ANTIANAEMIC POTENTIALS OF *THEOBROMA CACAO* L. STEM BARK IN PHENYLHDRAZINE TREATED FEMALE ALBINO WISTAR RATS

BY

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PHY/T/12/033

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SEPTEMBER, 2017.

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A PROJECT SUBMITTED TO THE DEPARTMENT OF PHSIOLOGY, FACULTY OF BASIC MEDICAL SCIENCES, MADONNA UNVERSITY, ELELE CAMPUS, RIVERS STATE, NIGERIA

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF BACHELOR OF SCIENCE (B.SC.HONS) DEGREE IN PHSIOLOGY

SEPTEMBER, 2017.

CERTIFICATION

This is to certify that UCHEGBU CHIBUEZE an undergraduate in the department of human physiology with the registration number PHY/T/12/033 has satisfactorily completed the research in partial fulfilment of the requirements for the award of a Bachelor of Science (B.Sc.) degree in physiology.

……. …….

UCHEGBU CHIBUEE DATE

STUDENT

……. …….

DATE

Mrs. B.O OLUWATAYO (Ph.D)

SUPERVISOR

…… ……

Dr. GODSPOWER ONYESO DATE

HEAD OF DEPARTMENT

……. …….

DATE

EXTERNAL EXAMINER

DEDICATION

This work is dedicated to the almighty God for his perfect protection and provision through the duration of my studies.

I also dedicate this work to my beloved parents Mr. and Mrs. Uchegbu and my dearest siblings.

ACKWOWLEDGEMENT

My immeasurable gratitude goes to God almighty for seeing me through and for the gift of wisdom, knowledge and understanding throughout the period of this research work.

My deep appreciation goes to my wonderful parents Mr. Uchegbu Donatus and Mrs. Uchegbu Clementina and to my siblings Ugo boss, Tasha, Chimeziri and Glory for always being there for me.

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ABSTRACT

Previous studies indicated that the stem bark of *Theobroma cacao L.* is enrinched with minerals like phosphorus, sodium, calcium, magnesium and vitamins. The present study investigated the effect of higher doses of aqueous extracts of *Theobroma cacao L.* stem bark on some haematological parameters (White Blood Cell, Red Blood Cell indices, hemoglogin concentration, hematocrit and platelet) of normal and Phenylhydrazine-induced anaemic albino wistar rats. Forty female albino wistar rats weighing 130-200g were randomly grouped into 8 groups of 5 rats each. Group 1 (normal control) were fed with standard feed and water only, group 2 (positive control) was induced with anaemia without treatment, groups 3, 4 and 5 were induced with anaemia and treated with 200mg/kg,500mg/kg and 800mg/kg b. wt., of *Theobromacacao L.* stem bark extract respectively. Groups 6, 7 and 8 were normal rats given 1000mg/kg,3000mg/kg and 5000mg/kg b. wt. of the extract respectively for 21 days. Phenylhydrazine was used to induce anaemia intraperitoneal at the dosage of 60mg/kg body weight for two days. The result obtained after the experiment showed a significant increase ($p<0.001$); ($p<0.05$) in the Red Blood Cell, and also in the White Blood Cell a significant increase ($p<0.001$) was observed, the Hemoglobin concentration and hematocrit equally showed a significant increase ($p<0.001$) both in anaemic and normal groups. The increases suggest that the aqueous extracts of the cocoa stem bark possesses haematinic properties which could be helpful substitutes in cases of blood shortage or other conditions which places high demand on the blood forming system of the body.

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CHAPTER ONE

1.0 INTRODUCTION

The Nigerian climate and its environment favours a wide variety of plant species many of which have varied medicinal and antimicrobial potential. (Adedeji *et al.,* 2006,Adesanya *et al.,* 2007).

In Africa many of these plants are abundant and untapped, while they are still been used in developing countries as medicinal plants for the treatment of sick individuals.Numerous plants have been tested for their therapeutic potential and among them is *Theobroma cacao L*. Major Studies indicated that the bioactive compound of these plants have potent anti-inflammatory, antimicrobial and other properties. (Aggarwal *et al., 2008,* Alkharfy *et al.,* 2011*,* Ahmed *et al.,* 2013). Recently, these bioactive compound has shown to counteract the development of drug induced anaemia as well as preventing oxidative stress and cell damage in circulating erythrocyte. (Harzallah *et al., 2012*).

Theobroma cocoa L. is one of the world's most magical and incredible trees. The botanical name is *Theobroma cacao L*. which roughly translated, means "food of the gods". It belongs to the family malvaceae, genus Theobroma L. and specie Theobroma cacao L. from where the botanical name is gotten.

Besides complications like cardiovascular disorders, diabetes mellitus, anaemia,and others. Anaemia is one of the raging causes of death worldwide. It is

a common syndrome observed in various pathological conditions such as genetic defects, infections, etc. (Assobayire *et al.,* 2001).

Anaemia may result in the reduction in hemoglobin, hematocrit or red cell number. In physiologic terms an anaemia is any disorder in which the patient suffers from tissue hypoxia due to decreased oxygen carrying capacity of the blood.

