS analysis of *Dennettia tripetala*Leaves

PEAK NO.	RETENTION TIME (MIN)	% COMPOSITION	NAME OF COMPOUND	STRUCTURE	MOLECULAR WEIGHT	BIOLOGICAL ACTIVITY	REFERENCE
7	7.179	3.219	Neophytadiene	$C_{20}H_{38}$ 	278	Diterpene, sesquiterpene /anti-inflammatory, antimicrobial (pubchem), antioxidant cardioprotective (medchem)	Pratama*et al*., 2019.
8	7.237	0.597	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	$C_{20}H_{40}O$ 	296	Diterpene alcohol/ Antimicrobial, Anti-inflammatory, Anticancer, Diuretic, Antifungal against S. typhi, modulate transcription in cells, antimalaria, precursor for the manufacture of synthetic forms of vitamin E and vitamin K1.	Banakar and Jayaraj, 2018.
9	7.282	1.824	1,2-15,16-Diepoxyhexadecane	$C_{16}H_{30}O_2$ 	254	Alkane, hydrocarbon/ antitumor and anti-inflammatory	Shareef *etal*., 2016
10	7.328	0.684	Nonadecane	$C_{19}H_{40}$ 	268	Alkane, hydrocarbon/Antimicrobial, Antioxidant, Anticancer, Anti HIV, Antioxidant, Antibacterial, Antimicrobial , Cytotoxic effect, Antimicrobial,	Banakar and Jayaraj, 2018.

- **Table 4.5a. GC-MS analysis of *Dennettia tripetala*Leaves**

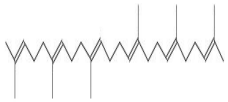
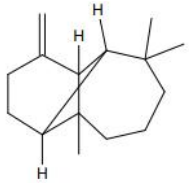
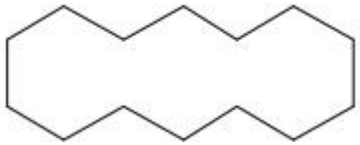
K NO.	N TIME (MIN)	COMPOSITION			MOLECULAR WEIGHT	BIOLOGICAL ACTIVITY	REFERENCE
11	7.763	0.099	Octatriacontylpentafluoropropionate	$C_{41}H_{77}F_5O_2$ 	696	Carboxylic acid ester/ antiviral(covid-19)	Elwakil*et al*., 2021.
12	7.843	0.203	Heneicosane	$C_{21}H_{44}$ 	296	Alkane, hydrocarbon/ antimicrobial,Anti-inflammatory, analgesic, antipyretic	Vanitha *et al,* 2020.
13	8.444	0.292	Eicosane	$C_{20}H_{42}$ 	282	Anti-inflammatory, analgesic, antipyretic, wound healing, antioxidant	Balachandran *et al*., 2023; Okechukwu, 2020.
14	11.385	0.627	Squalene	$C_{30}H_{50}$ 	410	Triterpene / Antibacterial, Antioxidant, Antitumor, Cancer preventive, Immunostimulant,Chemo preventive, Lipoxygenase-inhibitor, Pesticide)	Banakar and Jayaraj,2018.

Table 4.5b. GC-MS analysis of *Cola acuminata*Leaves

PEAK NO.	RETENTION TIME (MIN.)	% COMPOSITION	NAME OF COMPOUND	STRUCTURE	MOLECULAR WEIGHT	BIOLOGICAL ACITIVITY	REFERENCES
1	6.149	0.420	β-Longipinene	$C_{15}H_{24}$ 	204	Sesquiterpene/ antimicrobial and anti-insecticidal activity antioxidant and anti-inflammatory	Shukurova*et al*., 2020; Santana *et al*., 2012.
2	6.275	0.606	Pentadecane	$C_{15}H_{32}$ 	212	Alkane, hydrocarbon/anti-inflammatory, antipyretic and analgesic	Chuah *et al*., 2018;Okechukwu, 2020.
3	6.538	3.292	Cetene	$C_{16}H_{32}$ 	224	Alkene, hydrocarbon/antibacterial, antioxidant	Yogeswari*et al*.,2012; Elgorban*et al*., 2019

4	6.825	1.047	Heptadecane	$C_{17}H_{36}$ 	240	Alkane, hydrocarbon/ antibacterial	Keke *et al*., 2023; Togashi *et al*., 2007.

Table 4.5b. GC-MS analysis of *Cola acuminata*Leaves

PEAK NO.	RETENTION TIME (MIN)	% COMPOSITION	NAME OF COMPOUND	STRUCTURE	MOLECULAR WEIGHT	BIOLOGICAL ACTIVITY	REFERENCE
5	6.893	1.629	Cyclotetradecane	$C_{14}H_{28}$ 	196	Cycloalkane, hydrocarbon/ antimicrobial	Keke *et al*., 2023; Chuah *et al*., 2018.
6	7.065	2.625	1-Octadecene	$C_{18}H_{36}$ 	252	Alkene, hydrocarbon/ Antibacterial, antioxidant and anticancer activity	Keke, *et al*., 2023; Belakhdar*et al*., 2015.

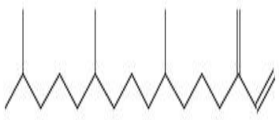
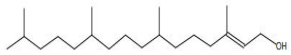
7	7.174	3.896	Neophytadiene	$C_{20}H_{38}$ 	278	Diterpene, an anti-inflammatory agent, an antimicrobial agent, antifungal and antioxidant properties, cardioprotective, inhibition of cyclooxygenase or lipoxygenase	Pubchem

Table 4.5b. GC-MS analysis of *Cola acuminata*Leaves

PEAK NO.	RETENTION TIME (MIN)	% COMPOSITION	NAME OF COMPOUND	STRUCTURE	MOLECULAR WEIGHT	BIOLOGICAL ACTIVITY	REFERENCE
8	7.231	1.357	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	$C_{20}H_{40}O$ 	296	Diterpene alcohol/ Antimicrobial, Anti-inflammatory, Anticancer, Diuretic, Antifungal against S. typhi, antimalaria,precursor for the manufacture of synthetic forms of vitamin E and vitamin K1 and modulates transcription.	(Banakar and Jayaraj, 2018),
9	7.391	4.148	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270	Alkane, Anti HIV, Antioxidant, Antibacterial,	Banakar and Jayaraj, 2018.

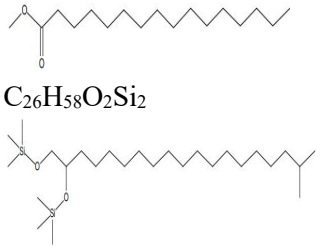
10	7.654	2.034	18-Methyl-nonadecane-1,2-dio, trimethylsilyl ether	 $C_{26}H_{58}O_2Si_2$	458	Antimicrobial , Cytotoxic effect, Antimicrobial, Antimalarial, Alkane, antifungal, antioxidant, anti-inflammatory, analgesic, and antipyretic	Okechukwu, 2020; Ahsan *et al*., 2017.

Table 4.5b. GC-MS analysis of *Cola acuminata*Leaves

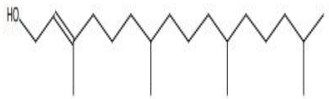
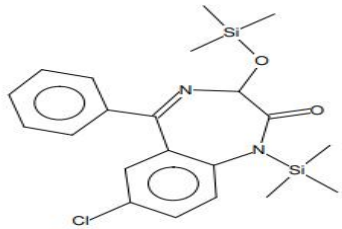
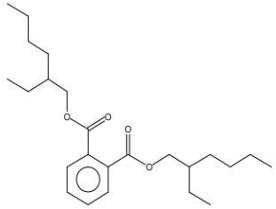
PEAK NO.	RETENTION TIME (MIN)	% COMPOSITION	NAME OF COMPOUND	STRUCTURE	MOLECULAR WEIGHT	BIOLOGICAL ACTIVITY	REFERENCE
11	7.889	3.677	Phytol	$C_{20}H_{40}O$ 	296	Antioxidant and antimicrobial activities	Gollo *et al*., 2020; El-sayed and Ismail, 2022
12	8.015	1.950	Oxazepam, 2TMS derivative	$C_{21}H_{27}ClN_2O_2Si_2$ 	430	Diterpenoid/Antimicrobial, Anti inflammatory, Anticancer, Diuretic, Antifungal against S. typhi, Resistant gonorrhea, Joint dislocation, Headache, Hernia, Stimulant and antimalaria	Banakar and Jayaraj, 2018.
13	8.696	0.949	1-Eicosene	$C_{20}H_{40}$ 	280	Antioxidant and Insecticidal Activities	Ganesh and Mohankumar, 2017.
14	9.468	7.500	Bis(2-ethylhexyl) phthalate	$C_{24}H_{38}O_4$ 	390	Antimicrobial activity	Ganesh and Mohankumar, 2017.

Table 4.5b. GC-MS analysis of *Cola acuminata*Leaves

PEAK	RETENTION	%	NAME OF	STRUCTURE	MOLECULAR	BIOLOGICAL	REFERENCE

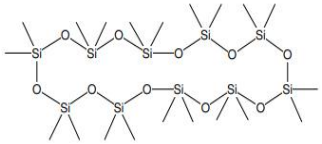
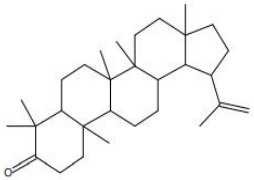
NO.	TIME (MIN)	COMPOSITION	COMPOUND		WEIGHT	ACTIVITY	
15	10.275	2.393	Eicosamethyl-cyclodecasiloxane,	$C_{20}H_{60}O_{10}Si_{10}$ 	740	Organic compound phthalate, antimicrobial, cytotoxic, anticancer antibacterial, larvicidal	Lofty *et al*., 2018; Javed *et al.*, 2022.
16	10.767	2.960	Eicosane	$C_{20}H_{42}$ 	282	Antimicrobial activity	Ganesh and Mohankumar, 2017.
17	21.141	12.279	Lup-20(29)-en-3-one	$C_{30}H_{48}O$ 	424	Anti-inflammatory, analgesic, antipyretic, wound healing, antioxidant	Balachandran *et al*., 2023; Okechukwu, 2020.

Table 4.6a. High Performance Liquid Chromatography (HPLC) Analysis of *Dennettia tripetala*Leaves

S/N	Component (*Dennettia tripetala*)	Retention	Area	Height	Concentration (µg/mL)
1	Kaempferol	0.856	7238.6163	167.440	8.9255
2	Epihedrine	3.093	3384.2110	114.610	8.7072
3	Catechin	5.483	2946.0368	99.913	4.5703
4	Anthocyanin	7.873	4954.9828	167.963	8.4991
5	Dihydrocytisine	9.350	3037.9009	103.290	7.8095
6	Aphyllidine	10.830	5995.0366	203.063	6.7536
7	Cyanogenic glycoside	14.036	3257.7420	110.237	2.9395
8	Steroid	19.976	5032.1969	168.293	8.6316
9	Narigenin	20.613	3392.0544	117.580	8.7274
10	Tannin	23.363	6937.3508	232.210	18.3972
11	Flavonones	25.960	7284.2334	246.540	12.4944
12	Flavone	29.326	5246.3590	177.219	8.9989
13	Proanthocyanidin	31.653	6803.7662	230.237	12.6660
14	Ribalinidine	34.150	30087.4534	104.110	3.8318
15	Spartein	36.770	4438.4062	150.474	11.4196
16	Oxalate	38.643	7991.6386	270.115	17.8200
17	Sapogenin	43.536	5028.2088	170.352	5.8400

Table 4.6b. High Performance Liquid Chromatography (HPLC) analysis of *Cola acuminata* Leaves

S/N	Component (*Cola Acuminata*)	Retention	Area	Height	Concentration (µg/mL)
1	Kaemferol	0.280	3426.8154	117.436	4.2254
2	Steroid	2.390	12252.8106	301.042	10.5084
3	Epihedrine	4.120	6344.5478	157.568	2.7206
4	Catechin	6.016	18153.9630	442.688	28.1631
5	Anthocyanin	7.470	8442.8722	206.427	7.2409
6	Dihydrocytisine	10.366	19598.0759	476.646	16.7936
7	Aphyllidine	12.970	6238.3258	152.341	7.0277
8	Cyanogenic glycoside	15.460	4967.5726	121.273	4.4824
9	Aphyllidine	17.966	11339.3364	276.425	7.6359
10	Narigenin	20.313	12756.4948	307.631	10.9404
11	Tannin	22.730	9573.3711	233.187	25.3877
12	Flavonones	25.650	10008.8176	245.115	17.1678
13	Ammodendrine	27.536	11458.0104	280.295	1.9287
14	Flavone	29.860	5478.6156	133.724	9.3973
15	Proanthocyanidin	32.996	14337.0482	348.877	8.8967
16	Ribalinidine	34.600	6059.7940	147.836	7.5207
17	Spartein	36.876	6988.5601	170.310	8.9904
18	Oxalate	39.200	10234.8247	249.264	22.8219
17	Sapogenin	42.276	3473.1416	85.310	4.0338
18	Phytate	44.170	10509.6912	256.782	7.7220

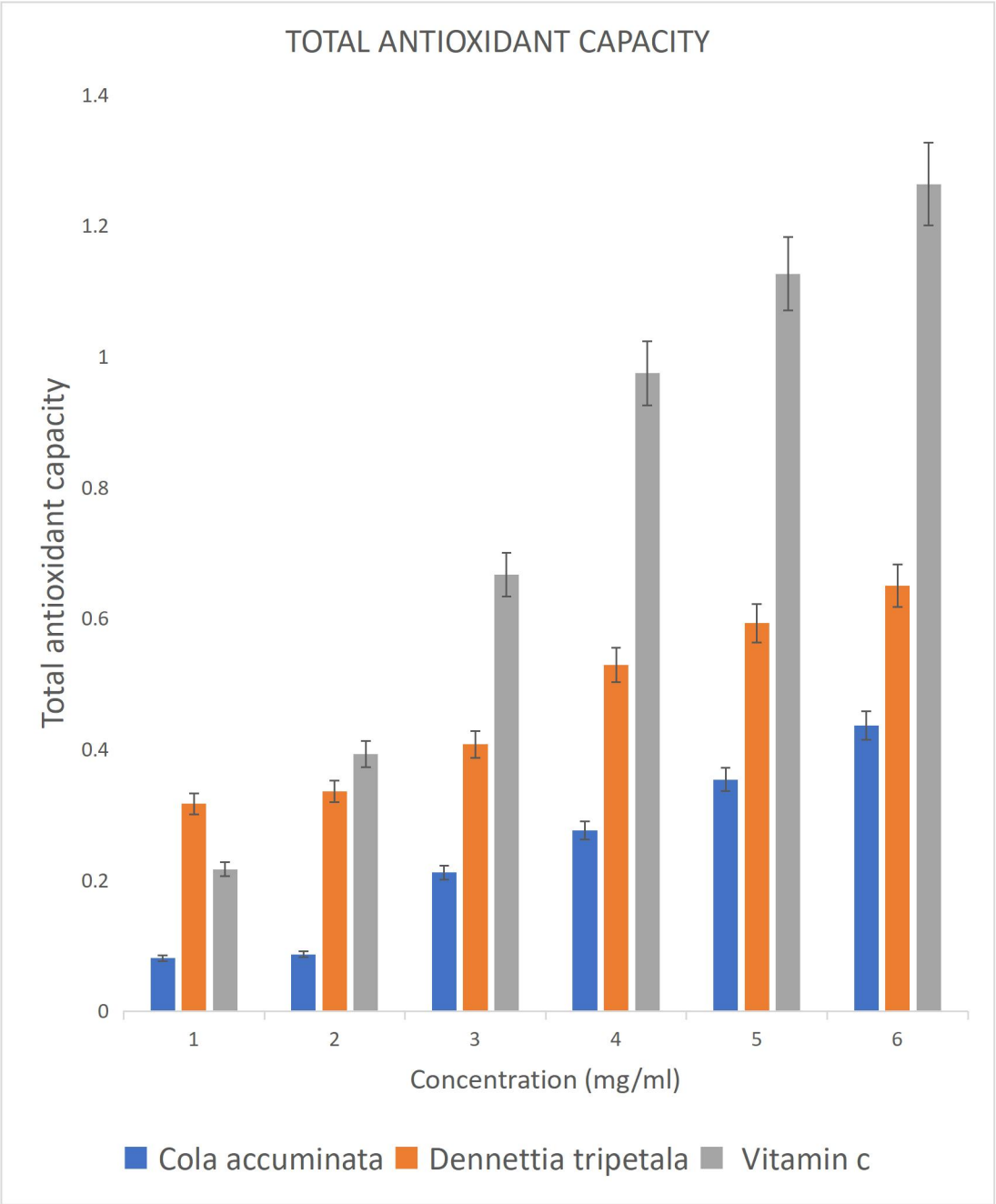


Fig. 4.1a. Total Antioxidant Capacity of *Dennettia tripetala* and *Cola acuminata* Leaves

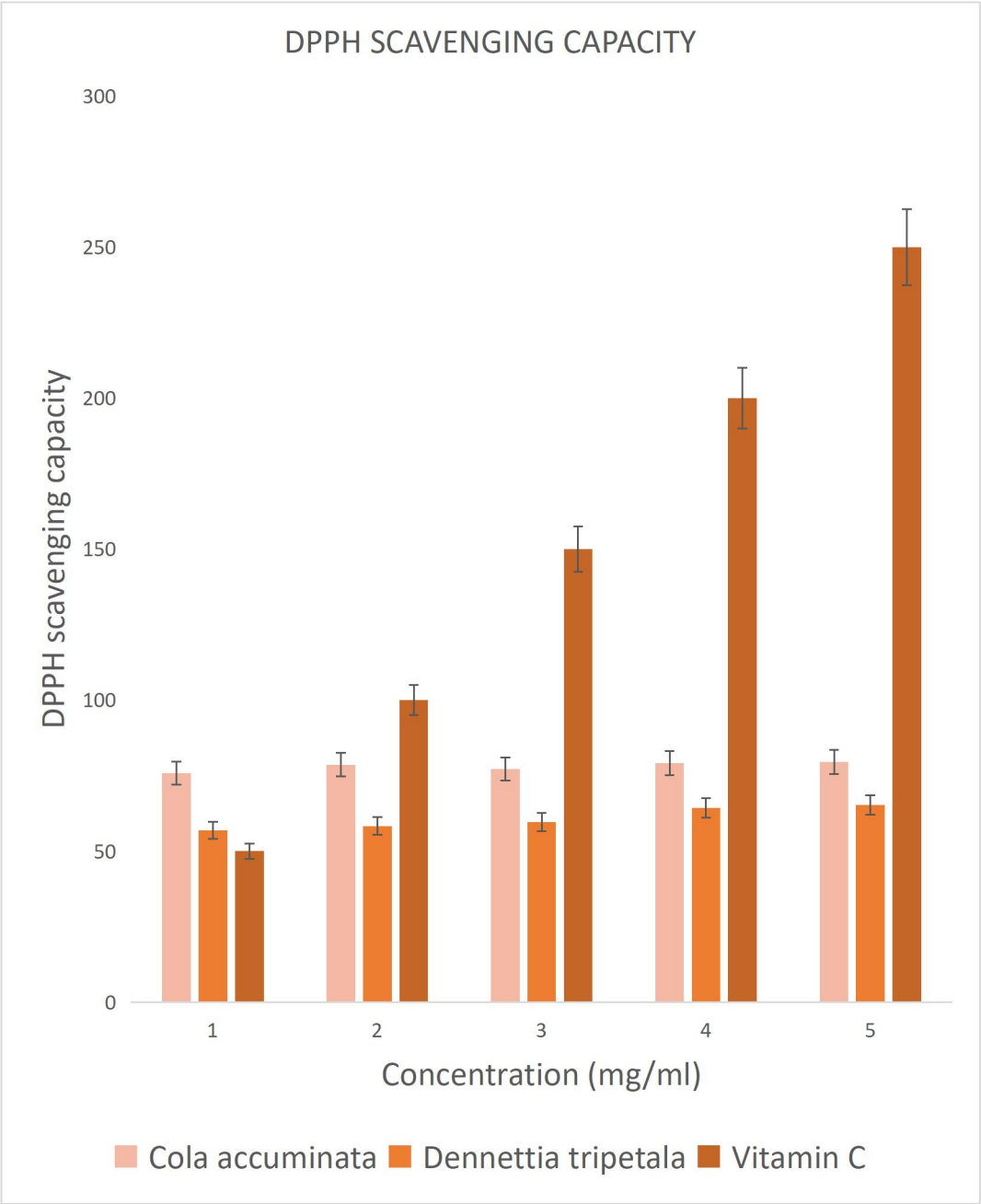


Fig. 4.1b. DPPH Scavenging capacity of *Dennettia tripetala* and *Cola acuminata* Leaves

4.8. Acute Toxicity of *Dennettia tripetala* and *Cola acuminata* Methanol Leaf Extracts

Table 4.7 express results for acute toxicity testing. There were no signs of toxicity or mortality observed within the period of twenty-four hours, seven days and fourteen days respectively. Animals were not reckless and did not lose appetite. The LD50 for both *Dennettia tripetala* and *Cola acuminata* methanol leaf extracts were greater than 5,000mg/kg body weight of albino rats.

4.9. Sub-Acute Toxicity of *Dennettia tripetala* and *Cola acuminata* Methanol Leaf Extracts on Kidney function markers

The results for the sub-acute toxicity testing of *Dennettia tripetala* and *Cola acuminata* methanol leaf extracts on plasma albumin, urea and creatinine concentrations in albino wistar rats are presented in tables 4.8a and 4.8b

Table 4.8a reveal that there was no significant difference in albumin and creatinine concentrations of the low dose and high dose *Dennettia tripetala* methanol leaf extract treated groups when compared with the control. However, the concentration of urea was significantly ($p<0.05$) lowered in group for group 4, and higher in group 6 when compared with the control.

Table 4.8b show that there was no significant difference in albumin, urea and creatinine concentrations of the low dose and high dose *Cola acuminata* methanol leaf extract treated groups when compared with the control.

Table 4.7. Acute toxicity of *Dennettia tripetala* and *Colaacuminata* of Methanol Leaf Extracts

Extracts	Phase 1 Dose(mg/kg Body weight)	Mortality	Phase 2 Dose(mg/kg Body weight)	Mortality
Methanol Extract of *Dennettia tripetala*	10	0/3	1600	0/1
	100	0/3	2900	0/1
	1000	0/3	5000	0/1
Methanol Extract of *Cola acuminata*	10	0/3	1600	0/1
	100	0/3	2900	0/1
	1000	0/3	5000	0/1

4.10. Sub-Acute Toxicity of *Dennettia tripetala* and *Cola acuminata* Methanol Leaf Extracts on Liver function markers

ALP, activities in *Dennettia tripetala* and *Cola acuminata* extract treated group reveal that there was no significant difference in the low dose group compared to the control, but higher doses induced a significant ($p<0.05$) higher activity compared with the control.

ALT activities in *Dennettia tripetala* extract treated group increased significantly ($p<0.05$) across all the groups compared with the control except for group 2. *Cola acuminata* treatment did not alter ALT activities significantly in the low dose groups but higher doses resulted in significant ($p<0.05$) increased activity.

Dennettia tripetala and *Cola acuminata* extract induced a significant ($p<0.05$) reduction in AST activity across all the groups.

Table 4.8a. Effect of *Dennettia tripetala* methanol leaf extract on Plasma Albumin, Urea and Creatinine

Group	ALBUMIN (g/L)	UREA (mg/dL)	CREATININE (mg/dL)
Group 1 Control (Normal saline)	39.10±4.00[a]	105.00±1.41[a]	2.14±0.37[a]
Group 2 (10 mg/kg body weight	37.90±3.32[a]	105.67±3.79[a]	2.12±1.00[a]
Group 3 (100 mg/kg body weight)	39.10±1.56[a]	107.25±0.35[a]	3.84±1.26[a]
Group 4 (1,000 mg/kg body weight)	39.95±5.52[a]	79.27±6.20[b]	2.15±1.601[a]
Group 5 (1,600 mg/kg body weight)	40.66±1.36[a]	105.00±2.83[a]	1.61±1.36[a]
Group 6 (2,900 mg/kg body weight)	38.35±1.41[a]	128.00±7.07[b]	2.90±1.36[a]
Group 7 (5,000 mg/kg body weight)	41.20±2.01[a]	105.50±4.95[a]	4.06±0.75[a]

Values are mean ± SD n=4 values with different alphabet are significantly ($p<0.05$) different.

Table 4.8b. Effect of *Cola acuminata* methanol leaf extract on Plasma Albumin, Urea and Creatinine

Group	ALBUMIN (g/L)	UREA (mg/dL)	CREATININE (mg/dL)
Group 1 Control (Normal saline)	39.10±4.00[a]	105.00±1.41[a]	2.14±0.36[a]
Group 2 (10 mg/kg body weight	40.13±3.57[a]	89.74±4.51[a]	2.12±1.62[a]
Group 3 (100 mg/kg body weight)	37.67±2.41[a]	85.74±4.51[a]	4.01±4.3[a]
Group 4 (1,000 mg/kg body weight)	36.20±0.00[a]	92.01±0.00[a]	2.08±0.00[a]
Group 5 (1,600 mg/kg body weight)	36.50±3.39[a]	116.61±4.07[a]	1.92±0.91[a]
Group 6 (2,900 mg/kg body weight)	40.66±3.21[a]	91.25±7.57[a]	4.28±2.43[a]
Group 7 (5,000 /kg body weight)	39.60±5.43[a]	100.93±4.06 [a]	1.49±1.48[a]

Values are mean ± SD n=4 values with different alphabet are significantly ($p<0.05$) different.

Table 4.8c. Effect of *Dennettia tripetala* methanol leaf extract on Plasma ALP, ALT and AST

Group	ALP (U/L)	ALT(U/L)	AST(U/L)
Group 1 Control (Normal saline)	223.00±4.24[a]	38.00±1.41[a]	43.50±7.07[a]
Group 2 (10 mg/kg body weight	219.33±1.16[a]	24.00±4.24[b]	19.33±3.51[b]
Group 3 (100 mg/kg body weight)	224.00±5.66[a]	45.5±3.54 [a]	8.00±1.41[b]
Group 4 (1,000 mg/kg body weight)	277.00±1.41[b]	82.00±3.61[b]	25.66±2.30[b]
Group 5 (1,600 mg/kg body weight)	276.67±1.16[b]	67.67±1.16 [b]	17.00±3.46[b]
Group 6 (2,900 mg/kg body weight)	222.67±4.62[a]	50.00±2.83 [b]	10.00±3.00[b]
Group 7 (5,000 mg/kg body weight)	259.00±4.58[b]	72.50±6.36 [b]	12.00±6.24[b]

Values are mean ± SD n=4 values with different alphabet are significantly ($p<0.05$) different.

Table 4.8d. Effect of *Cola acuminata* methanol leaf extract on Plasma ALP, ALT and AST

Group	ALP (U/L)	ALT(U/L)	AST(U/L)
Group 1 Control (Normal saline)	223.00±5.24[a]	38.00±1.41[a]	43.50±3.54[a]
Group 2 (10 mg/kg body weight	224.00±5.65 [a]	39.04±3.84[a]	14.33±8.08[b]
Group 3 (100 mg/kg body weight)	222.00±3.46 [a]	42.12±2.14[a]	15.33±6.00[b]
Group 4 (1,000 mg/kg body weight)	236.00±0.00[b]	32.01±2.82[a]	10.00±3.00[b]
Group 5 (1,600 mg/kg body weight)	189.00±4.94[b]	64.36±3.29[b]	22.00±2.10[b]
Group 6 (2,900 mg/kg body weight)	206.00±4.94[b]	42.32±3.00[a]	12.66±7.37[b]
Group 7 (5,000 mg/kg body weight)	224.00±5.65 [a]	57.23±2.30[b]	13.33±8.50[b]

Values are mean ± SD n=4 values with different alphabet are significantly ($p<0.05$) different.

4.11. Sub-Acute Toxicity of *Dennettia tripetala* and *Cola acuminata* Methanol Leaf Extracts in Albino Wistar Rats on some Hematological indices

Results for some hematological indices in *Dennettia tripetala* and *Cola acuminata* methanol extract treated groups are presented intables 4.8e and 4.8f. *Dennettia tripetala* and *Cola acuminata* methanol leaf extract showed no significant difference in the PCV, WBC, RBC, HBG and PLT concentrations across all the groups when compared with the control.

4.12. Determination of Effective Dose of Each Extract on Phenylhydrazine Toxicity

Table 4.9a indicates that phenylhydrazine resulted in low PCV levels and increases in creatinine and urea levels in the group administered only phenylhydrazine. *Dennettia tripetala* methanol leaf extract at 1,500 mg/kg body weight dose significantly (p>0.05) increased the PCV levels and decreased urea and creatinine levels in anemic rats compared with the other doses. Therefore 1,500 mg/kg body weight is considered the effective dose for *Dennettia tripetala* methanol leaf extract.

4.13. Comparative Effect of *Dennettia tripetala* and *Cola acuminata* Methanol Leaf Extracts on Liver Function Markers in Phenylhydrazine Induced Hemolytic Anemia

Table 4.10 reveals results for plasma and liver proteins. The table shows that there were no significant differences across all the groups when compared with normal control. Although there were slight alterations in the levels of protein in the liver and plasma.

AST, ALT and ALP activities are presented in table 4.11. AST activity increased significantly ($p<0.05$) in the phenylhydrazine group when compared with the normal control. *Dennettia tripetala* and *Cola acuminata* treated groups gave values that were significantly ($p<0.05$) lower than those of normal control and phenylhydrazine treated groups.

ALP Levels in phenylhydrazine treated group was higher than that of the control, although not significant. *Dennettia tripetala* and *Cola acuminata* treated groups values decreased compared with the phenylhydrazine treated group.

Alanine amino transferase (ALT) activity was significantly ($p<0.05$) increased in the phenylhydrazine treated group when compared with those of the normal control. Treatment with *Dennettia tripetala*, *Cola acuminata* methanol leaf extract and Vitamin C, significantly ($p<0.05$)reduced values compared with the phenylhydrazine treated group.

There was no significant difference between the two extract treated groups for ALT, AST and ALP when compared.

Table 4.8e. Effect of *Dennettia tripetala* methanol leaf extract on some Hematological indices

Group	PCV(%)	WBC(U/l)	RBC(U/l)	HBG(d/L)	PLT(U/l)
Group 1 Control (Normal saline)	70.30±3.68	5.50±2.8	9.68±1.40	14.60±2.96	366.00±237.00
Group 2 (10 mg/kg body weight	59.94±4.45	4.30±3.44	7.40±5.1	17.00±2.60	189.00±191.00
Group 3 (100 mg/kg body weight)	51.10±2.5	8.80±0.14	8.11±0.36	13.85±0.35	754.00±211.00
Group 4 (1,000 mg/kg body weight)	51.90±1.04	11.10±2.15	8.22±0.32	14.00±0.54	856.00±171.00
Group 5 (1,600 mg/kg body weight)	64.50±1.13	6.96±2.76	9.40±1.22	16.00±1.83	294.00±253.00
Group 6 (2,900 mg/kg body weight)	46.10±3.70	6.20±1.58	7.22±0.59	12.60±0.79	645.00±282.00
Group 7 (5,000 mg/kg body weight)	52.7±3.66	5.36±1.68	8.21±0.79	13.95±0.35	430.00±182.00

Values are mean ± SD n=4 values with different alphabet are significantly ($p<0.05$) different.

Table 4.8f. Effect of *Cola acuminata* methanol leaf extract on some Hematological indices

Group	PCV (%)	WBC(U/l)	RBC(U/l)	HBG(d/L)	PLT(U/l)
Group 1 Control (Normal saline)	64.80±9.87	5.50±2.80	9.68±1.40	14.60±2.95	366.00±137.00
Group 2 (10 mg/kg body weight	56.40±2.47	5.60±0.91	6.11±4.53	13.60±1.81	771.00±182.00
Group 3 (100 mg/kg body weight)	57.60±4.00	5.60±1.91	9.00±0.28	14.70±0.63	160.00±72.00
Group 4 (1,000 mg/kg body weight)	58.80±0.00	5.70±0.00	8.80±0.00	15.20±0.00	693.00±0.00
Group 5 (1,600 mg/kg body weight)	50.40±3.74	7.50±3.04	7.58±0.32	13.50±0.63	338.00±253.00
Group 6 (2,900 mg/kg body weight)	54.00±7.21	6.20±4.71	8.29±1.29	17.60±4.34	645.00±144.00
Group 7 (5,000 mg/kg body weight)	76.0±8.15	8.60±5.50	11.95±0.72	20.00±2.91	289.00±1.31

Values are mean ± SD n=4 values with different alphabet are significantly ($p<0.05$) different.

Table 4.9a. Effect of *Cola Acuminata* Methanol Leaf Extract on Phenylhydrazine Induced Hemolytic Anemia on kidney, liver and Pack cell volume (PCV)

Group	CREATININE (mg/dL)	UREA (mg/dL)	TOTAL PLASMA PROTEIN (µg/mL)	PCV (%)
Group 1 Control (Normal saline)	0.145±0.12	12.344±2.73	0.860±0.09	49.170±5.53
Group 2 PHZ 60 mg/kg body weight CA	0.944±0.27	13.839±4.46	0.940±0.18	22.000±2.55[a]
Group 3 PHZ + 500 mg/kg body weight CA	0.161±0.27[b]	9.875±5.13	0.740±0.12[b]	58.000±5.69[b]
Group 4 PHZ + 1,5000 mg/kg body weight CA	0.338±0.24[b]	5.106±2.79[b]	0.880±0.06	54.000±2.90[b]
Group 5 PHZ + 2,500 mg/kg body weight CA	0.836±0.28	9.674±2.01	0.810±0.19	55.000±3.52[b]
Group 6 PHZ + 3,500 mg/kg body weight CA	0.846±0.12	9.351±2.30	0.750±0.90[b]	51.600±6.58[b]
Group 7 PHZ + 4,500 mg/kg body weight CA	1.090±0.30	10.641±4.59	0.740±0.08[b]	51.200±4.09[b]

Values are mean ± SD n = 6 [a]= significantly different from control
[b]= significantly different from group 2 values without alphabets are notsignificantly different form control PHZ= Phenylhydrazine

Table 4.9b. Effect of *Dennettia tripetala* Methanol Leaf Extract on Phenylhydrazine Induced Hemolytic Anemia Albino on kidney, liver and Pack cell volume (PCV) pilot studies

Group	CREATININE (mg/dL)	UREA (mg/dL)	TOTAL PLASMA PROTEIN (µg/mL)	PCV (%)
Group 1 Control (Normal saline)	0.135±0.105	12.344±2.73	0.859±0.09	49.170±5.53
Group 2 PHZ 60 mg/kg body weight DT	0.944±0.270[a]	13.839±4.46[a]	0.880±0.06	22.000±2.55[a]
Group 3 PHZ + 500 mg/kg body weight DT	0.145±0.104[b]	11.958±0.84[b]	0.716±0.10	52.000±6.87[b]
Group 4 PHZ + 1,5000 mg/kg body weight DT	0.080±0.03[b]	9.136±2.22[b]	0.834±0.09	53.200±3.42[b]
Group 5 PHZ + 2,500 mg/kg body weight DT	0.084±0.07[b]	12.092±2.24[b]	0.749±0.09	50.500±3.62[b]
Group 6 PHZ + 3,500 mg/kg body weight DT	0.113±0.074[b]	11.993±2.68[b]	0.805±0.08	48.830±7.68[b]
Group 7 PHZ + 4,500 mg/kg body weight DT	0.338±0.08[b]	9.170±0.89[b]	0.814±0.03	54.800±2.86[b]

Values are mean ± SD n = 6 [a]= significantly different from control
[b]= significantly different from group 2. values without alphabets are not significantly different form control PHZ= Phenylhydrazine

Table 4.10. Comparative Effect of *Cola acuminata* and *Dennettia tripetala* Methanol Leaf Extracts on Plasma and Liver Protein concentrations in Phenylhydrazine Induced Hemolytic Anemia

Group	Serum Mean ±SD (µg/mL)	Liver Mean ±SD (µg/mL)
Group 1 Control (Normal saline)	2.864± 0.219	0.220±0.083
Group 2 PHZ only (125mg/kg body weight ip)	2.731±0.253	0.206±0.630
Group 3 *Dennettia tripetala* (1,500mg/kg body weight) + PHZ	2.651±0.097	0.247±0.037
Group 4 *Cola acuminata* (500mg/kg body weight) + PHZ	2.513±0.249	0.156±0.042
Group 5 Vitamin C (100mg/kg body weight) + PHZ	2.797±0.029	0.172±0.058

Values are mean ± SD n = 6 values without alphabets are not significantly different form control PHZ= Phenylhydrazine

Table 4.11. Effect of *Cola acuminata* and *Dennettia tripetala* Methanol Leaf Extracts on Plasma ALP, ALT and AST concentrations in Phenylhydrazine Induced Hemolytic Anemia

Group	ALP (U/L)	ALT (U/L)	AST (U/L)
Group 1 Control (Normal saline)	0.419±0.02[a]	29.657±2.88[a]	27.893±3.69[a]
Group 2 PHZ only (125mg/kg body weight ip)	0.568±0.04[a]	40.993±1.84[b]	121.197±0.37[b]
Group 3 *Dennettia tripetala* (1,500mg/kg body weight) + PHZ	0.409±0.02[a]	23.545±2.17[ac]	14.805±3.13[c]
Group 4 *Cola acuminata* (500mg/kg body weight) + PHZ	0.415±0.02[a]	24.570±2.14[a]	15.248±2.28[c]
Group 5 Vitamin C (100mg/kg body weight) + PHZ	0.403±0.02[a]	26.073±3.64[a]	30.150±0.21[a]

Values are mean ± SD n = 6 [a]= significantly different from control
[b]= significantly different from group 2 values without alphabets are not significantly different form control PHZ= Phenylhydrazine

4.14. Effect of *Cola acuminata* and *Dennettia tripetala* Methanol Leaf Extracts on Kidney Function Markers in Phenylhydrazine Induced Hemolytic Anemia

Table 4.12a shows that there was a significant ($p<0.05$) increase in the levels of urea and creatinine in group 2. *Dennettia tripetala* and *Cola acuminata* methanol leaf extract significantly ($p < 0.05$) reduced these values to near normal control values. *Dennettia tripetala* reduced the elevated levels of urea significantly ($p<0.05$) more than *Cola acuminata* in a manner comparable to that of Vitamin C. But there was no significant difference in the values of creatinine when both extracts were compared.

Table 4.12b indicates that there was no significant difference in the electrolyte's values obtained across the extract treated groups and Vitamin C groups when compared with the normal control.

4.15. Effect of *Cola acuminata* and *Dennettia tripetala* Methanol Leaf Extracts on Oxidative stress indices in Phenylhydrazine Induced Hemolytic Anemia

The result on table 4.13 reveals that malondialdehyde levels increased significantly ($p<0.05$) in group 2 compared with control. *Dennettia tripetala*, *Cola acuminata* and Vitamin C,significantly<0.05) reduced these values. *Dennettia tripetala* showed lower values in the kidney and spleen, while *Cola acuminata* showed lower values in the liver.

Table 4.14 shows that phenylhydrazine significantly ($p<0.05$) reduced the activity of catalase in the kidney, liver and spleen. Catalase activity was increased significantly ($p<0.05$) by the *Dennettia tripetala*, *Cola acuminata* and Vitamin C. *Dennettia tripetala* increased catalase activity in the liver and spleen better than *Cola acuminata,* but in the kidney, the reverse was the case.Vitamin C values were higher in the spleen and in the liver.

Table 4.12a. Effect of *Cola acuminata* and *Dennettia tripetala* Methanol Leaf Extracts on Plasma Albumin, Urea and Creatinine concentrations in Phenylhydrazine Induced Hemolytic Anemia

Group	ALBUMIN (g/L)	UREA (mg/dL)	CREATININE (mg/dL)
Group 1 Control (Normal saline)	0.309±0.02[a]	39.479±3.58[a]	1.267±0.78[a]
Group 2 PHZ only (125mg/kg body weight ip)	0.322±0.02[a]	48.343±2.01[b]	4.000±2.00[b]
Group 3 *Dennettia tripetala* (1,500mg/kg body weight) + PHZ	0.3119±0.03[a]	35.959±2.04[ac]	0.567±0.09[a]
Group 4 *Cola acuminata* (500mg/kg body weight) + PHZ	0.242±0.03[ab]	41.851±4.94[a]	0.508±0.06[a]
Group 5 Vitamin C (100mg/kg body weight) + PHZ	0.321±0.05[a]	35.009±4.04[ac]	1.222±0.19[a]

Values are mean ± SD n = 6 [b]= significantly different from control
values with similar alphabets are not significantly different PHZ=Phenylhydrazine

Table 4.12b. Effect of *Cola acuminata* and *Dennettia tripetala* Methanol Leaf Extracts on Plasma Electrolytes in Phenylhydrazine Induced Hemolytic Anemia

Groups	Sodium (mMol/L)	Potassium (mMol/L)	Chloride (mMol/L)	Bicarbonate (mMol/L)
Group 1 Control (Normal Control)	137.333±2.31[a]	4.467±0.32 [a]	105.333±0.58 [a]	14.667±4.16 [a]
Group 2 (Negative control) PHZ only(125mg/kg body weight ip)	137.500±0.71[a]	4.650±0.21 [a]	104.500±0.58 [a]	13.500±2.12 [a]
Group 3 *Dennettia tripetala* (1,500mg/kg body weight) + PHZ	137.000±1.00[a]	4.567±0.25 [a]	105.333.0.58 [a]	14.000±2.65 [a]
Group 4 *Cola acuminata* (500mg/kg body weight) + PHZ	136.333±2.08[a]	4.233±0.45 [a]	107.333±3.06 [a]	14.667±0.58 [a]
Group 5 Vitamin C (Positive control) (100mg/kg body weight) + PHZ	137.333±0.58[a]	3.633±0.45 [a]	107.667±3.51 [a]	12.333±4.04 [a]

Values are mean ± SD n = 6 values with similar alphabets are not significantly different PHZ= Phenylhydrazine

Table 4.13. Effect of *Cola acuminata* and *Dennettia tripetala* Methanol Leaf Extracts on Tissue Malondialdehyde concentrations in Phenylhydrazine Induced Hemolytic Anemia

GROUP	KIDNEY Mean±SD x10^-6 (µmol/mL)	LIVER Mean±SD x10^-7 (µmol/mL)	SPLEEN Mean±SD x10^-7 (µmol/mL)
Group 1 Control (Normal saline)	1.0192±0.15	3.686±0.80	5.304±2.46
Group 2 PHZ only (125mg/kg body weight ip)	1.989±0.49 [a]	10.817±2.96[a]	11.522±4.84[a]
Group 3 *Dennettia tripetala* (1,500mg/kg body weight) + PHZ	1.152±0.23 [b]	4.968±1.86 [b]	8.894±1.06[b]
Group 4 *Cola acuminata* (500mg/kg body weight) + PHZ	1.342±0.37 [b]	4.551±1.38[b]	9.316±2.11[b]
Group 5 Vitamin C (100mg/kg body weight) + PHZ	0.724±0.28 [b]	4.850±2.11[b]	7.596±0.91[b]

KEY: Values are mean ± SD n = 6 [a]= significantly different from control [b]= significantly different from group 2 PHZ=Phenylhydrazine

Table 4.14. Effect of *Cola acuminata* and *Dennettia tripetala* Methanol Leaf Extracts on Catalase activity in Phenylhydrazine Induced Hemolytic Anemia

GROUP	CATALASE (U/L)		
	KIDNEY	LIVER	SPLEEN
Group 1 Control (Normal saline)	0.074±0.02 [a]	0.075±0.00 [a]	0.062±0.02 [a]
Group 2 PHZ only (125mg/kg body weight ip)	0.038±0.01[b]	0.046±0.02[b]	0.046±0.01[b]
Group 3 *Dennettia tripetala* (1,500mg/kg body weight) + PHZ	0.054±0.01[ac]	0.082±0.00[ac]	0.075±0.01[ac]
Group 4 *Cola acuminata* (500mg/kg body weight) + PHZ	0.041±0.01[ac]	0.087±0.02 [ac]	0.060±0.02[ac]
Group 5 Vitamin C (100mg/kg body weight) + PHZ	0.047±0.01[ac]	0.089±0.01[ac]	0.084±0.02[ac]

KEY: Values are mean ± SD n = 6 [a]= not significantly different from control
[b]= significantly different from control [c]= significantly different from group 2
PHZ=Phenylhydrazine

4.15. Effect of *Cola acuminata* and *Dennettia tripetala* Methanol Leaf Extracts on Oxidative stress indices in Phenylhydrazine Induced Hemolytic Anemia cont.

The result for reduced glutathione (GSH)is presented in table 4.15. The concentrations of GSH in group 2 was significantly ($p<0.05$) lower than that of the control. Administration of *Dennettia tripetala*, and *Cola acuminata* methanol leaf extract significantly increased ($p<0.05$) GSH levels, when compared with group 2.GSH levels were also increased in the Vitamin C treated group, but not significantly different from the extract treated group. GSH levels were higher in the *Cola acuminata* group in the kidneys, but in the liver and spleen, *Dennettia tripetala* showed higher values.

Table 4.16 indicate result for Glutathione S-transferase (GST) activity. Phenylhydrazine significantly ($p<0.05$) reduced the activity of GST in the kidney, liver and spleen. Treatment with *Dennettia tripetala*, *Cola acuminata* and Vitamin C, showed significant ($p<0.05$) increase in the GSH levels. Vitamin C values were higher, followed by the two extracts. In the kidney, GST activity in the *Cola acuminata group* was significantly ($p<0.05$) higher than group 2, followed by *Dennettia tripetala* and Vitamin C. In the spleen, GST activity in Vitamin Cgroup was significantly ($p<0.05$) higher when compared with group 2, followed by *Dennettia tripetala* and *Cola acuminata.*

4.16. Effect of *Dennettia tripetala* and *Cola acuminata* Methanol Leaf Extracts on Histology of Kidney, Liver and Spleen of in Albino Wistar Rats induced with Phenylhydrazine Hemolytic Anemia

Plates4.1-4.5 show photomicrograph of the spleen. Administration of phenylhydrazine induced remarkable damage to the spleen, including: Atrophy of lymphoid follicles (white pulp), decreased sequestration of red blood cells (red pulp), vascular stenosis and hypertrophy.

Table 4.15. Effect of *Dennettia tripetala* and *Cola acuminata* Methanol Leaf Extracts on Glutathione (GSH) activity in Phenylhydrazine Induced Hemolytic Anemia

GROUP	REDUCED GLUTATHIONE (GSH) (µg/mL)		
	KIDNEY	LIVER	SPLEEN
Group 1 Control (Normal saline)	6.617±1.66[a]	7.574±1.33 [a]	12.610±3.10 [a]
Group 2 PHZ only (125mg/kg body weight ip)	4.915±1.01[b]	5.660±2.16[b]	4.879±1.17[b]
Group 3 *Dennettia tripetala* (1,500mg/kg body weight) + PHZ	6.015±1.09[ac]	10.199±2.37[ac]	15.589±1.83[ac]
Group 4 *Cola acuminata* (500mg/kg body weight) + PHZ	6.652±1.81[ac]	8.319±0.45[ac]	14.596±3.61[ac]
Group 5 Vitamin C (100mg/kg body weight) + PHZ	6.723±1.66[ac]	8.284±1.60[ac]	14.312±1.73[ac]

KEY: Values are mean ± SD n = 6 [a]= not significantly different from control
[b]= significantly different from control [c]= significantly different from group 2
PHZ=Phenylhydrazine

Table 4.16. Effect of *Dennettia tripetala* and *Cola acuminata* Methanol Leaf Extracts on Glutathione-S-Transferase (GST) activity in Phenylhydrazine Induced Hemolytic

Group	GST(µmol/ml/min)		
	KIDNEY (mean±SD)x10^-2	LIVER (mean±SD)x10^-3	SPLEEN (mean±SD)x10^-2
Group 1 Control (Normal saline)	1.04±0.28[a]	7.80±1.70[a]	1.65±0.33 [a]
Group 2 PHZ only (125mg/kg body weight ip)	0.43±0.17[b]	4.30±1.70[b]	1.16±0.20 [b]
Group 3 *Dennettia tripetala* (1,500mg/kg body weight) + PHZ	0.87±0.20[ac]	5.20±2.00[ac]	1.62±0.20 [ac]
Group 4 *Cola acuminata* (500mg/kg body weight) + PHZ	1.39±0.75[ac]	5.20±2.50[ac]	1.50±0.20 [ac]
Group 5 Vitamin C (100mg/kg body weight) + PHZ	0.61±0.33[ac]	6.10±1.70[ac]	1.62±0.20 [ac]

KEY: Values are mean ± SD n = 6 [a]= not significantly different from control [b]=significantly different from control [c]= significantly different from groupPHZ=Phenylhydrazine

4.16. Effect of *Dennettia tripetala* and *Cola acuminata* Methanol Leaf Extracts on Histology of Kidney, Liver and Spleen of in Albino Wistar Rats induced with Phenylhydrazine Hemolytic Anemia cont.

Treatment with Vitamin C and Phenylhydrazine induced moderate follicular activation, moderate increase in red cell sequestration and activation of sinus histocytes. Co-administration of *Dennettia tripetala* and phenylhydrazine induced marked follicular activation and marked increased red blood cell sequestration. Coadministration of *Cola acuminata* with phenylhydrazine induced mild follicular activation, mild increase in red blood cell sequestration and activation of sinus histiocytes. *Dennettia tripetala* appeared to be the most potent, while *Cola acuminata* was the least.