Hemolytic Anaemia is a type of anaemia which brings about hemolysis which is the premature destruction of erythrocytes. The severity of the anaemia depends on whether the onset of hemolysis is gradual or abrupt and on the extent of erythrocyte destruction.

Phenylhydrazine (PHZ) is an antipyretic drug that was first characterized by Herman Emil Fisher in 1875.

This drug is well known for its ability to produce hemolysis in rats and humans (Dornfest*et al.,* 1983; Dornfest*et al.,* 1992; Ogiso*et al.,* 1989).

PHZ has been reported to cause vascular thrombosis as a side effect in clinical use (Reinhardt et al., 1981). PHZ is known to induce acute thrombosis in rat lung (Sato *et al.,* 2012).

PHZ decreases hemoglobin level, red blood cell concentration, and packed cell volume and impairs erythrocyte deformability. The phenylhydrazine-induced hemolysis is due to oxidative stress (Berger, 2007).

Mechanisms of Action of phenylhydrazine is its reaction with oxygenated haemoglobin (oxidative stress) to form reactive product, phenylhydrazine metabolites react with plasma membrane to cause lipid peroxidation and protein

oxidation resulting in the destruction of RBCs and hemolytic anemia (Beaven*et al*.,1954).

1.1 AIMS AND OBJECTIVES

The aim of this work is to investigate the effect of an aqueous extract of *Theobroma cacao* stem bark on the haematologic profile of both (normal) and on drug-induced anaemic female wistar albino rats.

1.2 SPECIFIC OBJECTIVES

To determine some haematological profiles of female albino wistar rats (normal) and anaemic rats treated with *Theobroma cocoa* stem bark extract.

- Total red cell count

  Red blood cell indices (MCH, MCV, and MCHC).

  - ✓ Mean corpuscular hemoglobin (MCH)
  - ✓ Mean corpuscular hemoglobin concentration(MCHC)
  - ✓ Mean corpuscular volume(MCV)

- Hematocrit
- Hemoglobin concentration
- Total White blood cell and differential count
- Platelet count

1.3 JUSTIFICATION

Theobroma cocoa had been used in many occasions as herbal medicine for the treatment of many diseases, although this work is majorly on cocoa stem bark, to determine the effect of higher doses on normal rats and drug induced anaemiaobtained in the earlier studies.

CHAPTER TWO

2.0 LITERATURE REVIEW

SCIENTIFIC CLASSIFICATION OF *THEOBROMA CACAO*

Kingdom Plantae

Phylum/Division Angiosperms

Order Malvalea

Family Malvaceae

Genus Theobroma L.

Specific epithet cacao

Species*Theobroma cacao*

BOTANICAL NAME: *Theobroma cacao*L.


T. cacao tree.

2.0.1 DESCRIPTION OF THEOBROMA CACAO

T. cacao Leaves have an unlobed average size of, 10–40 cm long and 5–20 cm broad. The flowers are produced in clusters directly on the trunk and older branches, this is known as cauliflory. Neufingerl, N.; Zebregs, Y.E.; Schuring, E.A.; Trautwein, E.A.The flowers are small, 1–2 cm in diameter, with pink calyx. The floral formula is ✶ K5 C5 A (5°+5²) G (5). (Ronse *et al.,* 2010).The young fruits are green before gradually turning yellow, orange, red or purple as they mature. Maturation takes about five to six months. Depending on the species the fruits may be ribbed and thick-skinned or smooth and thin-skinned. They are a favorite food of monkeys, who love the sweet and sour fruit pulp in which the seeds are embedded.

While many of the world's flowers are pollinated by bees (Hymenoptera) or butterflies (Lepidoptera), cacao flowers are pollinated by tiny flies,

Forcipomyiamidges in the subfamily Forcipomyiinae. (Hernández 1965). The fruit, called a cacao pod, is ovoid, 15–30 cm long and 8–10 cm wide, ripening yellow to orange, and weighs about 500 g when ripe. The pod contains 20 to 60 seeds, usually called "beans", embedded in a white pulp. The seeds are the main ingredient of chocolate, while the pulp is used in some countries to prepare refreshing juice, smoothies, jelly, and nata. (Figueira, *et al*., 1993). Cacao is self-incompatible and thus cannot pollinate itself (Baker *et al.,* 1997)

2.0.2 HISTORY OF THEOBROMA CACAO

 CarolusLinnaeusCarolus Linnaeus

The name *Theobroma cacao* was first applied to the cocoa tree by Carolus Linnaeus—the father of modern-day taxonomic plant classification. The name was published in his classic work systemanaturae in the mid-1700s.

The Maya believed the kakaw (cacao) was discovered by the gods in a mountain that also contained other delectable foods to be used by them. According to Maya mythology, the Plumed Serpent gave cacao to the Maya after humans were created from maize by divine grandmother goddess Xmucane. (Coe, *et al.,* 1996).

2.1.0 GENERAL USES

At present Cacao's main use is as a source material for Cocoa powder and Chocolate. A by-product of this multi-billion dollar industry is the cocoa butter, expressed from the roasted seeds. Cacao butter is heavily used in the cosmetic and pharmaceutical industries. However, a recent study has shown that a number of other products could be derived from the Cacao tree without infringing on seed yields. (Figueira, *et al.,* 1993).