Plates4.6- 4.10 reveals the photomicrograph of the Liver. Phenylhydrazine induced severe hepatocyte necrosis, biliary ductal and vascular wall degeneration in the liver of albino rats. Treatment with *Dennettia tripetala*, *Cola acuminata* and Vitamin C, all grouped separately, restored the normal liver architecture. However, there was the additional beneficial effects of mobilization of plasma cells in the portal region of the liver, with the resultant boost in the local immune system of the liver as well as increase in the portal blood flow in the liver in the groups given *Dennettia tripetala* and Vitamin C.

Plate 4.11-4.15 shows photomicrograph of the kidneys. In the kidneys, phenylhydrazine induced severe tubular necrosis and moderate glomerular degeneration. Administration of *Dennettia tripetala*, *Colaacuminata* and Vitamin C, in separate groups restored normal nephron architecture However, the groups given *Dennettia tripetala* and Vitamin C had the additional beneficial vasoactive and hemodynamic activities of vasodilatation and increased interstitial blood flow. Thus *D.*

tripetala activity was comparable to that of Vitamin and appeared to be more potent than *Cola*. *acuminata.*

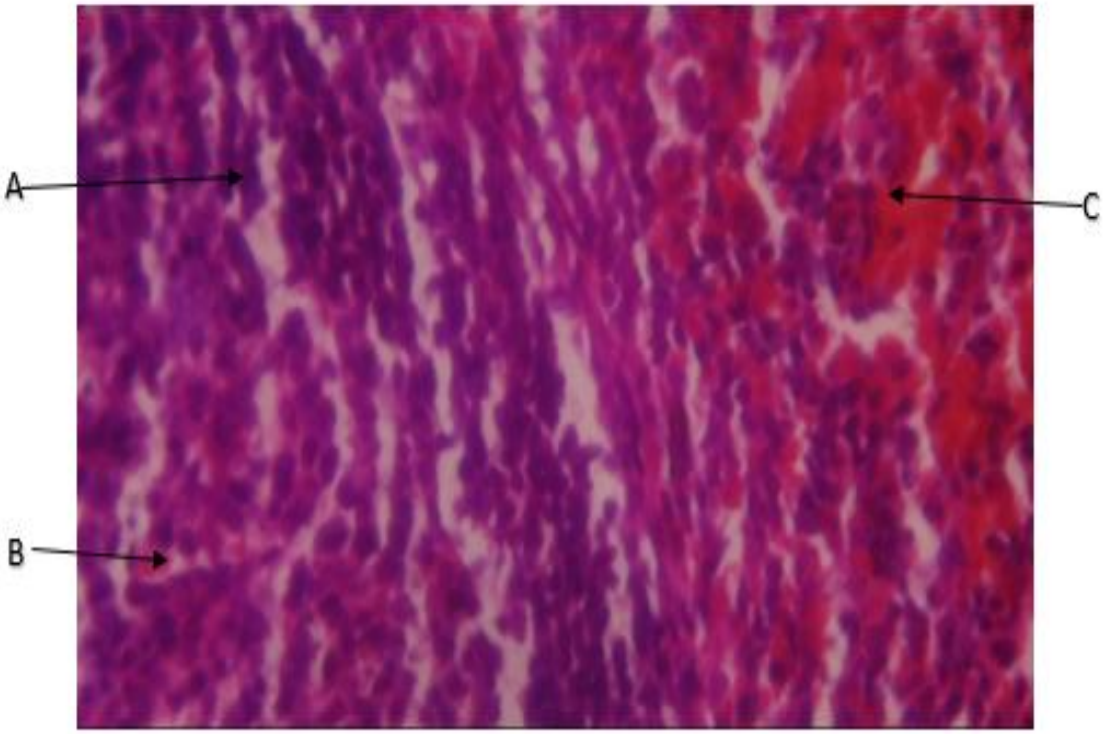


Plate 4.1. Rat spleen. GROUP 1: Composed of: A, normal lymphoid follicle, B, capillary and C, red pulp (H&E x 400)

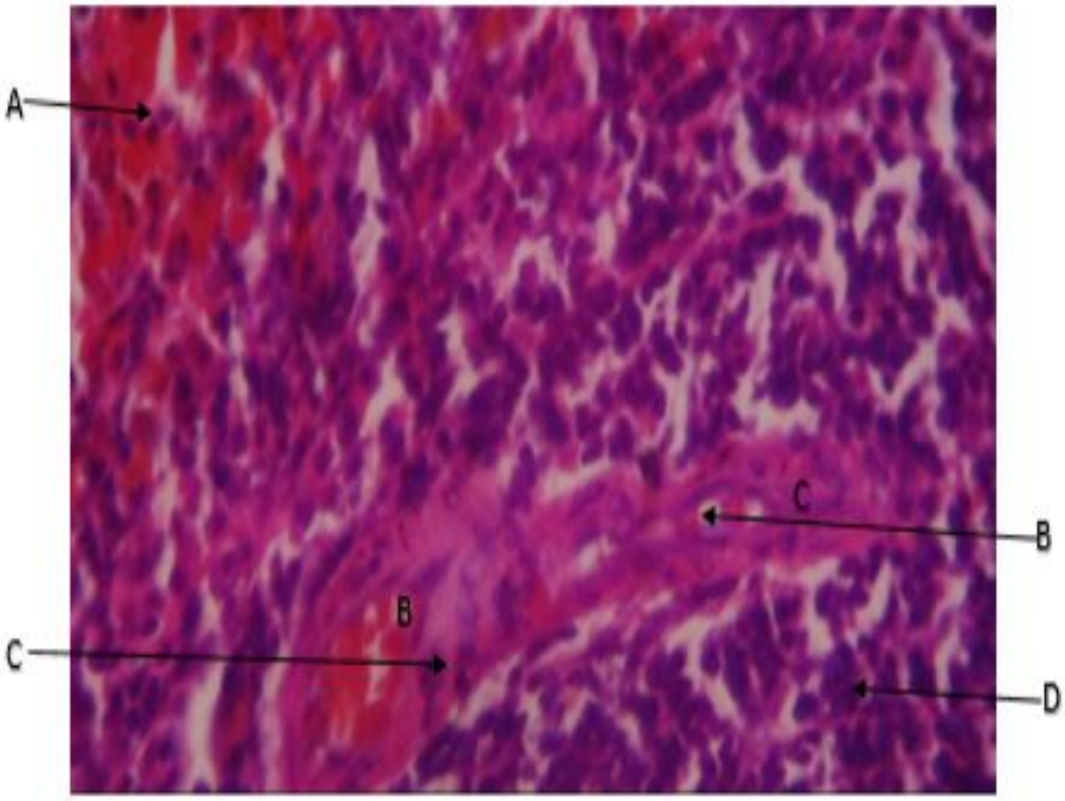


Plate 4.2.GROUP 2:Spleen of rat treated with Phenylhydrazine (PHZ) showing A: decreasedred blood cell sequestration , B, arteriolar stenosis and C, hypertrophy and D, follicular atrophy (H&E x 400)

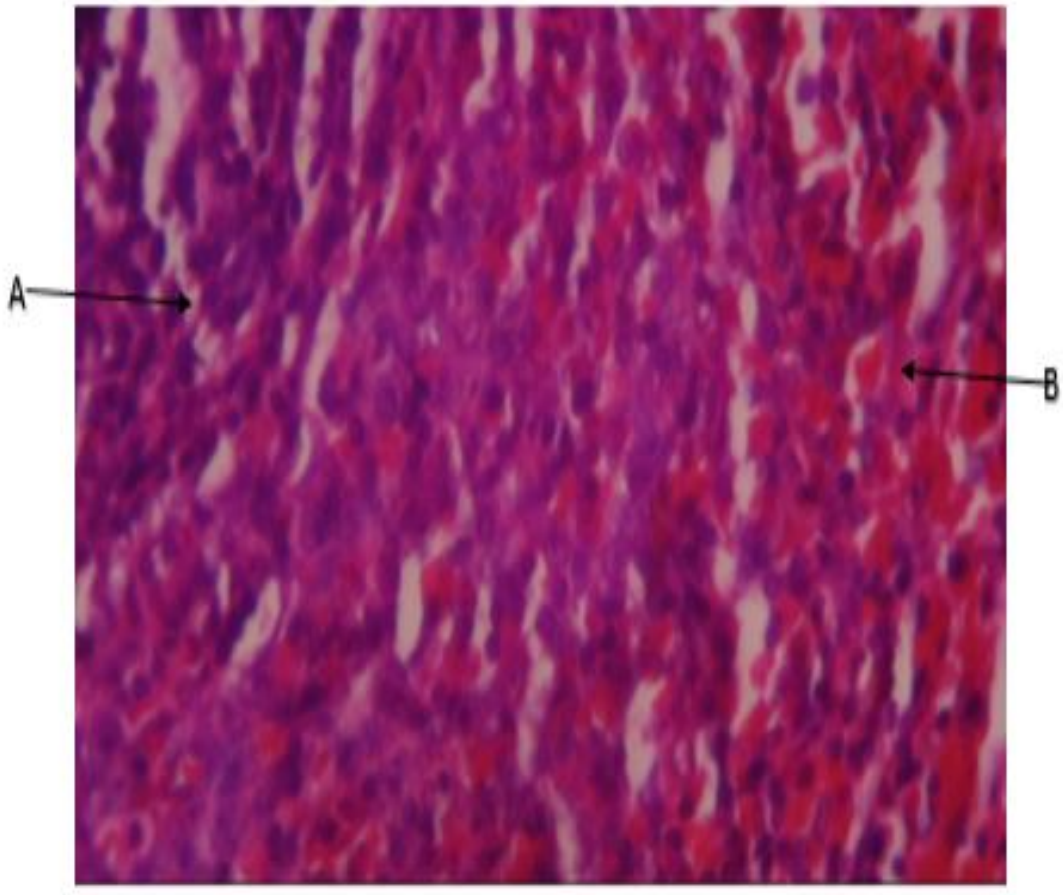


Plate 4.3. GROUP 3: Spleen of rat given Methanol extract of *Dennettia tripetala* + PHZ showing:A, marked follicular activation and B, marked increase in red cellsequestration (H&E x 400)

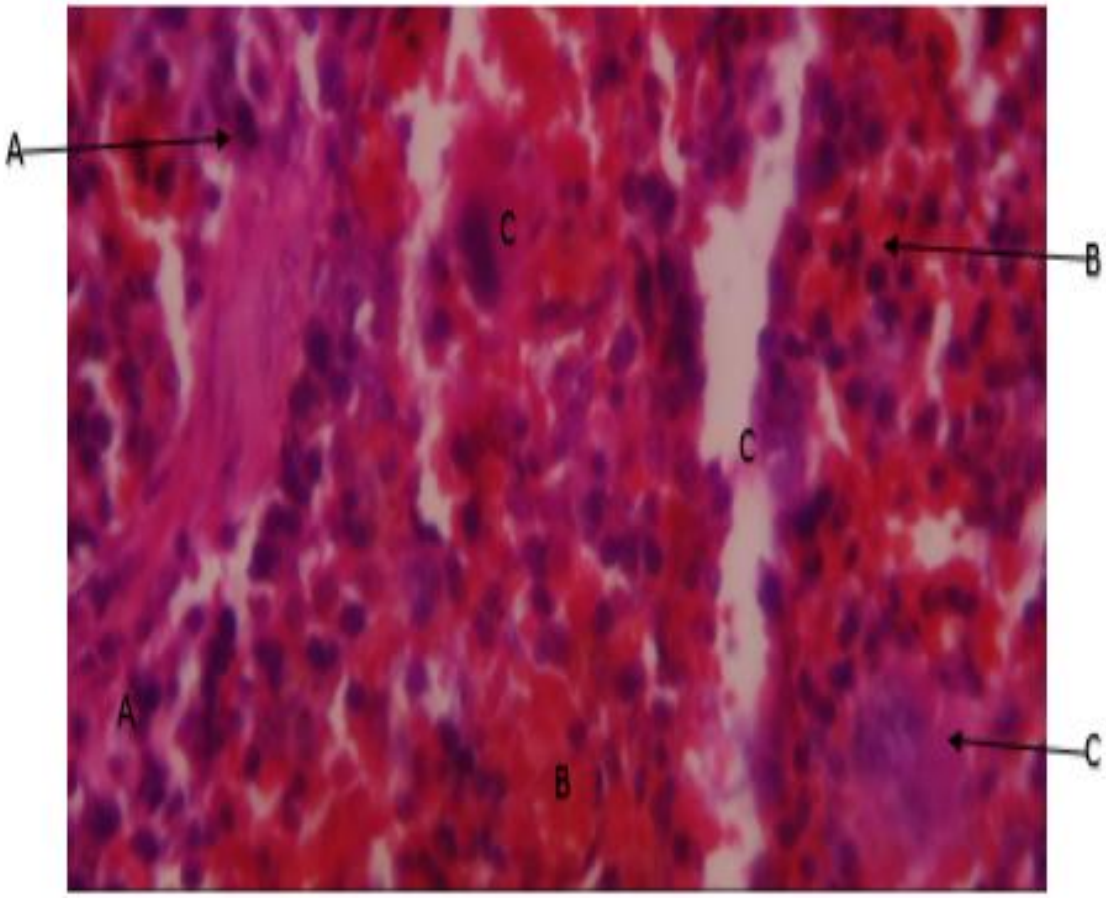


Plate 4.4. GROUP 4: Spleen of rat given Methanol extract of *Cola acuminata* leaves + PHZ showing: A, mild follicular activation, B, mild increase in red cell sequestration and C, mobilization of sinus histiocytes (H&E x 400)

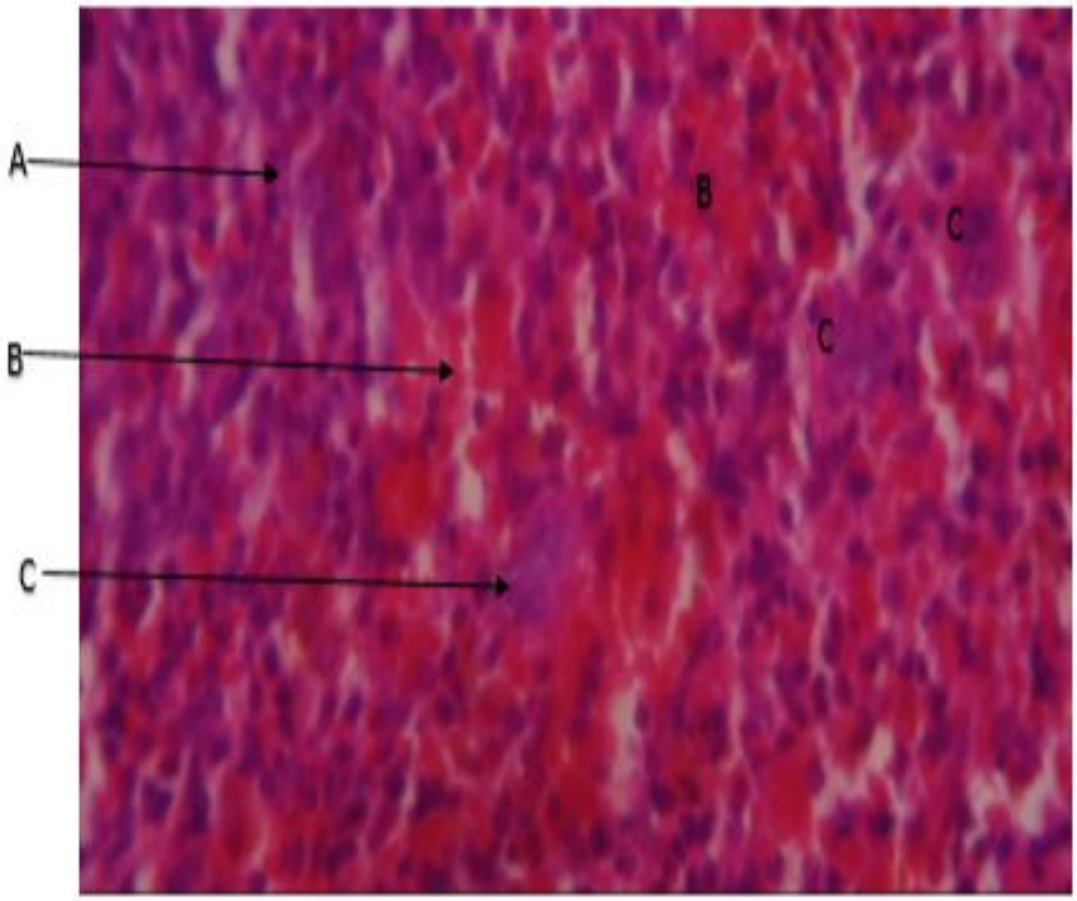


Plate 4.5. GROUP 5:Spleen of rat given Vitamin C + PHZ: A, moderate follicular activation, B, moderate increase in red cell sequestration and C, mobilization of sinus histiocytes (H&E x 400)

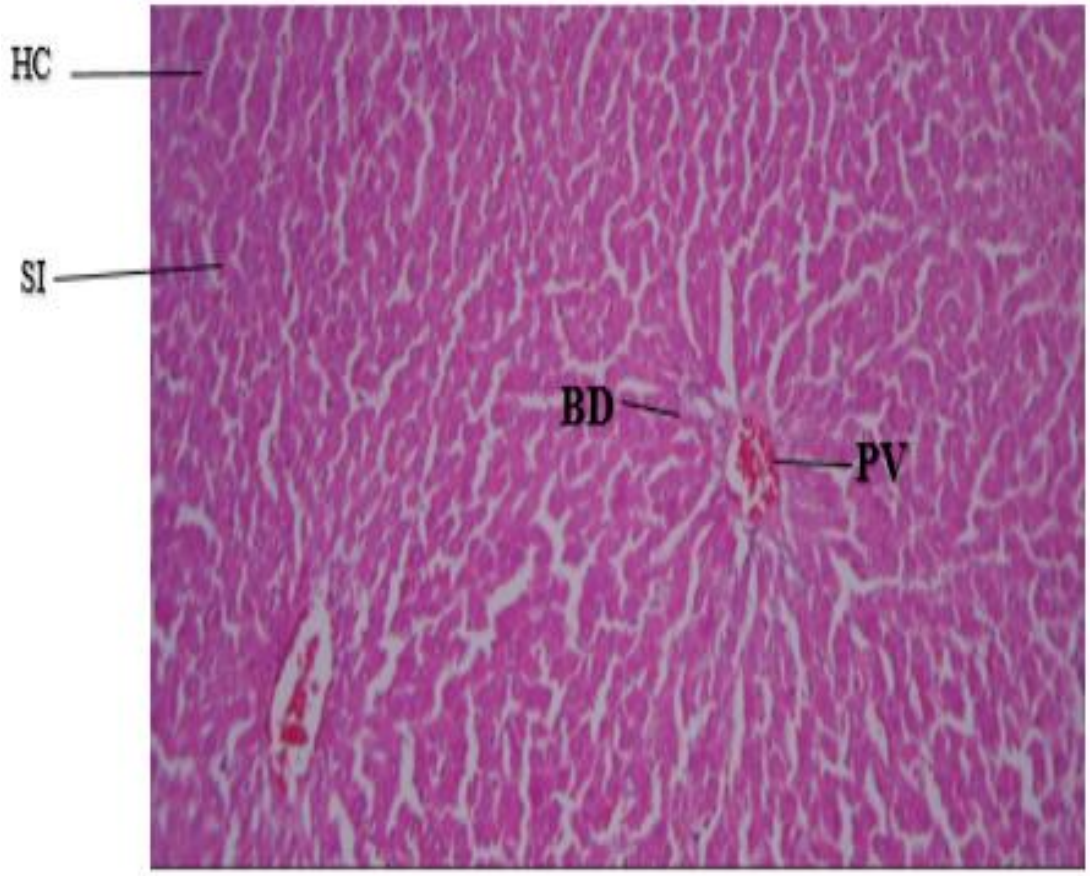


Plate 4.6. GROUP 1. Rat liver of Control group. Composed of normal tissue architecture: hepatocytes (HC), sinusoids (SI), bile ducts (BD), portal vein(PV): H&E x 100

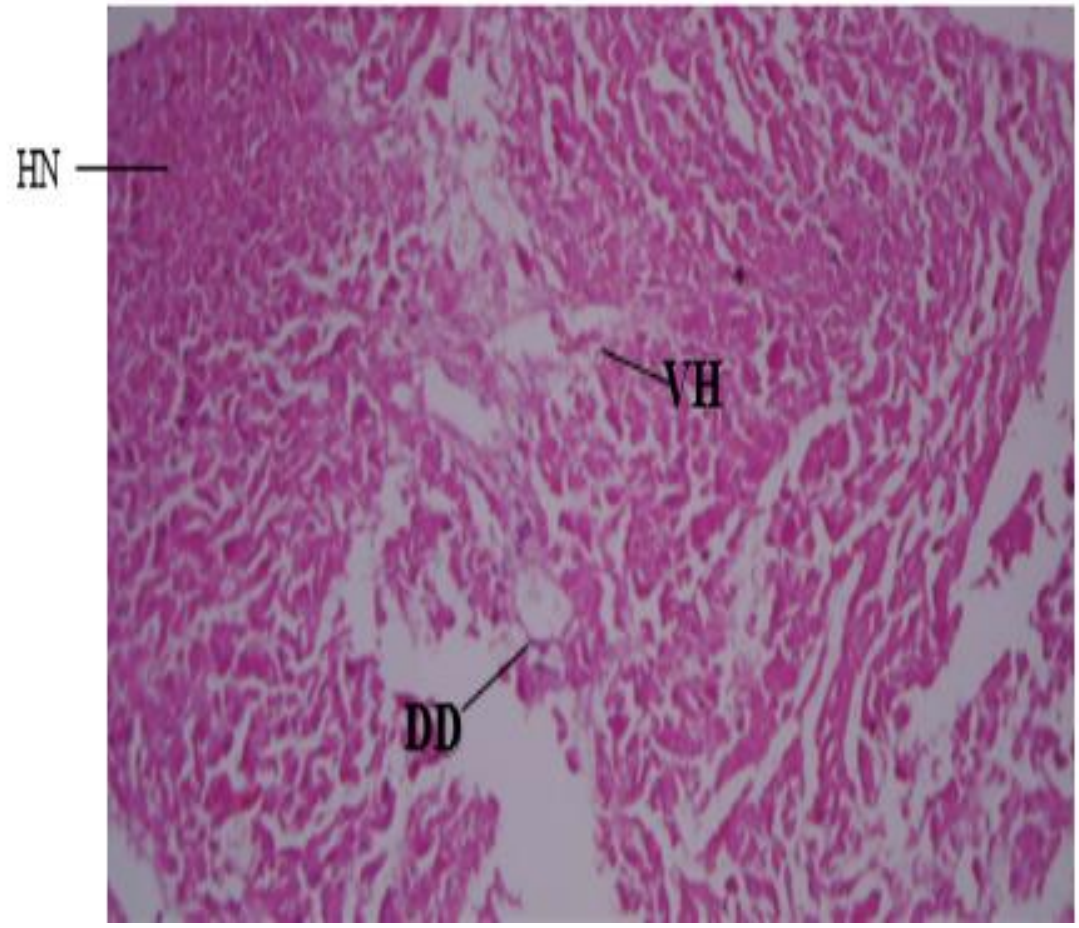


Plate 4.7. GROUP 2. Liver of rat given Phenylhydrazine only showing: severe hepatocyte necrosis (HN), biliary ductal degeneration (DD), vascular mural hyalinization (VH) H&E x 100

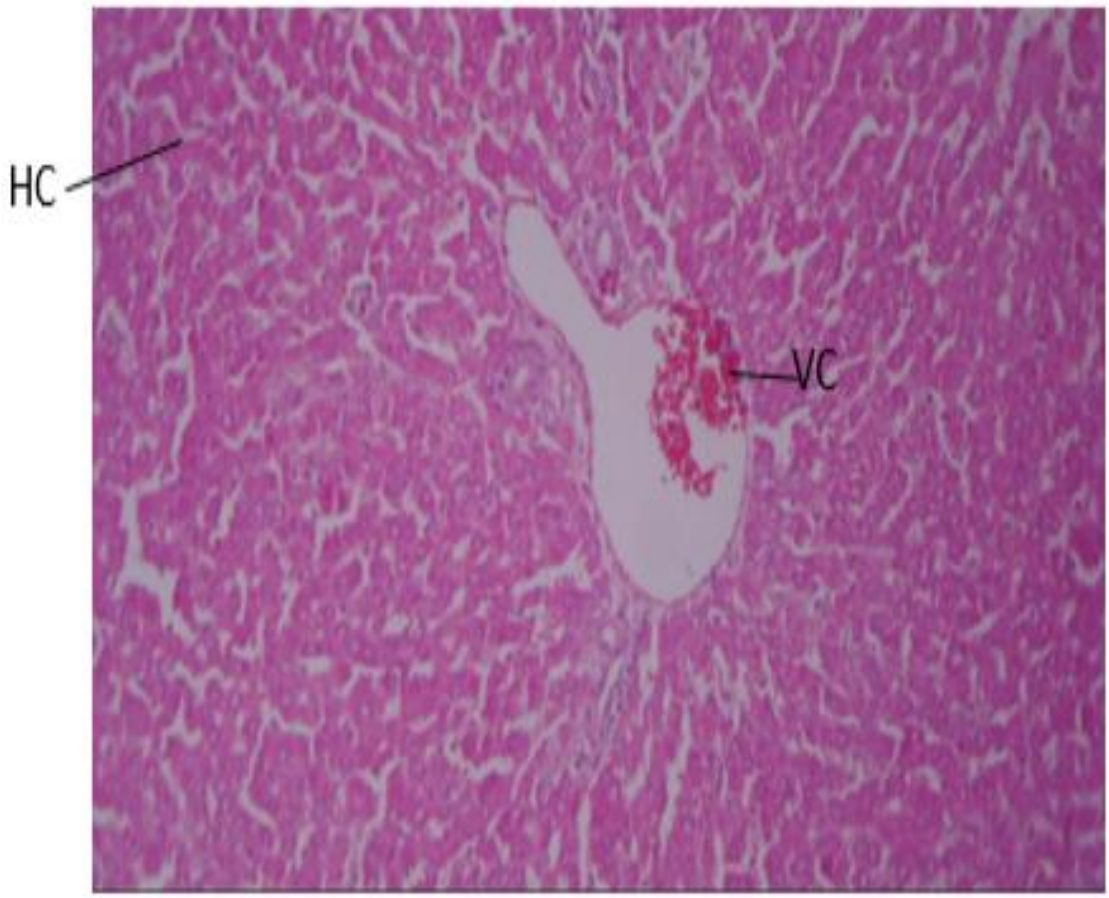


Plate 4.8. GROUP 3. Liver of rat given phenylhydrazine + 500mg/kg *Cola acuminata*showing normal architecture: hepatocytes (HC), active portal congestion (VC)H&E x 100

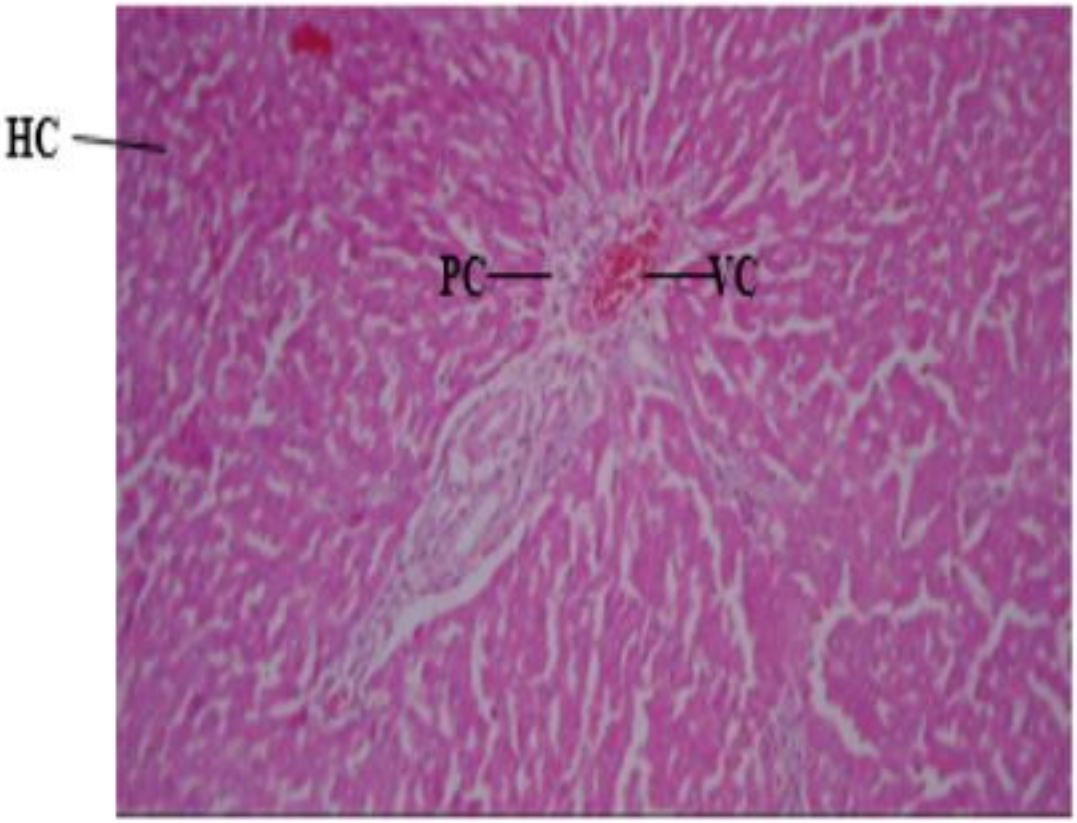


Plate 4.9. GROUP 4. Liver of rat given phenylhydrazine + 1500mg/kg *Dennettia tripetala* showing normal architecture: hepatocytes (HC), periportal mobilization of plasma cells (PC), active portal congestion (VC) H&E x 100

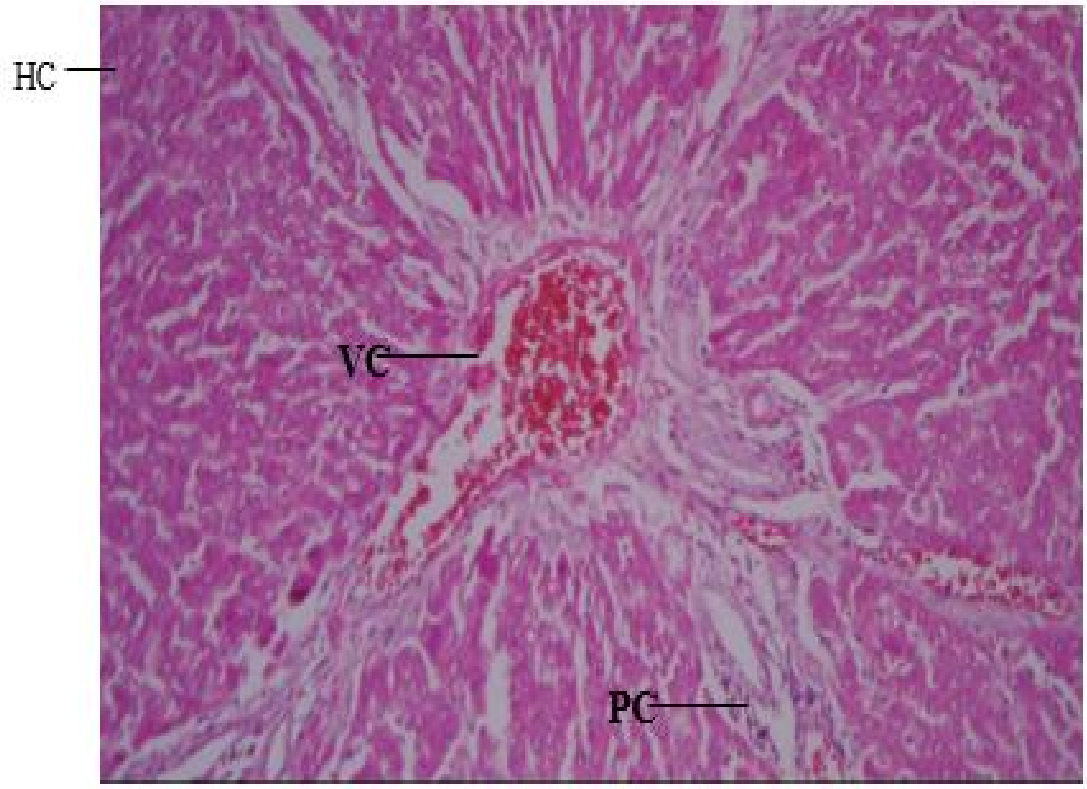


Plate 4.10. GROUP 5. Liver of rat given Phenylhydrazine + 100mg/kg Vit C: showing normal architecture: hepatocytes (HC), periportal mobilization of plasma cells (PC), active portal congestion (VC) : (left) H&E x 100

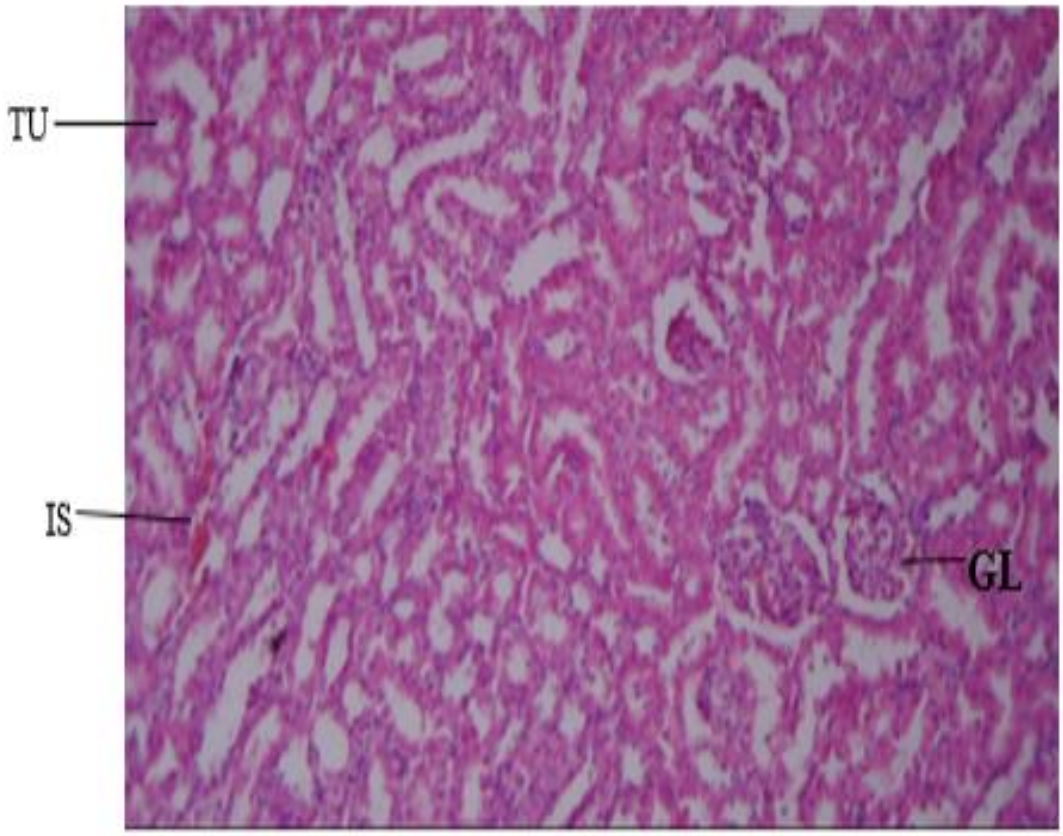


Plate 4.11. GROUP 1. Rat kidney of Control. Composed of normal architecture: tubules (TU), interstitial space (IS), glomeruli (GL): H&E x 100

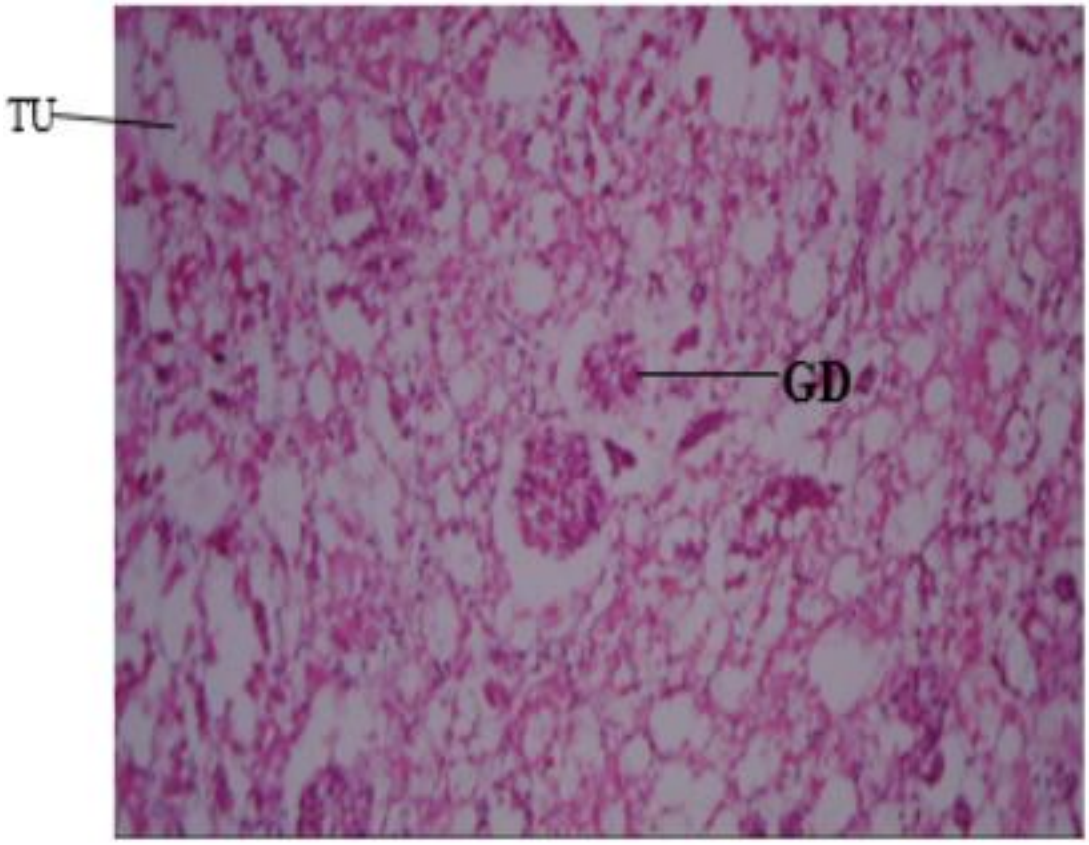


Plate 4.12. GROUP 2. Kidney of rat given Phenylhydrazine only, showing: severe tubular necrosis (TU), glomerular degeneration GD) : H&E x 100

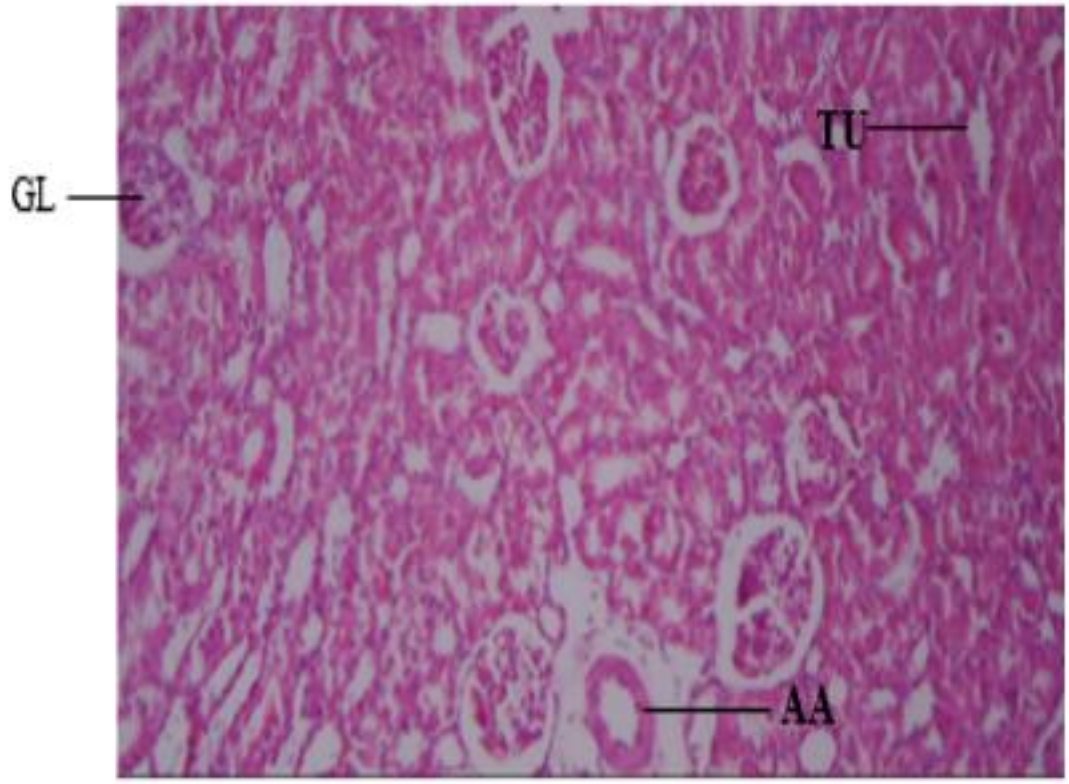


Plate 4.13. GROUP 3. Kidney of rat given phenylhydrazine + 500mg/kg *Cola acuminata*showing normal architecture: tubules (TU), glomeruli (GL), arcuate artery (AA):H&E x 100

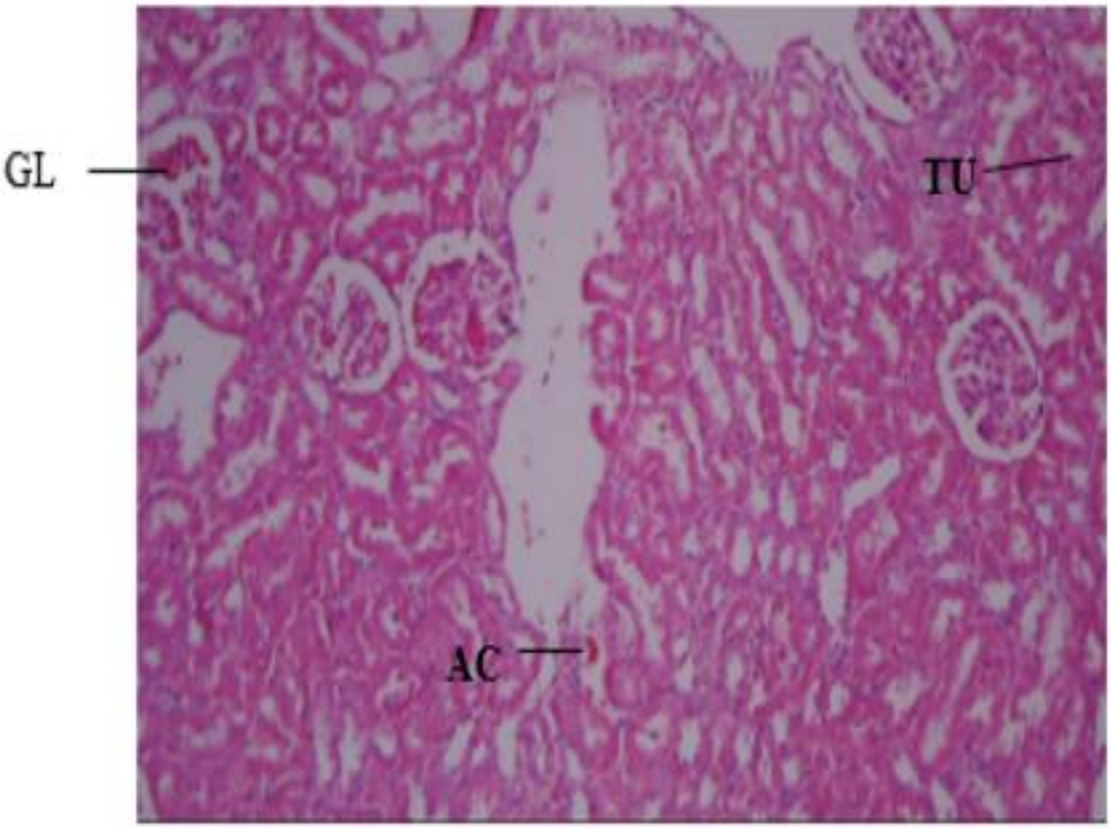


Plate 4.14. GROUP 4. Kidney of rat given phenylhydrazine + 1500mg/kg *Dennettia tripetala* showing normal architecture: glomeruli (GL), tubules (TU), active e interstitial congestion (AC): H&E x 100

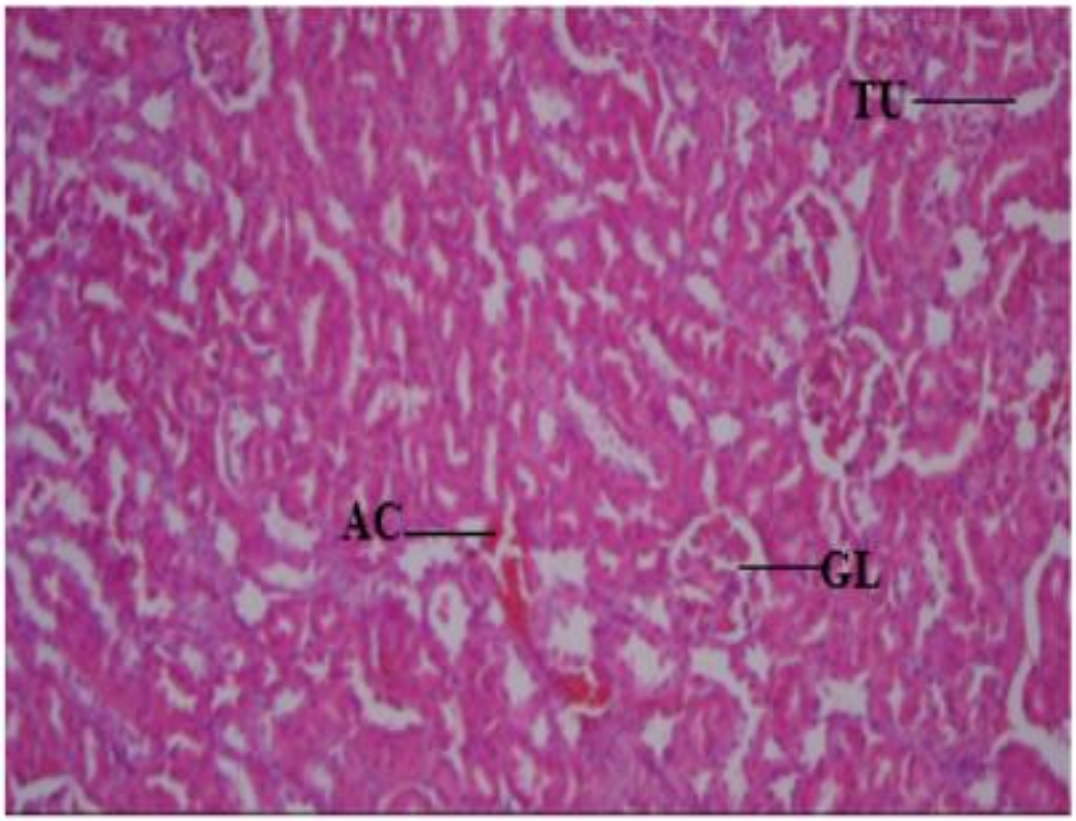


Plate 4.15. GROUP 5. Kidney of rat given phenylhydrazine + 100mg/kg Vit. C showing normal architecture: tubules (TU), glomeruli (GL), active interstitial congestion (AC) : H&E x 100

4.17. Effect of *Dennettia tripetala* and*Cola acuminata* Methanol Leaf Extracts on Histological Changes Induced by the Phenylhydrazine-Induced Toxicity on Bone Marrow Iron Sequestration

Photomicrograph of the bone marrow are shown in plates 16-20. Phenylhydrazine resulted in depletion of iron stores in the reticulocytes of the bone marrow. *Dennettia tripetala*, *Cola acuminata* and Vitamin C effectively restored the iron stores. It was observed that *Cola acuminata* restored iron stores better than *Dennettia tripetala* and Vitamin C.

4.18. Effect of *Cola acuminata* and *Dennettia tripetala* Methanol Leaf Extracts on Hematological indices in Phenylhydrazine Induced Hemolytic Anemia

Hematological analysis of whole blood is presented in Tables 4.16a-c. It indicates that phenylhydrazine resulted in a significant ($p<0.05$) decrease in RBC, HBG, HCT and LYM while MCV was significantly ($p<0.05$) increased. *Dennettia tripetala* and *cola acuminata* methanol leaf extract, including Vitamin C, significantly ($p<0.05$) increased these values to control levels.There was reduction in the values of platelet, WBC, MCH, MCHC, MPV, PCT and P-LCR values, which were not significant. *Dennettia tripetala* and *cola acuminata* methanol leaf extract, including Vitamin C, significantly ($p<0.05$) increased these values when compared with group 2.

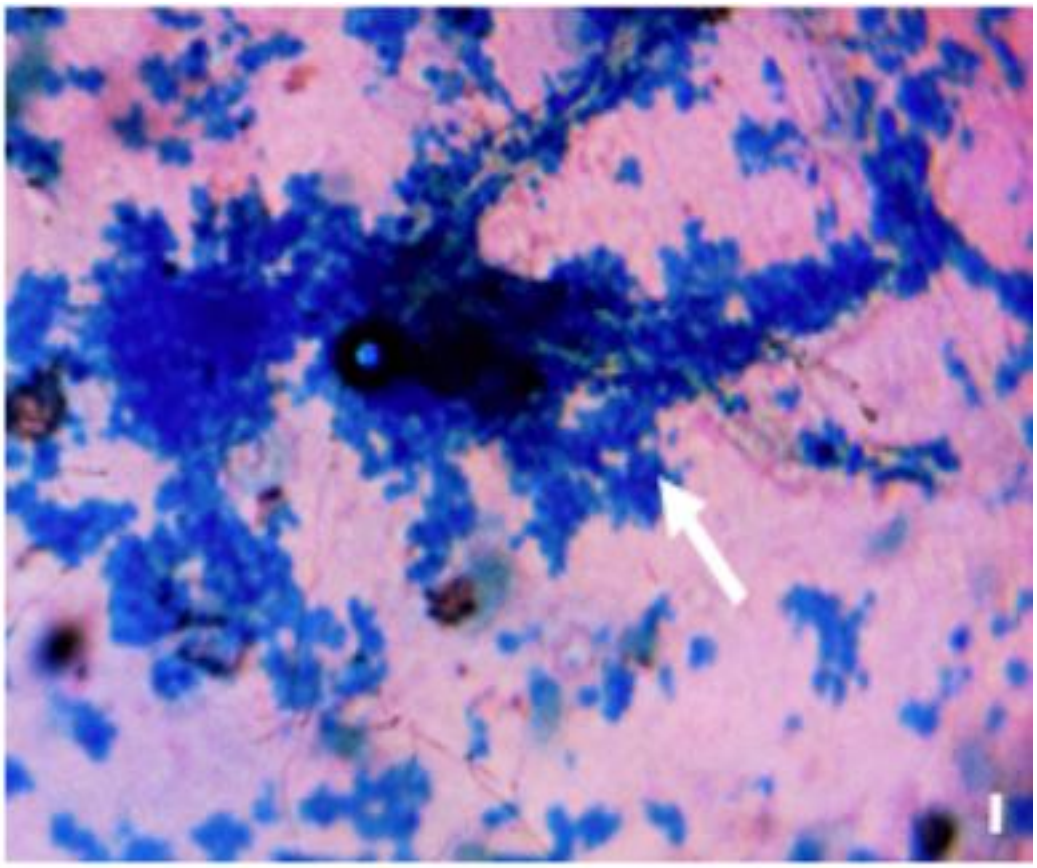


Plate 4.16: GROUP 1:Rat bone marrow of control showing normal bone marrow reticulum cells with normal distribution of iron. **(X100)**

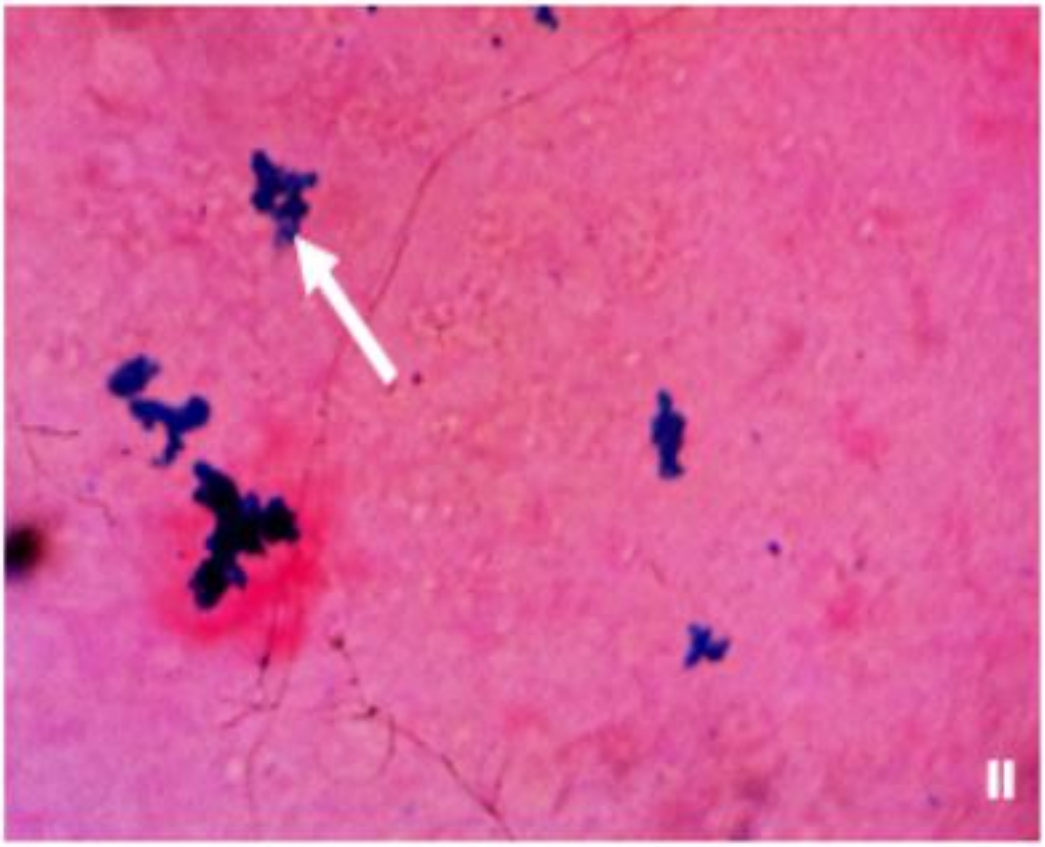


Plate 4.17: GROUP 2:Bone marrow of rat given Phenylhydrazine only showing Grade 1iron status, with very slight iron storage(X100)

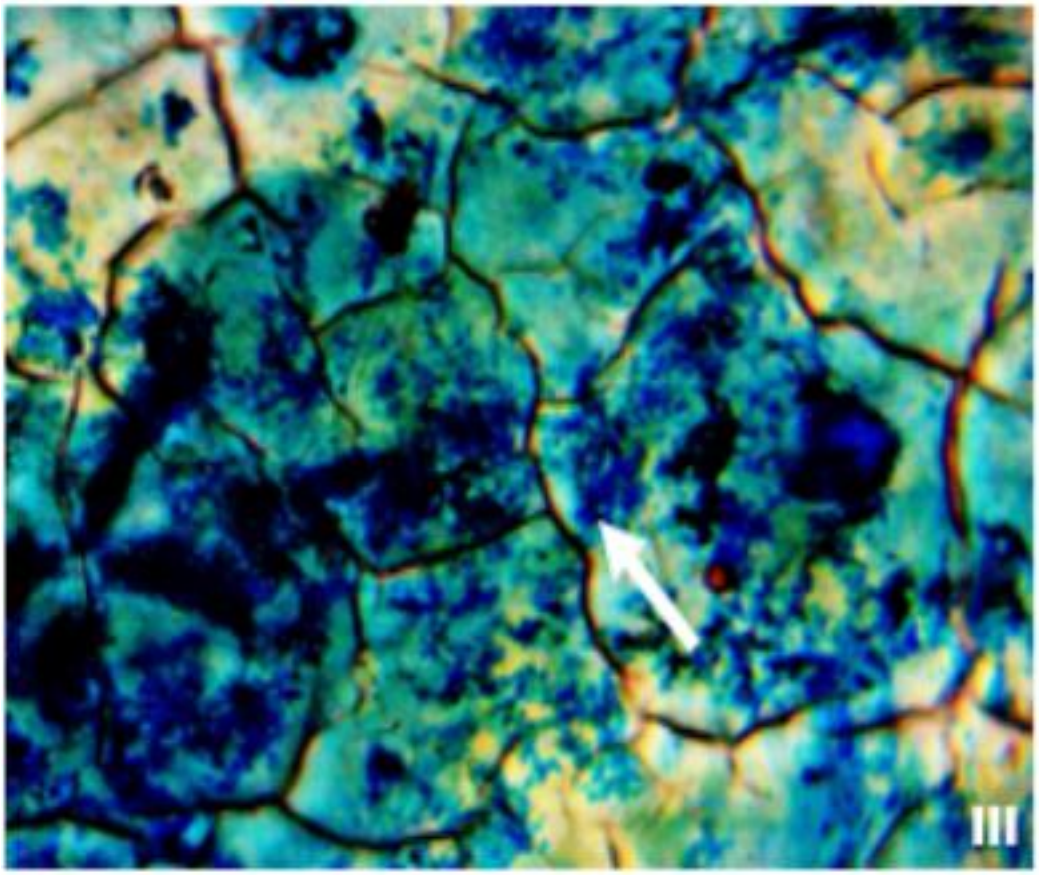


Plate 4.18: GROUP 3:Bone marrow of rat given PHZ +500mg/kg *Cola acuminata* showing grade 3 iron status, with moderate iron distribution(X100)

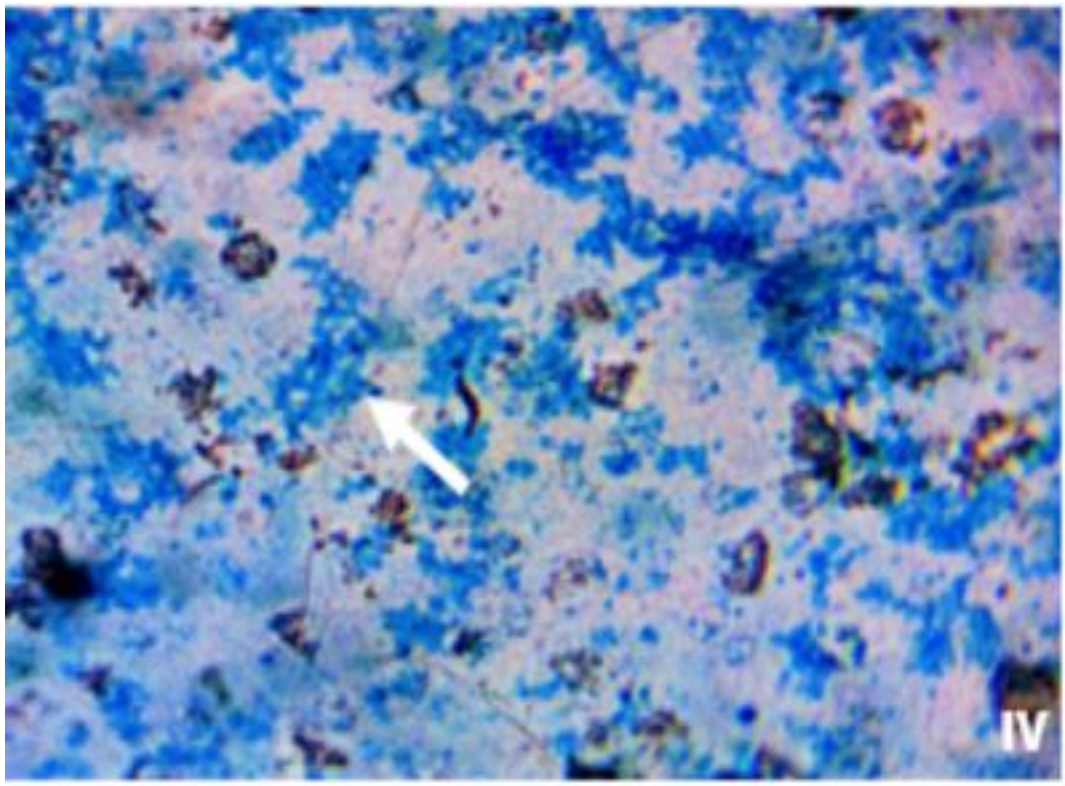


Plate 4.19: GROUP 4:Bone marrow of rat given phenylhydrazine + 1500mg/kg *Dennettia tripetala*Showing grade 4 iron status, with moderate heavy iron distribution(X100)

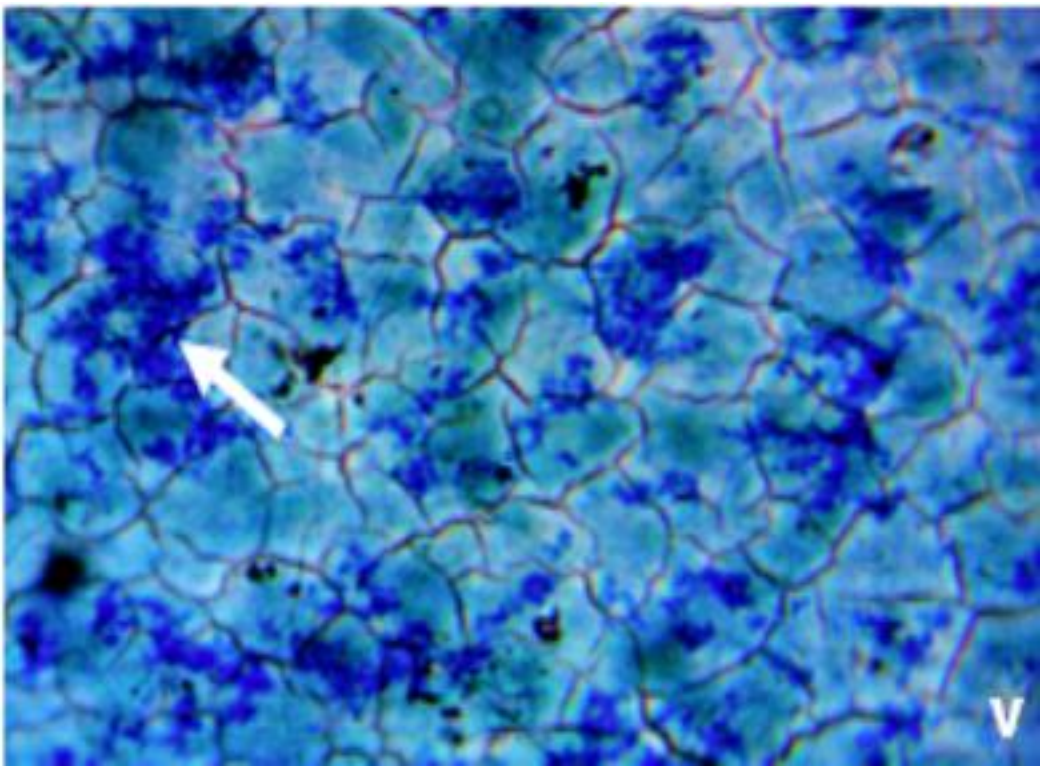


Plate 4.20: GROUP 5:Bone marrow of rat given phenylhydrazine + 100mg/kg Vit. Cshowing grade 3 iron status with moderate iron distribution(X100).

Table 4.17a. Effect of *Cola acuminata* and *Dennettia tripetala* Methanol Leaf Extracts on Hematological indices in Phenylhydrazine Induced Hemolytic Anemia

	RBC (10^6UL)	HBG (g/dl)	HCT %	WBC (10^3/UL)	PLATELET (10^3/uL)	LYM(%)
GROUP						
GRP1 CONTROL	7.84±0.25[a]	16.1±0.92[a]	43.23±2.31[a]	7.23±1.55[a]	600±398.62[a]	83.4±1.66[a]
GROUP2 PHZ	6.04±0.50[b]	13.57±1.86[b]	43.2±1.06[a]	7.033±2.98[a]	284.67±167.92[a]	75.47±4.62[b]
GROUP3 PHZ+DT	6.42±0.19[bc]	14.83±0.85[b]	43.7±3.00[a]	12.33±4.24[a]	539.33±126.93[a]	78.3±3.17[b]
GROUP4 PHZ+CA	6.94±0.4[bc]	15.47±1.96[b]	46.43±4.68[a]	11.80±4.12[a]	550.33±99.12[a]	82.05±3.04[b]
GROUP5 PHZ +VTC	6.68±0.29[bc]	15.67±0.49[b]	44.3±1.8[a]	9.33±3.76[a]	570.67±123.43[a]	78.50±2.26[b]

KEY: Values are mean ± SD n = 6 [a]= not significantly different from control
[b]=significantly different from control [c]= significantly different from group 2
PHZ=Phenylhydrazine

Table 4.17b. Effect of *Cola acuminata* and *Dennettia tripetala* Methanol Leaf Extracts on Hematological indices in Phenylhydrazine Induced Hemolytic Anemia

	MID (%)	GRAN (%)	MCV(fL)	MCH (pg)	MCHC (g/dl)	RDW-SD (fL)
GRP1 CONTROL	12.07±1.25[a]	4.53±0.70a	55.13±1.33[a]	23.80±5.74[a]	37.2±0.6[a]	29.2±1.21[a]
GROUP2 PHZ	15.97±3.15[b]	8.57±5.23[a]	71.90±5.29[b]	22.37±1.29[a]	31.33±3.95[b]	57.73±7.39[b]
GROUP3 PHZ+DT	15.33±3.00[b]	6.37±1.10[a]	68.23±6.49[b]	23.10±2.00[a]	33.63±0.95[ac]	52.73±23.45[a]
GROUP4 PHZ+CA	19.77±9.03[b]	5.50±3.83[a]	66.93±3.81[b]	22.17±1.64[a]	33.20±1.31[ac]	37.73±4.97[a]
GROUP5 PHZ +VTC	12.57±6.59[b]	4.33±1.70[a]	66.53±2.91[b]	23.40±0.36[a]	35.36±1.59[ac]	52.03±3.26[a]

KEY: Values are mean ± SD n = 6 [a]= not significantly different from control
[b]=significantly different from control [c]= significantly different from group 2
PHZ=Phenylhydrazine

Table 4.17c. Effect of *Cola acuminata* and *Dennettia tripetala* Methanol Leaf Extracts on Hematological indices in Phenylhydrazine Induced Hemolytic Anemia

	RDW-CV (%)	MPV (fL)	PDW (%)	PCT (%)	P-LCR (%)
GRP1 CONTROL	14.1±0.44[a]	8.13±1.36[a]	8.60±1.21[a]	0.52±0.43[a]	6.73±11.66[a]
GROUP2 PHZ	22.50±2.51[b]	7.87±0.75[a]	9.20±2.25[a]	0.22±0.14[a]	4.53±7.85[a]
GROUP3 PHZ+DT	21.00±7.73[bc]	7.70±0.44[a]	9.10±0.66[a]	0.41±0.07[a]	3.67±3.60[a]
GROUP4 PHZ+CA	15.63±2.04[ac]	7.33±0.25[a]	8.17±0.46[a]	0.40±0.08[a]	0.00±0.00[a]
GROUP5 PHZ +VTC	21.53±0.59[bc]	7.47±0.46[a]	8.17±0.40[a]	0.42±0.12[a]	0.00±0.00a

KEY: Values are mean ± SD n = 6 [a]= not significantly different from control
[b]=significantly different from control [c]= significantly different from group 2
PHZ=Phenylhydrazine

4.19. Effect of *Dennettia tripetala* and*Cola acuminata* Methanol Leaf Extracts on Expression of some Iron metabolism genes in Changes Induced by the Phenylhydrazine-Induced Hemolytic Anemia

Figures 4.2, 4.3, 4.4 and 4.5 show melt peak, melt curve and amplification curve for the expression of IREG, DMT-IRE, HO1 and TFR1 genes in the liver and spleen of anemic albino Wistar rats, treated with *Cola acuminata* and *Dennettia tripetala* methanol leaf extracts.

In the liver, HO1 was downregulated in the phenylhydrazine group, while IREG was upregulated. *Dennettia tripetala* and *Cola acuminata* methanol leaf extract downregulated HO1 expression and downregulated IREG. There was no change in the expression of TFR1 and DMT-IRE across all the groups.

In the spleen, HO1 was upregulated by phenylhydrazine. *Dennettia tripetala* and *Cola acuminata* methanol leaf extract downregulated this gene. There was no significant difference in the other genes IREG,DMT-IRE and TFR1 was down regulated by both extracts and Vitamin C. IREG was downregulated by *Dennettia tripetala,* but upregulated by *Cola acuminata.* TFR1 showed no change with both extract and vitamin c, compared with the control and group 2.

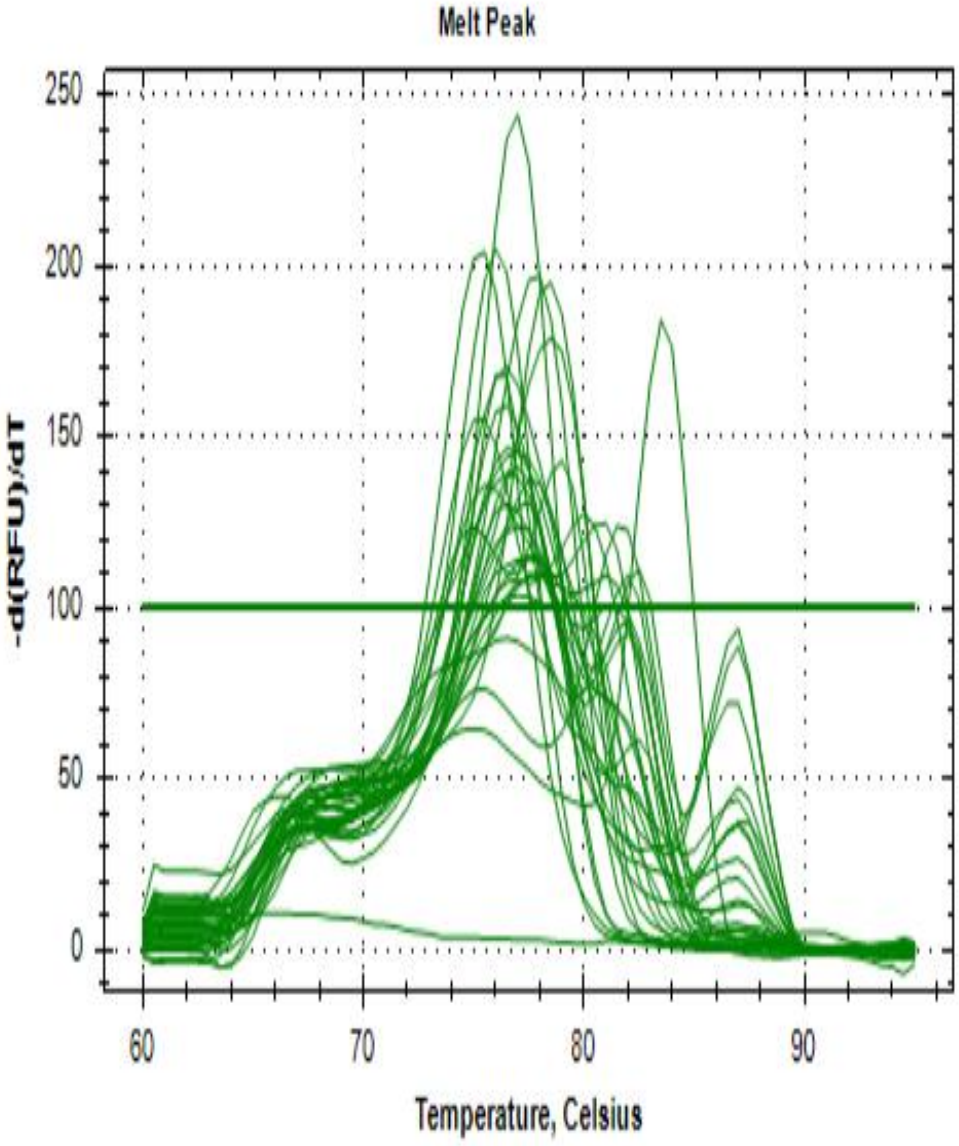


Fig. 4.2a Melt Peak for DMT1-IRE

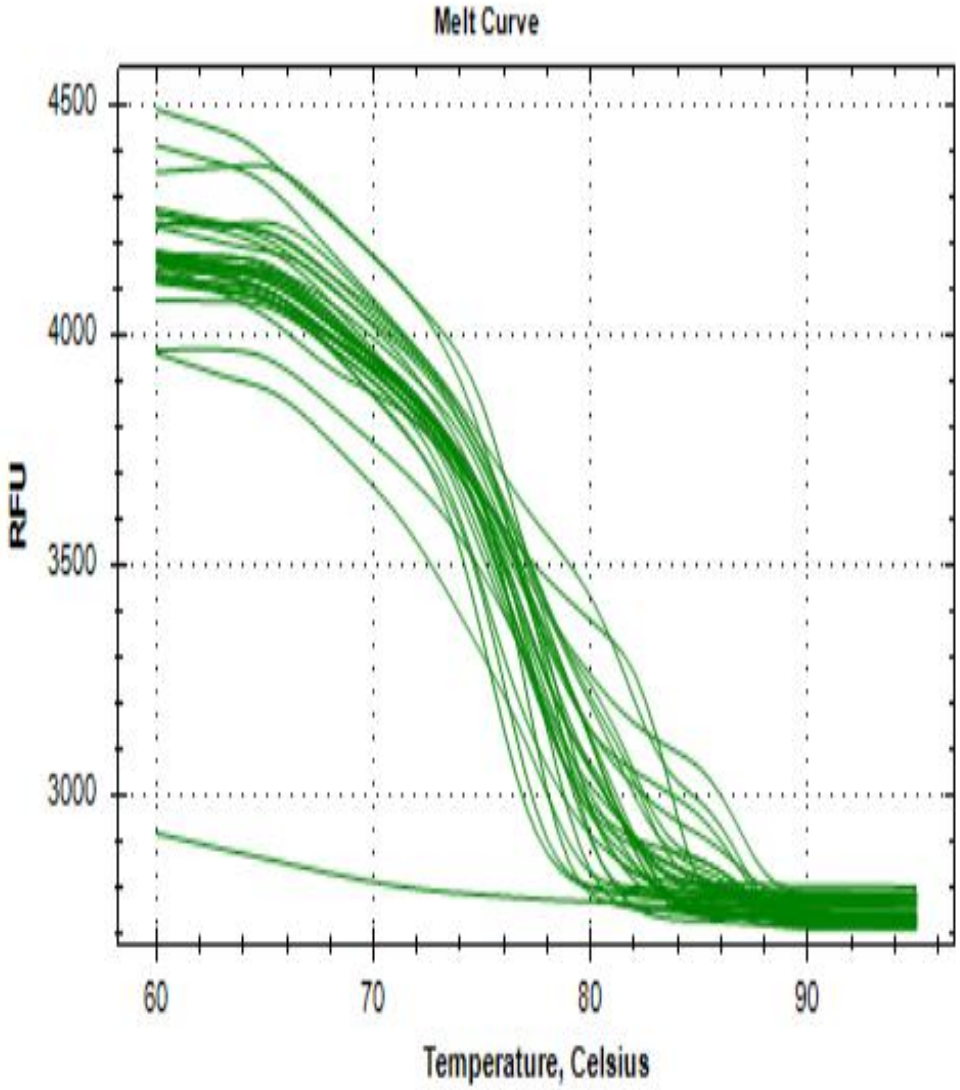


Fig. 4.2b Melt curve for DMT1-IRE

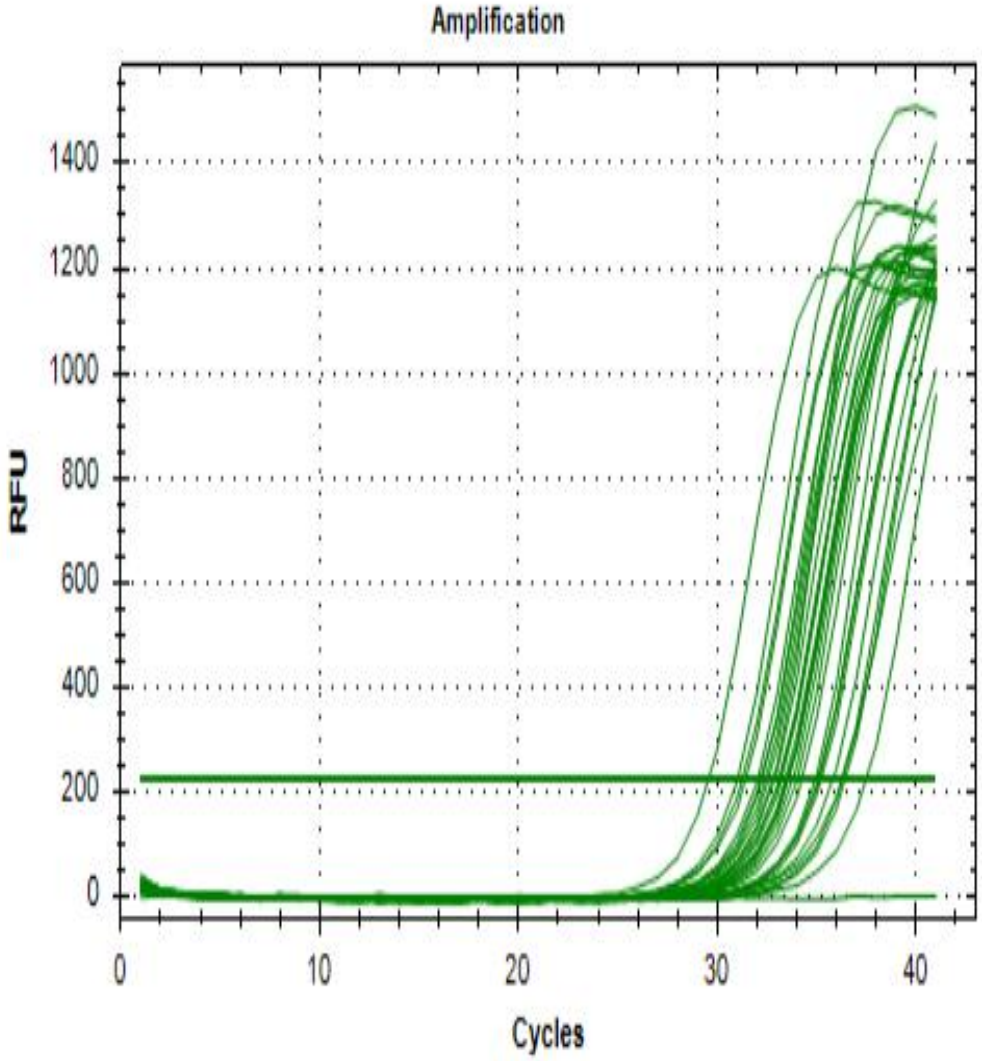


Fig. 4.2c Amplification curve for DMT1-IRE

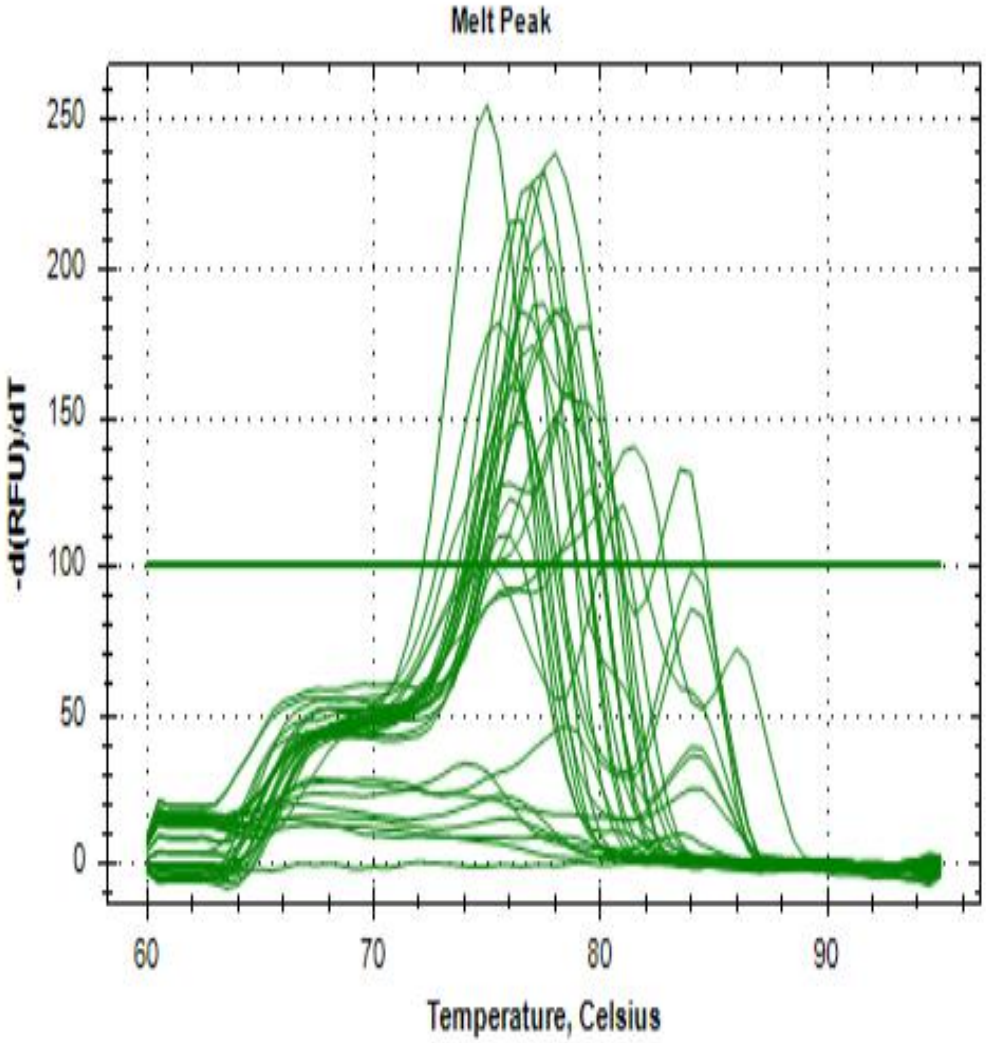


Fig. 4.3a Melt peak for TFR1

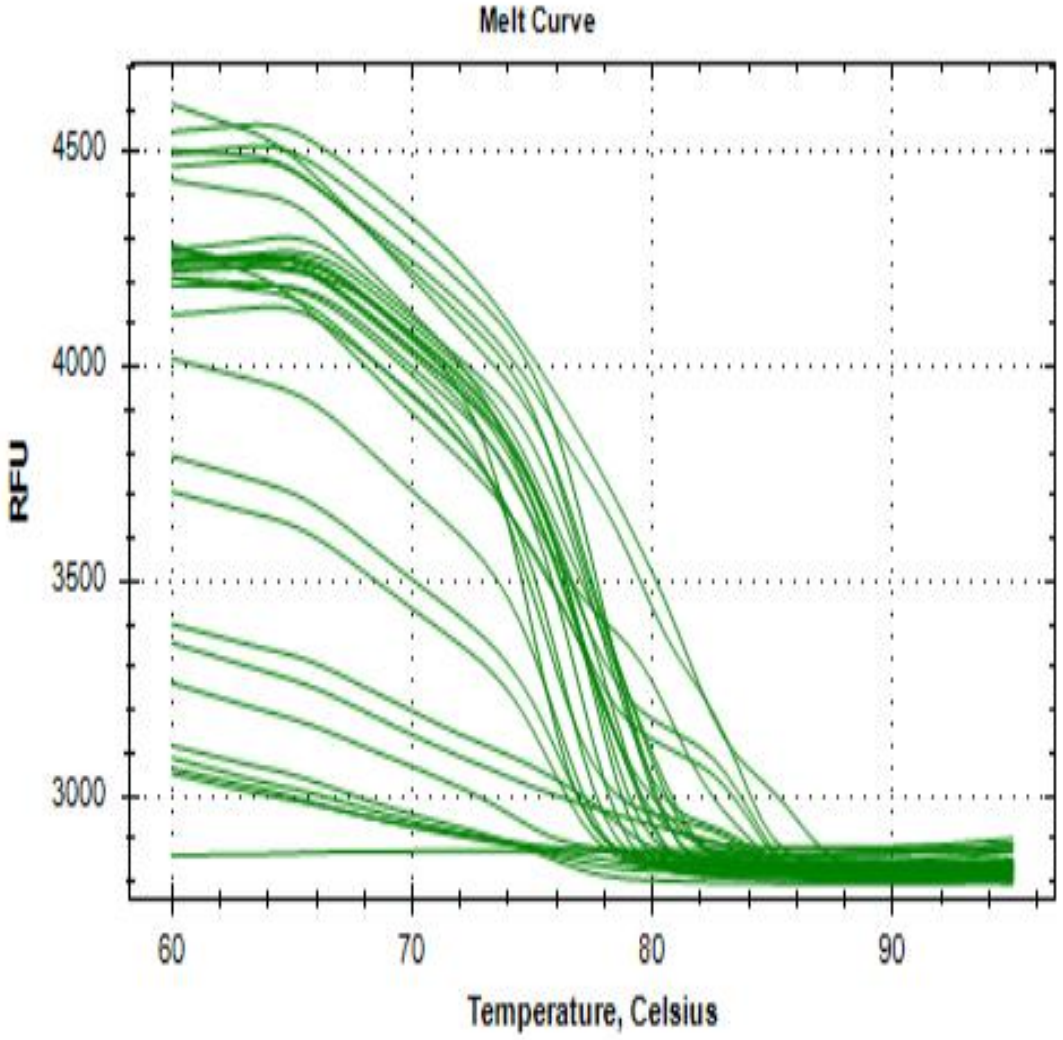


Fig. 4.3b Melt curve for TFR1

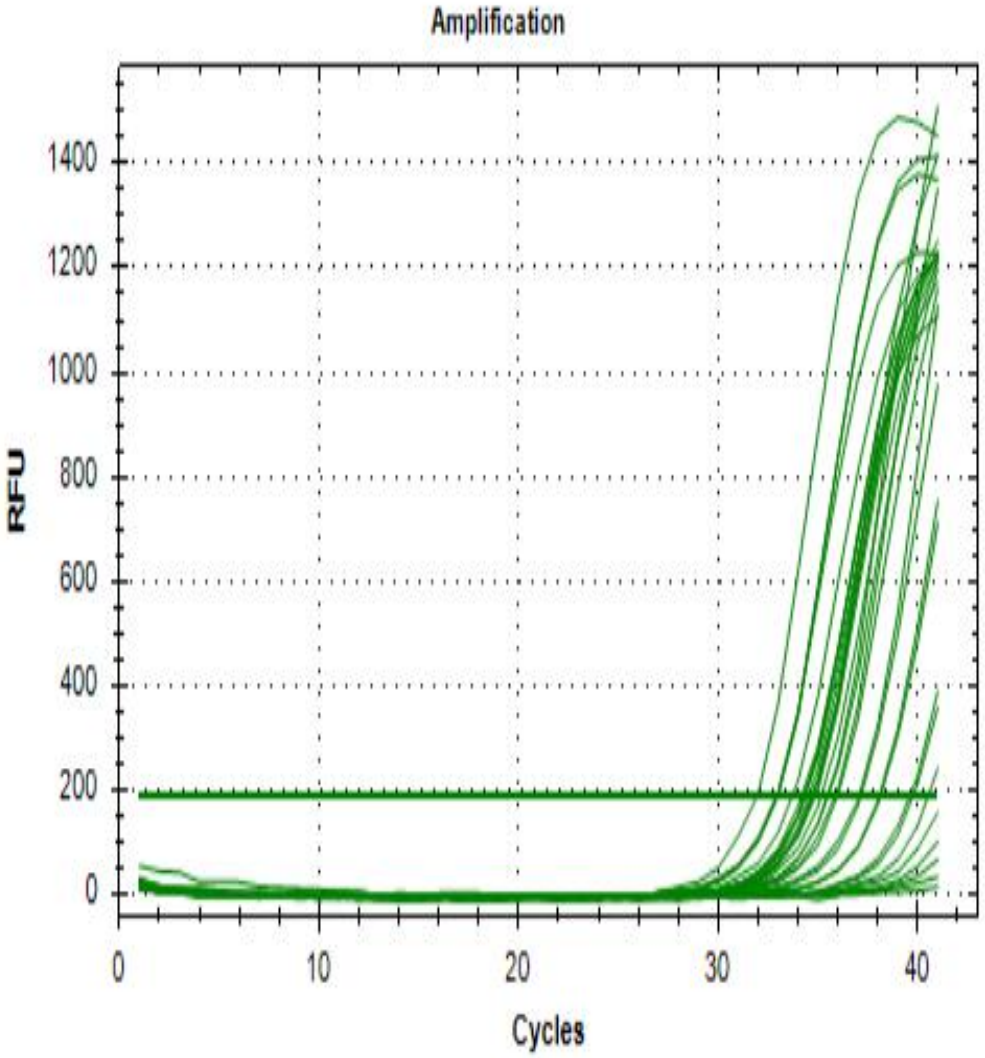


Fig. 4.3c Amplification curve for TFR1

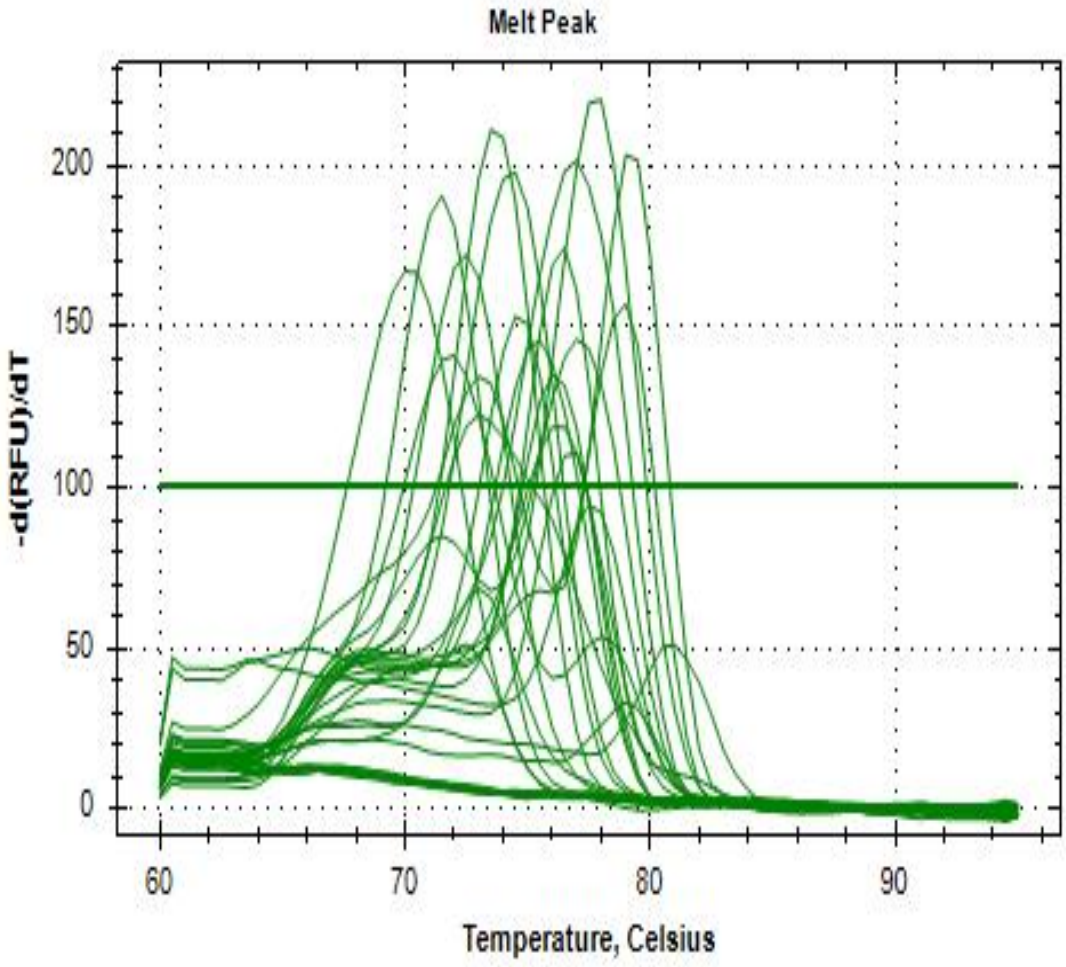


Fig. 4.4a Melt peak for HO1

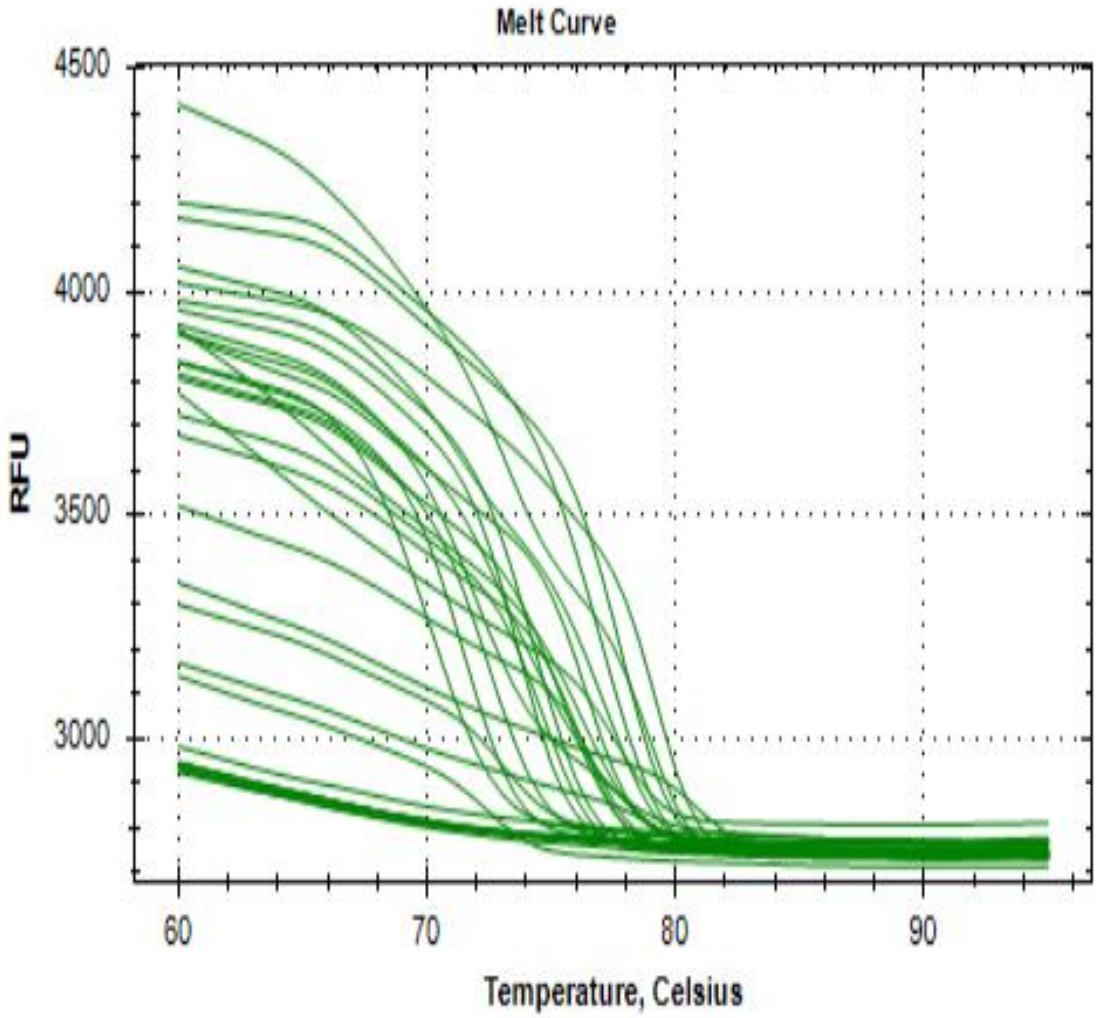


Fig. 4.4b Melt curve for HO1

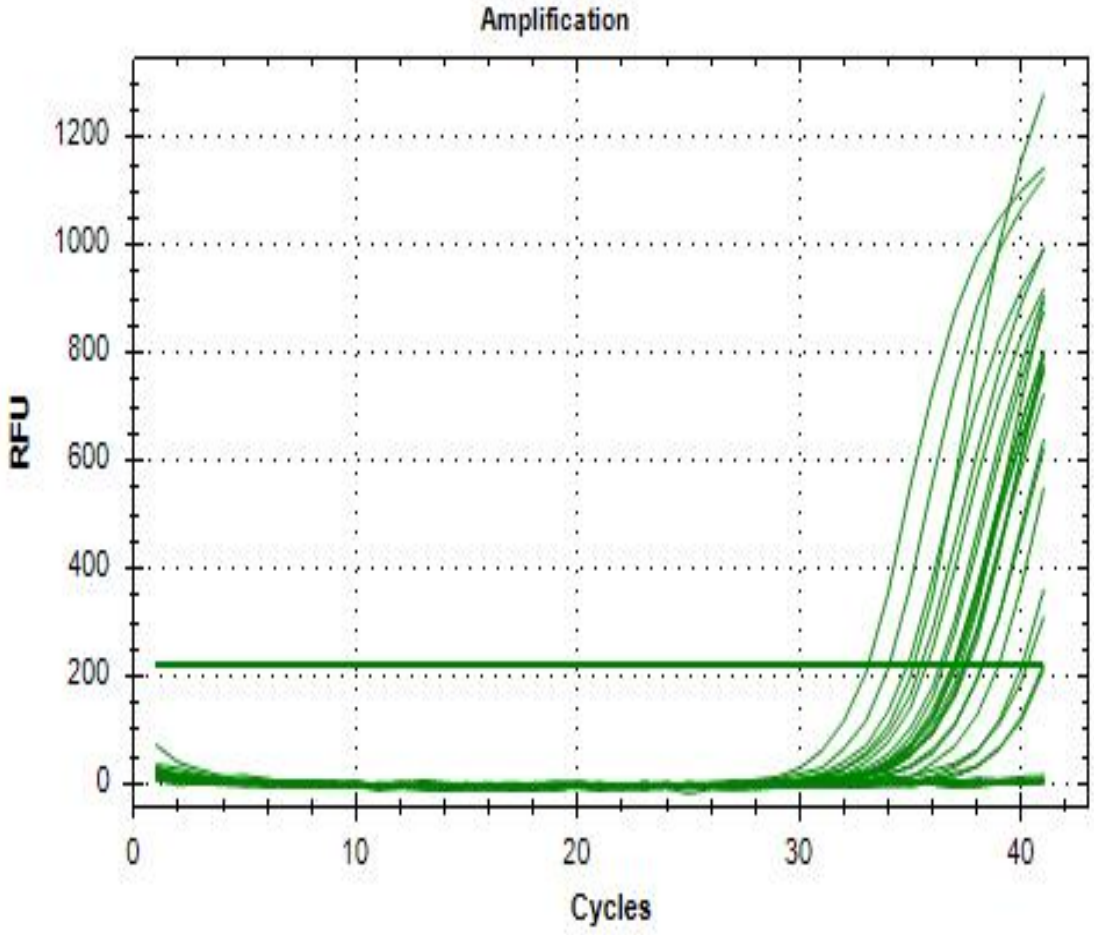


Fig. 4.4c Amplification for HO1

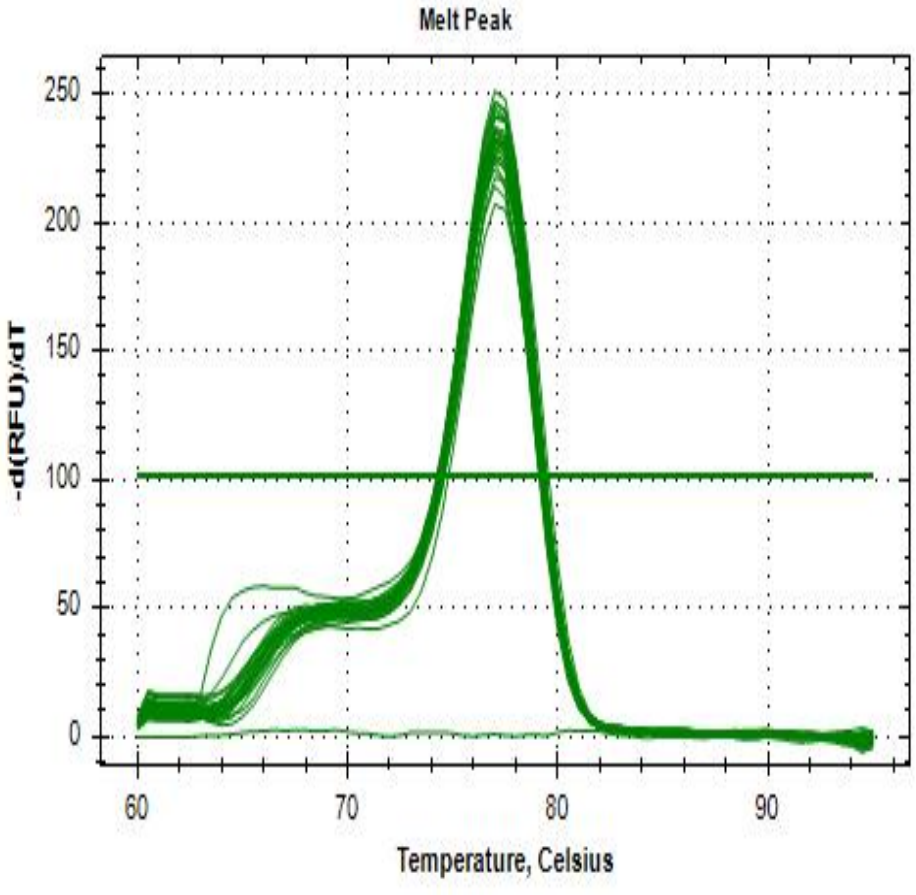


Fig. 4.5a Melt peak for 18S

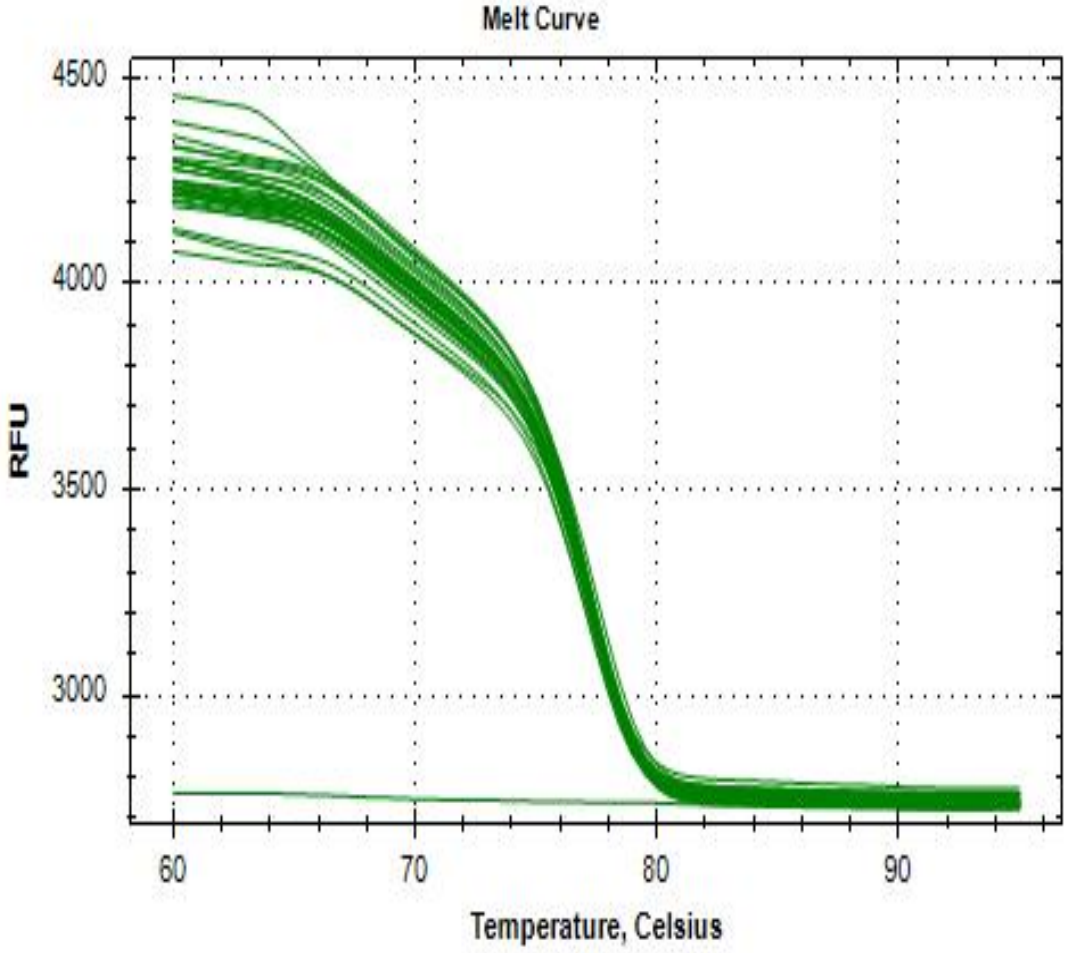


Fig. 4.5b Melt curve for 18S

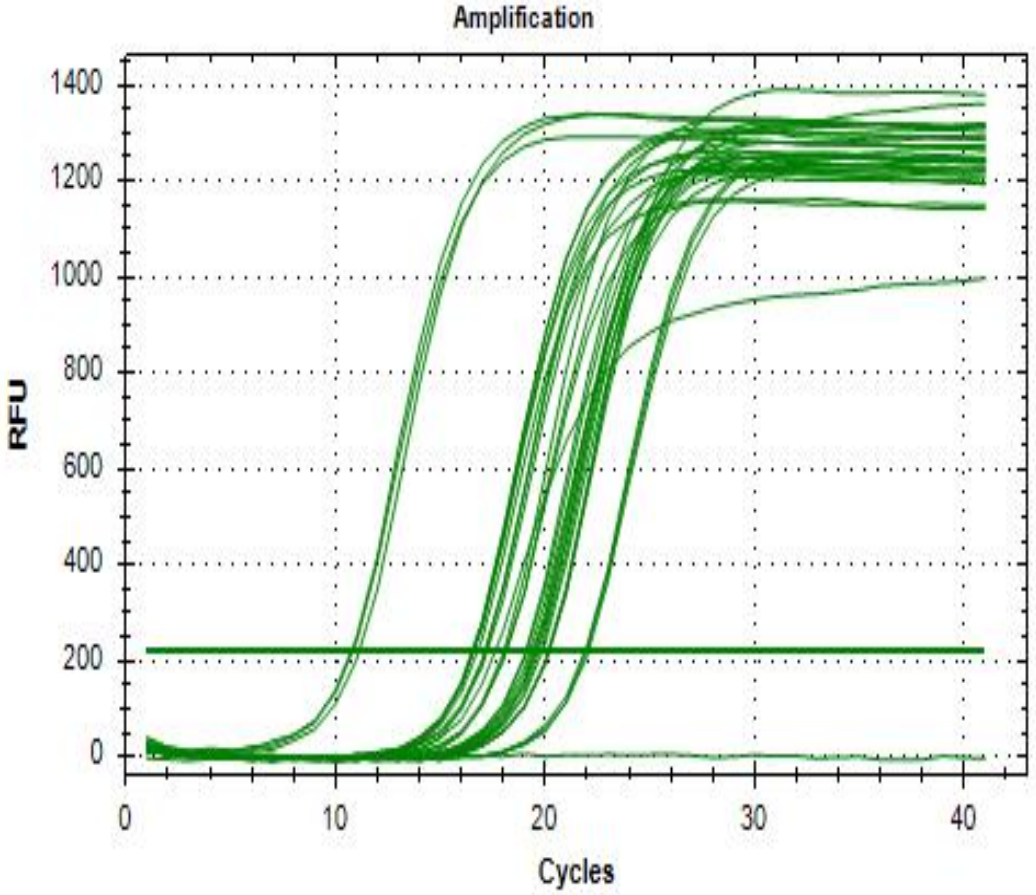


Fig. 4.5c Melt peak for 18S

CHAPTER 5
DISCUSSION AND CONCLUSION

5.1. Discussion

5.1.1. Proximate and mineral content of *Dennettia tripetala* and *Cola acuminata* Leaves

Carbohydrates, proteins, lipids, fibre and minerals are important component of food which need to be present in the right proportion to confer health benefits to individuals. While proteins serve several biochemical functions including enzymatic, structural, transport, etc, carbohydrates and lipids provide the needed energy for cellular functions. Fibre aids easy digestion through the digestive system, minerals are important for proper absorption of nutrients, and as cofactors for some enzymes. They are also useful in health and disease states.

The results for proximate analysis showed that both extracts contain minerals, proteins, lipids, fibre, and carbohydrate with low moisture content. DT contained higher content of proteins and ash while CA had higher amounts of lipids, fibre, carbohydrate and moisture.Low moisture content of the leaves reduce the chances of being spoilt by microbes (Adeyeye and Adejuyo 1994), thus elongating the shelf life. The results for proximate analysis reveal that the leaves of DT and CA exhibit lower moisture contents when compared with *Celosia argentea* (80%), *Amaranthus cruentus* (86%) reported by Mensah *et al*. (2008),*Vernonia calvaona* (37.67%) reported by Igile*et al*. (2013), *Gnetumafricanum* (9.18%) and *Telfairia occidentalis* (8.64%) reported by Dike (2010), *Gossypium hirsurum* (15.04%) and *Mormordicacharanta* (13.08%) by Ayeni *et al*., (2015), *Dennettia tripetala* leaves (13.11%) reported by Evuen and Kpomah, (2023) and lemon grass leaves reported by Adegbegi*et al*., (2012). Evuen and Kpomah, (2023) results for *D.tripetala* leaves ash content was lower (6.52%) than that for the present research. They also reported much

higher values for crude fibre, lipids and moisture compared to the present study but lower values for carbohydrates.

The mineral analysis of both DT and CA, reveal that DT possesses a higher concentration of phosphorus, zinc and iron, while CA contains a higher concentration of sodium, potassium, calcium and magnesium.

5.1.2. Qualitative phytochemical composition

Phytochemicals are important secondary metabolites necessary for the general wellbeing of the cell. Flavonoids and polyphenolic compounds having at least two iron binding sites chelate iron effectively. Flavonoids may be categorized as lipophilic and hydrophilic chelators. Lipophilic chelators enhance iron absorption, lowers iron excretion, and improve the deposition of excess iron in tissues. This makes them good option for the treatment of iron deficiency anemia. Hydrophilic chelators, on the other hand, induce the elimination of excess iron, hinders iron absorption, and improve antioxidant and anti-inflammatory activity, without having other side effects (Kontoghiorghes and Kontoghiorghe, 2020).Flavonoids decrease oxygen toxicity, via the inhibition of HO • production from the fenton reaction by chelating iron (Vanhees, *et al*., (2011); Guelfi*et al*., (2023)).

It is assumed that flavonoids also chelate non-hemic iron, as a consequence as their biounavailability(Vanhees, *et al*., 2011;Petry, 2014).The rapid scavenging ability of flavonoids against pathogens is a remarkable role vital role in the plantsdefense system (Panche*et al*.2016).

Results for the qualitative phytochemical composition of the two plants reveal that they contain copious number of phytochemicals including flavonoids, cardiac glycosides, saponins, terpenoids, alkaloids, coumarins, tannins, reducing sugars, amino acids and phenols. Eromosele and Kehinde, (2018); Zailani*et al*., (2020)

reported similar findings for *Cola acuminata*'s phytochemical content. In concordance with this research, Okungbowa *et al*., (2017) reported the presence of saponins in *A. fistulosum*, *B. pinnatum*, *C. citratus* and *H. crinita.*Mordi *et al*., (2021); Evuen and Kpomah, (2023) reported similar phytochemical results for *D.tripetala* leaves content of phytochemicals.

DT and CA contain large amounts of flavonoids, an appreciable amount of saponins, while CA had higher composition of terpenoids, alkaloids and tannins. These findings are similar to that of Mordi *et al*., (2021); Evuen and Kpomah, (2023) for *D.tripetala* leaves.

The abundance of alkaloids found in the leaves of the two plants indicates potential benefits such as pain relief, reduction of inflammation, and enhanced resistance to diseases and stress (Gupta,1994). Mordi *et al*., (2021); Evuen and Kpomah, (2023) in their study with *D.tripetala* leaves reported similar observations as with this study.

According to Sodipo*et al.* (2000), saponins lowers cholesterol levels, boosts immunity and protect against carcinogenic agents. Conversely, high amounts of saponins couldresult in gastro-enteritis (Awe and Sodipo, 2001). In the two plants, saponins were found to be low in quantity making their consumption safe. Studies on*D.tripetala* leaves by Mordi *et al*., (2021) as well as Evuen and Kpomah, (2023) are in line with findings in the study.

Tannins have been reported to possessantibacterial, anti-tumour, antiviral, antifungal, and properties (Andzouana and Mombouli, 2012). Mordi *et al*., (2021);Evuen and Kpomah, (2023) reported similar results for *D.tripetala* leaves.

Phenols, flavonoids, alkaloids, tannins and saponins content in *Cola acuminata* leaves was reported by Eromosele and Kehinde, (2018). Zilani*et al*., (2020) extracted alkaloids, phenolics and flavonoids from the leaves of *Cola acuminata* which they

confirmed to effectively treat malaria infected mice. Phenolic, alkaloid and flavonoid compounds in plant extracts indicate that the plant might have antioxidant potentials to scavenge free radicals (Tonisi*et al.*, 2020).

5.1.3. GC-MS and HPLC analysis

HPLC

HPLC results show that the leaves of DT and CA contain flavonoids, catechin, proanthocyanidine and anthocyanin which are potent antioxidants present in plants. However, CA exhibited higher concentrations of all these except for proanthocyanidin and anthocyanin which were higher in DT compared with CA. *Cola acuminata* had higher concentrations of Tannins, flavones, flavanones, catechins, steroids, narigenin and cyanogen glycosides, while *Dennettia tripetala* had higher concentrations of proanthocyanidins, anthocyanidin and spartein.

Flavonoids are the most common class of polyphenols and are present in a wide variety of plants, imparting many of these plants with their specific colors. Their primarily functions are to protect the plant and to act as chemical messengers, physiological regulators, and cell cycle inhibitors (Imam, *et al*., 2017). Flavonoids have already been shown to have both anti-inflammatory and antioxidant effects (Rathee*et al*., 2009).

GC-MS

A number of bioactive compounds having biochemical and structural importance were found in the leaves of *Cola acuminata* and *Dennettia tripetala.* GC-MS results show the presence of terpenes, including β-Longipinene, neophytadiene, phytol, 3,7,11,15-Tetramethyl-2-hexadecen-1-ol, lup-20(29)-ene-3-one, nerol methyl ether and squalene in DT. Both DT and CA have neophytadiene, and phytol, in common. These compounds demonstrate several beneficial properties, such as antioxidants, antifungal,

antimicrobial, and anti-inflammatory activities. Additionally, DT and CA contain other bioactive compounds like eicosane, known for its anti-inflammatory, antioxidant, antitumor, immunostimulant, anticancer, and lipoxygenase-inhibitor properties. Moreover, Bis(2-ethylhexyl) phthalate, pentadecane, tetradecane, hexadecane, cetene, heptadecane, and octadecane exhibit antioxidant, antimicrobial, and anti-inflammatory effects (Banakar and Jayaraj, (2017 and 2018); Yogeswari*et al.,*(2012a); Elgorban*et al*., (2018); Kim *et al*., (2013); Pratama *et al*., (2019)).

In *Cola acuminata,* bioactive compounds found are as follows: β-Longipinene a sesquiterpene has been reported to possess antimicrobial and anti-insecticidal, antioxidant, anti-inflammatory activities (Shukurova*et al*., 2020) and cytotoxicity (Santana *et al*., 2012).Pentadecane, an alkane, proven to exhibitantipyretic, anti-inflammatory, and analgesic properties (Chuah,*et al.*, 2018;Okechukwu, 2020).Cetene is an alkene, with antibacterial and antioxidant capacity (Yogeswari*etal*.,2012; Elgorban*et al*., 2019). Heptadecane reported to have antibacterial effects (Keke,*et al*., 2023, Togashi *et al*., 2007). Cyclotetradecane, exhibits antimicrobial capacity (Keke *et al*., 2023; Chuah *et al*., 2018), 1-Octadecene has been found to exert antibacterial, antioxidant and anticancer potentials (Keke, *et al*., 2023;Belakhdar*et al*., 2015). Neophytadieneis reported to possessantimicrobial, anti-inflammatory, antioxidant and antifungal properties in addition to cardioprotective,inhibition of cyclooxygenase or lipoxygenase. 3,7,11,15-Tetramethyl-2-hexadecen-1-ol displays antimicrobial, anti-inflammatory, anticancer, diuretic, antifungal against S. typhi, resistant gonorrhea, Joint dislocation, headache, hernia, stimulant and antimalariaproperties (Banakar and Jayaraj, 2018). 9-octadecyne enhances the activity of cytochrome P450, modulates insulin and cortisol action. Nonadecane is an alkane, which has been reported to haveseveral properties amongst which areanti-HIV, antioxidant, antibacterial,

antimicrobial, cytotoxic effect, antimicrobial, antimalarial, unani uses such asweakness of the principal organs like brain, heart,liver, general weakness, palpitation, haemoptysis, conjunctivitis, stomatitis, earache, (Banakar and Jayaraj, 2018). Eicosane exhibits its effect as an antioxidant, antifungal, analgesic, anti-inflammatory, and antipyretic (Okechukwu, 2020;Ahsan *et al*., 2017). 11-Octadecenoic acid, methyl ester shows antioxidant and antimicrobial activities (Gollo *et al*., (2020), El-sayed and Ismail, (2022)). Phytol is a diterpenoid revealed to possess antimicrobial, anti-inflammatory, anticancer, diuretic, antifungal against S. typhi, resistant gonorrhea, joint dislocation, headache, hernia, stimulant and antimalaria (Banakar and Jayaraj, 2018). Oxazepam, 2 Trimethylsilyl (TMS) derivative is a sedative, anxiolytic, anticonvulsant, hypnotic, sedative, anterograde amnesia effects. Docosane is an antimicrobial agent. Octadecamethyl-cyclononasiloxaneexhibit antioxidant and insecticidal activities.Eicosamethyl-cyclodecasiloxane also displays antioxidant activity (and Mohankumar, 2017). Bis(2-ethylhexyl) phthalate shows antimicrobial, cytotoxic, anticancer (Lofty *et al.,* 2018), antibacterial and larvicidal properties (Javed *et al*, 2022). Eicosane has been reported to exhibit analgesic, anti-inflammatory, wound healing, antipyretic, antioxidant (Balachandran *et al*., 2023, Okechukwu, 2020,). Lup-20(29)-en-3-oneis a dietary pentacyclic triterpenoid which portrays anti-inflammatory, antioxidant and anti-carcinogenic effects (Liu *et al.*, 2021) Bioactive compounds found in *Dennettia tripetala* are numerous and include; Pentadecane which exhibitsanti-inflammatory, antimicrobial, analgesic, antipyretic activity (Balachandran *et al*., 2023, Okechukwu, 2020; konovalova*et al*, 2013). Tetradecane and hexadecane have proven to have cytotoxic, antimicrobial, anthelmentic, antipyretic, and anti-tumour activities andis applied in the management of bronchitis, asthma, tuberculosis, dyspepsia, constipation, anemia, throat diseases,

elephantiasis, antidiabetic, anti-inflammatory, anti-diarrhoeal (Banakar and Jayaraj, 2017). Cetene displays antibacterial and antioxidant (Yogeswari*et al*.,2012; Elgorban*et al*., 2019). Heptadecane has anti-inflammatory effect (Kim *et al*., 2013, Pratama*et al*., 2019). Octadecane shows antioxidant, anticorrosive, antisepsis and anti-inflammatory effects. It is effective against cough, lung diseases, cold and fever (Banakar and Jayaraj, 2018). Neophytadiene is a diterpene havingantimicrobial, anti-inflammatory, antioxidant (Pratama*et al*., 2019)cardioprotective effects.

3,7,11,15-Tetramethyl-2-hexadecen-1-ol (Phytol) is a diterpene alcohol which displays several biological effects including antimicrobial, anti-inflammatory, anticancer, diuretic, antifungal against S. typhi, resistant gonorrhea, headache, hernia, stimulant and antimalaria (Banakar and Jayaraj, 2018), precursor for the manufacture of synthetic forms of vitamin E and vitamin K1. Furthermore, phytol also was shown to modulate transcription in cells via transcription factors Peroxisome proliferator-activated receptor alpha (PPAR-alpha) and retinoid X receptor (RXR). An *In vitro* study carried out by Santos *et al*. (2013) to assess the antioxidant activity of phytol showed that this compound was able to scavenge hydroxyl radical and nitric oxide. 1,2-15,16-Diepoxyhexadecane displays anti-inflammatory and antitumor properties(Shareef *etal*., 2016).

Nonadecane has anti-HIV, antioxidant, antibacterial, antimicrobial, cytotoxic, and antimalarial effects. It also hasunani uses like weakness of the principal organs like heart, brain, liver, general weakness, hemoptysis, conjunctivitis, palpitation, earache, stomatitis (Banakar and Jayaraj, 2018). Octatriacontyl-pentafluoropropionate has antiviral (covid-19) (Elwakil*et al*., 2021). Heneicosane possess antimicrobial, anti-inflammatory, analgesic, antipyretic (Vanitha *et al*., 2020). Eicosane shows anti-inflammatory, analgesic, antipyretic, wound healing, antioxidant properties

(Balachandran *et al*., 2023, Okechukwu, 2020). Squalene is a triterpene possessing antioxidant, antitumor, cancer preventive, immune-stimulant, Chemo-preventive, Lipoxygenase-inhibitor, Pesticidal activities (Banakar and Jayaraj, 2018).

5.1.4. *In vitro* antioxidant analysis

Antioxidants are compounds that are able to scavenge free radicals and pro-oxidants by several mechanisms including iron chelation, donation of protons, etc. Plant phytochemicals have been shown to effectively curtail the effects of free radicals in biological systems thereby preventing the numerous diseases associated with oxidative stress. This makes antioxidants key targets for drug design. DPPH radical scavenging assay is the most generally accepted antioxidant assay usedaccess foods and herbal medicine because its procedure is easy and fast with dependable outcome (Dontha, 2016). DPPH is ahydrophobic radical that reacts with less polar antioxidants transferring capacity (Serpen*etal*., 2012).The DPPH scavenging potential of bioactive compounds in an extract is directly dependent on their hydrogen donating ability thus conferring antioxidant power (Yu *et al*. 2002; Jimoh *et al*. 2010).

The results of the present study reveal that *Dennettia tripetala* has a higher total antioxidant capacity than *Cola acuminata* when compared with vitamin C, the standard drug. Also, *Cola acuminata* scavenged DPPH radical better than *Dennettia tripetala* when compared with vitamin C. The total antioxidant capacity of DT being higher than that of CA can be attributed to the numerous antioxidant compounds present in the leaves as shown by GCMS results such as squalene, 6,10,14-trimethyl-5,9,13-Pentadecatrien-2-one, 3,7,11,15-Tetramethyl-2-hexadecen-1-ol, and hexadecane. CA on the other hand contains antioxidant compounds that have better capacity to scavenge the DPPH radical such as the β-Longipinene, Lup-20(29)-en-3-one, and Octadecamethyl-cyclononasiloxane, which are absent in DT.

5.1.5. Acute toxicity studies of extracts

The LD_{50} for both *Dennettia tripetala* and *Cola acuminata* methanol leaf extracts were greater than 5000mg/kg body weight of albino rats. At all doses, there were no signs of toxicity or mortality observed within the period of twenty-four hours, seven days and fourteen days respectively. Alaebo*et al*., (2022) reported similar observations with DT methanol leaf extract.

5.1.6. Sub-acute toxicity studies of each extract

i. D*ennettia tripetala*

Urea, Creatinine and electrolytes are significant indicators of kidney function. The results of this study for both DT and CA, are in line with research findings of Alaebo*et al*. (2022), Ogbonna *et al*., (2020) and Salawu *etal*., (2019) but at variance with studies done by Mordi *et al.,* (2021). The slight increase in creatinine may have resulted from glomerular inflammation and interstitial nephritis (Kodner and Kudrimoti, 2003). The present study establishes that the methanol leaf extract of *Dennettia tripetala* and *Cola acuminata* although not toxic to the kidneys altered kidney enzymes activity. However, CA showed lower values than DT. These effects may be attributed to the presence of certain biological constituent such as alkaloids and saponins. Care must be taken as continuous exposure to high doses for a longer period of time may result in kidney injury.

Total protein and albumin are markers of the liver function. Albumin is a carrier protein in the blood. Its assay and that of certain enzymes namely ALT, AST, ALP are common liver marker enzymes that serve as indicators of the health of the liver (Sun *et al*., 2019). Increased levels of these proteins and enzymes in the plasma or serum

are indicative of liver damage, which allows them to sip out of the hepatocytes, thereby disrupting effective functioning of the organ.

Albumin concentrations in the plasma across all groups was not significantly different from the normal control in this study for both CA and DT. Although CA gave lower values than DT. Alaebo*et al*. (2022);Salawu *et al*. (2019) and Ogbonna *et al*. (2020), reported similar results with 100 and 200mg/kg body weight. However, unlike the resent study, they reported that the 400mg/Kg body weight extract significantly ($p<0.05$) reduced the serum albumin concentration in the rats.

In this study,plasma ALP activitywas not significantly different from the normal control in the groups treated with low dose DT extracts but higher doses resulted in significant ($p<0.05$) increase in the activity of this enzyme. However, 2,900mg/kg body weight dose showed similar activity with that of the control. Aleabo*et al*. (2022) findings were in synchrony with results of this study. They showed that low dose DT resulted in significantly ($p<0.05$) increased ALP activities, compared with the control.Similarly, plasma ALP activity was not significantly different in the *Cola acuminata* low dose groups when compared with the control, but the higher doses resulted in a significant ($p<0.05$) reduction of ALP activity, with the lowest activity observed in the 1,600mg/kg body weight extract group. However, 1,000mg/kg body weight dose resulted in a significant ($p<0.05$) increase in ALP activity when compared with the control).Salawu *et al*. (2019) and Ogbonna *et al*. (2020) reported similar alterations of kidney and liver parameters by plant leaves alone.

ALT activities in *Dennettia tripetala* extract treated group increased significantly ($p<0.05$) across all the groups given high doses, compared with the control. Group 2 (10mg/kg body weight) which reduced significantly ($p<0.05$), while the 100mg/kg body weight group showed increased activity but not significantly different compared

with the control. Aleabo*et al*. (2022) also reported similar results with 100, 200 and 400mg/kg body weight extract.ALT activity in the *Cola acuminata* low dose groups resulted in increases which were not significantly different from the control. Higher doses of 1,600mg/kg and 5,000mg/kg body weight resulted in significant ($p<0.05$) increases in ALT activities when compared with the control. 1,000 and 2,900 mg/kg body weight on the other hand, gave rise to activities which were not significantly different from the normal control. Although, at 1,000mg/kg body weight, ALT activity decreased but not significantly.

Dennettia tripetala extract induced a significant ($p<0.05$) reduction in AST activity across all the groups, with lowest activity observed in the group given 100mg/kg body weight extract. This finding is not in line with that reported by Aleabo*et al*. (2022). *Cola acuminata* extract induced a significant ($p<0.05$) reduction in AST activity across all the groups, with the lowest activity observed at 1,000mg/kg body weight dose, when compared with the normal control.Obasi *et al*., (2022), stated results that are different from the present study. The disparity may be due to differences in plants.These findings indicate that both DT and CA are non-toxic to the liver and kidneys but high doses result in modulation of their function.

Heamatology of leaves alone on healthy animals

Dennettia tripetala and *Cola acuminata* methanol leaf extract did not induce significant differences in the PCV, WBC, RBC, HBG and PLT concentrations across all the groups when compared with the control. Although, both extracts resulted in alterations of the values of the given parameters.

In DT, PCV values were reduced across all the extract groups compared to the control, WBC reduced in the 10 and 5,000mg/kg body weight group, but increased in other groups, RBC values were lower than that of control. HBG increased in the 10 and

1,600m/kg body weight groups but decreased in the other groups. PLT increased across all groups except or the 10 and 1,600mg/kg body weight groups. Report by Mordi *et al*., (2022) is at variance with the present study, as their results showed significant ($p<0.05$) increases in PCV, PLT and HBG while significant ($p<0.05$) reductions in total white blood cell count at a dose of 200 and 500mg/kg body weight DT. They however reported similar findings in the results for RBC.

In CA. PCV values decreased across all the groups except for the 5,000mg/kg body weight group. WBC increased slightly across all the groups, RBC decreased across all the groups, while HBG values were similar to that of the control. PLT on the other hand showed highest values at 10mg/kg body weight extract and lowest values at 100mg/kg body weight extract. These results reveal that both DT and CA altered hematology parameters but values were not statistically significant. Plant chemicals may interfere with biomolecules to modulate their concentrations in the blood. Care must therefore be taken when these extracts are consumed in normal healthy state.

5.1.7. Effect of graded doses of each extract on phenylhydrazine-induced hemolytic anemia

PHZ resulted in anemic conditions in rats as seen in the significantly lowered PCV values, compared with the normal control. Treatment with graded doses of both CA and DT, improved PCV values when compared with that of the normal control. Ichipi-Ifukor*et al.,* (2013); Mordi *et al.,* (2021a) have reported similar findings.

In CA treated groups, the highest PCV was observed in the groups treated with 500mg/kg body weight extract. While creatinine and urea values reduced slightly in the higher doses treated groups, the reductions were significantly ($p<0.05$) different in the low dose groups compared with the negative control. There was no significant difference across the groups for total protein results.

In DT treated groups, the highest PCV was obtained at the highest dose of 5,000mg/kg body weight extract, followed by the groups treated with 1,500mg/kg body weight extract. Therefore, DT and CA effectively improved the PCV in spite of PHZ assault. This may be attributed to the plants content of iron which is needed for heamoglobin synthesis, a vital component of the red blood cells. In addition, phytochemicals present in the extracts protected the red blood from the reactive oxygen species and free radicals generated from PHZ metabolism, thereby preserving the architecture of the red blood cells. Kolawale*et al.,* (2017); Kale *et al*., (2019); Ousaaid*et al*., (2022) have reported results which are consistent with the present study.

5.1.8. Comparative effect of both extract on phenylhydrazine-induced anemia

i. Biochemical effects on kidney and liver function enzymes

The kidney is the main organ for the production of urine. Creatinine and urea are non-protein nitrogenous metabolites cleared by the kidneys via glomerular filtration. Estimates of plasma or serum levels of these metabolites are common indicators of kidney function (Tietz,2008). However, alterations to the structure of the kidneys may affect its ability to efficiently filter urea and creatinine, leading to their elevation in the blood (Ravnskov, 2005). PHZ induced kidney and liver damage in line with published researches by Kolawale*et al.,* (2017); Kale *et al*., (2019); Allahmoradi*et al*., (2019) Ezeigwe*et al*., (2020); El-Shafey, *et al*., (2023). Albumin which is a transport protein of the blood was slightly increased by PHZ. This was reversed by the administration of both CA and DT. Although, these changes were not statistically significant. The electrolytes, sodium, potassium, chloride and bicarbonate ions were assayed for in the plasma of toxicant treated group and those of the extract treated group. It was observed that there was no significant difference ($P< 0.05$) between the toxicant treated group and the extract treated groups. Vitamin C treated group also

showed no significant difference either. This finding is similar to those reported by Oluwafemi *et al*., (2019) and Nicolas *et. al*., (2018).

Alanine amino transferase (ALT) activity were significantly (P<0.05) increased in the phenylhydrazine treated group when compared with those of the control, which is indicative of liver damage. Treatment with *Dennettia tripetala* , *Cola acuminata* methanol leaf extract and vitamin c, reversed the damage significantly (P<0.05)as revealed by the reduced values compared with the phenylhydrazine treated group.Results obtained were similar to those reported by Oluwafemi *et al*., (2019), Obayuwana*et al*., (2022); Obayuwana and Obayuwana, (2022); Umoren *et al.,* (2023); Okafor and Atsu, (2022). *Dennettia tripetala* gave lower values followed by *Cola acuminata* and vitamin c. This shows that the extracts are more potent in reversing the liver damage when compared with Vitamin C.

DT and CA leaf extracts significantly (p<0.05) reversed kidney and liver damage arising from PHZ toxicity as effectively as vitamin C. Ezeigwe*et al*., (2020) reported similar results for kidney and liver function parameters with the aqueous extract of *Ficuscapensis* combined with *Cnidoscolusaconitifolius*. Also, Omage*et al*., (2021) reported similar findings in Carbon tetrachloride induced hepatorenal damage. Kale *et al*., (2019) demonstrated similarities with *Acridocarpussmeathmannii*root extract against PHZ induced renal and hepatic injury. El-Shafey, *et al*., (2023) reported similar findings with wheat germ oil.

.ii. Effect on oxidative stress in liver, kidney and spleen

Vitamin C is a water-soluble molecule which regenerates the radical form of alpha-tocopherol, thus playing a very vital role in regulating redox state. In addition, vitamin C influences intestinal iron absorption by interfering with the reduction of Fe^{3+} to Fe^{2+} (Imam, *et al*., 2017). Fe^{2+} is imported into duodenal enterocytes via DMT1, and it

exits the enterocytes through the basolateral membrane via Fpn1, which can be negatively regulated by hepcidin. Thereafter, transferrin transports the iron as Fe^{3+} to the bone marrow (for hematopoiesis), liver, and other organs (for storage). Besides its role in iron reduction prior to intestinal absorption, vitamin C regulates iron homeostasis by inhibiting expression of hepcidin (for example, in HepG2 cells), thereby attenuating iron deficiency (Imam, *et al*., 2017).

PHZ induced marked oxidative stress in the liver, kidney and spleen of rats. This was evident in the elevated levels of MDA, decreased activities of endogeneous antioxidant enzymes, catalase and GST, while GSH levels were significantly reduced. Kale *et al*., (2019): Baker *et al*., (2021) reported similar findings in the spleen of rats given PHZ. These results are in agreement with Bansode *et al*., (2019); Ashour, (2014); Kolawale*et al*., (2017); Ezeigwe*et al*., (2020) and El-Shafey, *et al*., (2023). Both DT and CA significantly ($p<0.05$) reduced the elevated MDA concentrations resulting from PHZ toxicity, in the same manner as vitamin C. In the same vein, catalase and GST activity, including GSH concentrations were significantly ($p<0.05$) increased. The results from this study are in consonance with those reported by Kolawale*et al*., (2017); Ezeigwe*et al*., (2020); Omage*et al*., (2021). The antioxidant effect exhibited by DT and CA may be attributed to the copious amount of flavonoids, terpenoids, and other organic compounds known to possess antioxidant properties. They are able to quench free radicals generated by PHZ metabolism, thereby preventing oxidative stress associated with PHZ toxicity.Plant phytochemicals have also been implicated in induction of endogenous antioxidant enzymes, thus enhancing the cells ability to resist oxidative stress.

iii. Effect on hematological parameters

White blood cells are a major game player in inflammatory responses (Ichipi-Ifukor*et al.,* 2013; Mordi *et al.,* 2021a). Neutrophils concentration have been reported to rise in response to recurring infections. On the other hand, reduced lymphocyte, monocyte and eosinophils have been observed to be involved in increased risk of developing infection (Cekici, *et al*., 2019; Koc and UnalliOzmen, 2022; Terradas*et al*., 2012). Also low basophil count has been reported in several clinical conditions in response to the development of anaphylactic reactions, a condition characterized by drop in blood pressure and sudden blockage of airways. (Korosec *et al*., 2018; Miyake and Karasuyama, 2017; Miyake *et al.,* 2021; Siracusa *et al*., 2013).

Phenylhydrazine resulted in significant ($p<0.05$) decrease in RBC, HBG, HCT and LYM while MCV was significantly ($p<0.05$) increased. Also, there was reduction in the values of platelet, WBC, MCH, MCHC, MPV, PCT and P-LCR , which were not significant. *Dennettia tripetala* and *cola acuminata* methanol leaf extract, including Vitamin C, significantly ($p<0.05$) increased these values when compared with group 2. Results from this study are fairly consistent with previous reports of reductions in HBG levels, RBC counts, PCV and an impairment in erythrocyte deformability following PHZ administration in rats (Berger, (2007); Ashour, (2015); Kolawale*et al.,* (2017); Kale *et al*., (2019); Ousaaid*et al*., (2022)).

Platelet indices are useful indicators of inflammatory diseases, blood clotting and in recovery of injuries (Pogorzelska *et al*., 2020; Verma *et al*., 2018) while plateletcrit count is involved in the screening and detection of quantitative platelet abnormalities related to hemostasis and thrombosis.

Administration of CA and DT reversed the effect of PHZ on RBC, HBG, HCT and PCV parameters of the blood. *Dennettia tripetala* and *cola acuminata* methanol leaf extract, including Vitamin C, significantly ($p<0.05$) increased these values to control

levels. Kolawale*et al.,*(2017) reported similar finding with *Citruluslanatus*rind whileOusaaid*et al*., (2022) also reported similar results with Apple-vinegar. Researches by Ashour, (2014); Berger, (2007); Kolawale*et al.,* (2017); Kale *et al*., (2019) also support the present study. The improvement in blood parameters may be attributed to the presence of alkaloids in plants. Alkaloids have been peoven to inhibit cyclic adenosine monophosphate (cAMP) phosphodiesterase, causing an accumulation of cAMP levels (Ndem*et.al*., 2013). This stimulates protein phosphorylation and synthesis with a possible enhancement of erythropoiesis (Ndem*et. al*., 2013). Flavonoids are also known to modulate hematological indices.

iv. Effect on histology of the kidney, liver and spleen

Administration of phenylhydrazine induced remarkable damage to the spleen, including: Atrophy of lymphoid follicles (white pulp), decreased sequestration of red blood cells (red pulp), vascular stenosis and hypertrophy. Treatment with Vitamin C and Phenylhydrazine induced moderate follicular activation, moderate increase in red cell sequestration and activation of sinus histocytes. Coadministration of *Dennettia tripetala* and phenylhydrazine induced marked follicular activation and marked increased red blood cell sequestration. Coadministration of *Cola acuminata* with phenylhydrazine induced mild follicular activation, mild increase in red blood cell sequestration and activation of sinus histiocytes. *Dennettia tripetala* appeared to be the most potent, while *Cola acuminata* was the least. This study is consistent with research by Kale *et al*., (2019).

Phenylhydrazine induced severe hepatocyte necrosis, biliary ductal and vascular wall degeneration in the liver of albino rats. Treatment with *Dennettia tripetala*, *Cola acuminata* and Vitamin C, all grouped separately, restored the normal liver architecture. However, there was the additional beneficial effects of mobilization of

plasma cells in the portal region of the liver, with the resultant boost in the local immune system of the liver as well as increase in the portal blood flow in the liver in the groups given *Dennettia tripetala* and Vitamin C. Similar findings have been reported by Kale*et al*., (2019) and Allahmoradi*et al*., (2019).

In the kidneys, phenylhydrazine induced severe tubular necrosis and moderate glomerular degeneration. Administration of *Dennettia tripetala*, *Colaacuminata* and Vitamin C, in separate groups restored normal nephron architecture However, the groups given *Dennettia tripetala* and Vitamin C had the additional beneficial vasoactive and hemodynamic activities of vasodilatation and increased interstitial blood flow. Thus *D. tripetala* activity was comparable to that of Vitamin C and appeared to be more potent than *Cola*. *acuminata.* Kale *et al*., (2019) gives supporting evidence to this study.

Histopathology examinations of the liver, kidney and spleen, prove the efficacy of DT and CA in reversing the oxidative stress injuries in these organs. Although in the three organs, DT and CA exhibited varying effects, their antioxidant effect was similar to that of Vitamin C.

v. Effect on bone marrow iron sequestration

Iron is an essential trace element required for maintaining physiological homeostasis (Imam *et al*., 2017). The balance between oxidative stress and antioxidants in various physiological processes is influenced by iron metabolism. Both insufficient and excessive iron levels can disrupt the redox equilibrium, which can be corrected through iron supplementation for deficiency and iron chelation for overload (Imam *et al*., 2017). Iron functions as a crucial element within protein cofactors like iron-sulfur (Fe-S) clusters and heme in various tissues apart from the liver. These components participate in the electron transport chain, facilitating ATP production during oxidative

phosphorylation within the inner mitochondrial membrane. Hence, iron is vital for sustaining sufficient ATP reserves during this metabolic process (Oexle, *et al*., 1999). The primary role of iron lies in its incorporation into heme within red blood cells (RBCs) for oxygen transportation. In muscle tissue, iron is predominantly found as heme myoglobin, while in the liver, it is mainly stored as ferritin (Kohgo, *et al*., 2008). Iron isstored in the form of ferritin, mostly in the bone marrow, liver, and spleen (Restrepo-Gallego *et al*., 2021). In the bone marrow, iron is used for hemoglobin formationWithin the liver, iron acts as the primary reserve, while reticuloendothelial cells in the spleen are responsible for recycling iron from aging red blood cells. Additionally, a minor proportion of iron (around 400 mg) is found in cellular proteins like myoglobin and cytochromes, with even less (approximately 3–4 mg) circulating and bound to transferrin (Kohgo, *et al*., 2008).

Phenylhydrazine resulted in depletion of iron stores in the reticulocytes of the bone marrow. This is evidenced in the increased demand for iron in the liver and the spleen during PHZ assault. *Dennettia tripetala*, *Cola acuminata* and Vitamin C effectively restored the iron stores. It was observed that *Cola acuminata* restored iron stores better than *Dennettia tripetala* and Vitamin C. The two extracts contain substantial amount of dietary iron, which explains their effect in restoring iron stores in the bone. The extracts contain flavonoids, terpenoids, saponins, alkaloids, and various organic compounds. These components have the ability to neutralize PHZ radicals, which could otherwise trigger the oxidation of biomolecules and the iron in the Fenton reaction.

.vi. Effect on iron metabolism genes expression

The primary role of the spleen lies in orchestrating the intricate pathways of hemoglobin metabolism to ensure a steady supply of iron throughout the body. This

regulation involves the modulation of key genes associated with iron metabolism, including transferrin receptor (TFR1), haem oxygenase (HO1), Dcytb, Ireg1, and various isoforms of DMT1. During acute hemolysis, the spleen responds by upregulating the expression of these genes, particularly those involved in iron transport, regulation, and hemoglobin degradation. Notably, there is a heightened expression of DMT1 isoforms, suggesting a local demand for iron to support increased erythropoietic activity within the spleen itself.Heam Oxygenase 1 (HO1) gene is a noteworthy inducible enzyme responsible forhaem degradation (Maines, 1997) and is likely to also participate in facilitating the efflux of iron from cells (Ferris, *et al*., 1999).DMT1 has been reported to be expressed and regulated by iron status in other tissues besides the duodenum (Trinder, *et al*., 2000). As Dcytb and Ireg1 are also assumed to be involved in iron metabolism in other tissues, besides the duodenum.

Recent research indicates the liver's crucial role in maintaining iron balance within the body, prompting an investigation into hepatic gene expression after PHZ-induced hemolysis. The study observed significant changes in gene expression in the livers of PHZ-treated mice. Notably, transferrin receptor 1 (TFR1) expression increased, indicating heightened iron uptake by the liver.

In the liver, HO1 was downregulated in the phenylhydrazine group, while IREG was upregulated.DMT-IRE and TFR1 were unchanged.Latunde-Dada *et al*., (2004); Tchernitchko, *et al*., (2002) and Canonne-Hergaux, *et al*., (2000) reported similar results for IREG, but is at par with results for HO1, DMT-IRE and TFR1. The difference may be as a result of different species of animals as they reported findings with mice. *Dennettia tripetala* and *Cola acuminata* methanol leaf extract downregulated HO1 and IREG expression. There was no change in the expression of

TFR1 and DMT-IRE across all the extract treated groups. This study showed that there were increased HO1 mRNA levels in the spleen of PHZ-treated rats, suggesting that there is adaptation of the capacity for heme degradation in the spleen, when massive hemolysis is induced. Studies indicate that the transporter Ireg1 also mediates the major efflux pathway in peripheral tissues (Abboud, and Haile, (2000); Yang, *et al*., (2002)). Consequently, Ireg1 expression tended to increase in the liver of rats treated with PHZ.

In the spleen, HO1 was upregulated while DMT-IRE, IREG and TFR1 were unchanged.by phenylhydrazine. Latunde-Dada *et al*., (2004); Tchernitchko, *et al*., (2002) and Canonne-Hergaux, *et al*., (2000) reported similar results for HO1, but is at par with results for IREG, DMT-IRE and TFR1. They reported increased expression of HO1, DMT-IRE, IREG and TFR1 genes in mice given PHZ. The disparity in the results may be as a result of the different animals used for the study. *Dennettia tripetala* and *Cola acuminata* methanol leaf extract downregulated HO1 gene. There was no significant difference in the other genes IREG, DMT-IRE and TFR1 was down regulated by both extracts and Vitamin C. IREG was downregulated by *Dennettia tripetala,* but upregulated by *Cola acuminata.* TFR1 showed no change with both extract and vitamin c, compared with the normal control and negative control groups. The results from this study portrays that both DT and CA restored the normal status of the genes, thereby restoring splenic and liver iron metabolism which was distorted by phenylhydrazine toxicity.

Bayele*et al*. (2015) reported that the transcription factor nuclear factor erythroid 2–related factor 2(Nrf2) regulates hepcidin expression as part of an antioxidant regulatory network, whereas several flavonoids modulate iron homeostasis by blocking Kelch-like ECH-associated protein 1(Keap1)-mediated transcriptional

repression, providing additional evidence that Nrf2 modulates the activity of the Hepcidin antimicrobial peptide (HAMP) promoter when activated as part of an antioxidant response (Bayele, *et al*., 2015).

5.2. Contribution to Knowledge

The study has contributed to knowledge in the following ways:

1. *Cola acuminata*and*Dennettia tripetala*methanol leaf extract both improved health status in PHZ induced anemic conditions.
2. *Cola acuminata* methanol leaf extract improved hematological parameters in anemic conditions better than *Dennettia tripetala.*
3. *Cola acuminata* extract effectively enhanced iron stores depleted in hemolytic anemia conditions better than *Dennettia tripetala*
4. *Dennettia tripetala* and *Cola acuminata* downregulated mRNA expressions of HO-1 gene (in spleen) and IREG gene (in liver).
5. *Dennettia tripetala* showed better antioxidant capacity than *Cola acuminata.*
6. *Dennettia tripetala* restored kidney and splenic morphology and enzymatic function better than *Cola acuminata,* but in the liver, the reverse was observed.

5.3. Conclusion and Recommendation

This study has provided ample scientific evidence supporting the traditional use of *Cola acuminata* and *Dennettia tripetala* leaves in the management of anemia and other ailments induced by oxidative stress. It has proven that the leaves are non-toxic but are also good sources of nutrients for maintenance of health. Both plants have shown efficacy in effectively reversing the deleterious effects of phenylhydrazine on the blood, bone marrow iron stores, kidney, liver and spleen, by improving

endogenous antioxidant enzyme armory and altering expression of iron metabolism genes. These leaves are therefore potent sources of biologically active compounds which may be used for drug design against oxidative stress-induced diseases, hemolytic anemia resulting from assaults that mimic phenylhydrazine mechanism, iron deficiency anemia and other related health conditions.

I therefore recommend that further research may be done with these plants, extracting and purifying the active principles and applying them to hemolytic anemia and other related ailments.

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