2.1.1 ENERGY

For every kilogram of dry beans, there can be 2 kg of pod meal; indicating a 1:2 seed: pod ratio. To convert production figures into pod waste figures, this suggests we multiply by two. Pod meal contains ca 12.6% moisture, 7.6% ash, 8.1% protein, 34.8% crude fiber, 3.3% fat, and 33.6% N-free extract. One hundred kg cacao pod meal has the same feeding value as 96–97 kg chopped corn (including husks).

Comparison between cocoa stem bark, leaves, husk and seed is the presence of flavonoids, saponins, tannins, alkaloids, glycosides and terpens, which provide health benefit through cell signaling pathways and antioxidant effects (Hamburger *et al*., 1991).

2.1.2 ANAEMIA

Anaemia is from Ancient Greek, meaning *lack of blood*; it is a decrease in number of red blood cells (RBCs) or less than the normal quantity of haemoglobin in the blood. However, it can include decreased oxygen-binding ability of each haemoglobin molecule due to deformity or lack in numerical development as in some other types of haemoglobin deficiency (Haltermane*t al*., 2001).

Anaemia is a disease characterized by the reduction in the concentration of Hemoglobin, circulating RBC and its indices (MCV, MCH and MCHC) and PCV per unit of the peripheral blood below the normal (Aguwa, 1996; Oma, 1991). Anaemia impairs normal development in children and it constitutes a major public health problem in the young children in the developing countries with wide social and economic implications (Montalemberk*et al*., 1996). Main function of RBC is the transportation of oxygen into the tissues of body. At such, any pathological or physiological condition affects the RBC alters its function and this may be detrimental to the body.

A significant correlation with diagnostic values has been demonstrated between RCB, Hemoglobin, PCV and the other RBC indices (MCV, MCH, MCHC) in both humans and rats (Archer *et al.,* 1982).

Anaemia is a common blood disorder affecting people of all ages and posing a great threat to global healthcare. There are several types of anaemia many of which are rare but in all cases there is a reduction of number of circulating hemoglobin (Holden *et al.,* 2007).

2.2 CLASSIFICATION OF ANAEMIA

Several kinds of anaemia are produced by a variety of underlying causes. It can be classified in a variety of ways, based on the morphology of RBCs, underlying etiologic mechanisms, and discernible clinical spectra, etc. The three main classes include excessive blood loss (acutely such as a hemorrhage or chronically through low-volume loss), excessive blood cell destruction (haemolysis) or deficient red blood cell production (ineffective hematopoiesis) (Halterman*et al*., 2001).

2.2.1 SIGNS AND SYMPTOMS OF ANAEMIA

The clinical manifestation and severity of anaemia vary considerably among individual patients. Moderate anaemia can typically cause signs and symptoms such as headache, palpitations, tachycardia and shortness of breath. Chronic anaemia can result in severe organ damage affecting the cardiovascular system, immune system, lungs, kidneys, and the central nervous system (Ludwig *et al.,* 2001). A common anaemia-related problem is fatigue, which impairs the patient's ability to perform normal daily activities. As fatigue is a multifactorial syndrome not only caused by anaemia, trying to correlate the relative contribution of anaemia to fatigue is complex. Even when anaemia is improved, the full symptoms of fatigue might not be relieved, because fatigue can be present independent of anaemia (Ahlberg, 2003). Patients with severe anaemia are often symptomatic at rest. When signs and symptoms of anaemia occur, the most commonly observed include pallor of the skin and mucous membranes, soft systolic murmurs, palpitations of the heart, dyspnea (shortness of breath), lethargy, and fatigability (Bunn, 2012).

2.2.2 HEMOLYTIC ANAEMIA

Hemolysis is the destruction or removal of red blood cells from the circulation before their normal life span of 120 days. While haemolysis can be a lifelong asymptomatic condition, it most often presents as anaemia when erythrocytosis cannot match the pace of red cell destruction. Haemolysis also can manifest as jaundice, cholelithiasis, or isolated reticulocytosis (Ucar, 2002).

2.2.3 MECHANISMS OF HEMOLYSIS

There are two mechanisms of hemolysis. Intravascular haemolysis is the destruction of red blood cells in the circulation with the release of cell contents into the plasma. Mechanical trauma from a damaged endothelium, complement fixation and activation on the cell surface, and infectious agents may cause direct membrane degradation and cell destruction .The more common extravascular haemolysis is the removal and destruction of red blood cells with membrane alterations by the macrophages of the spleen and liver. Circulating blood is filtered continuously through thin walled splenic cords into the splenic sinusoids (with fenestrated basement membranes), a sponge like labyrinth of macrophages with long dendritic processes. A normal 8-micron red blood cell can deform itself and pass through the 3-micron openings in the splenic cords. Red blood cells with structural alterations of the membrane surface (including antibodies) are unable to traverse this network and are phagocytosed and destroyed by macrophages (Ucar, 2002).

2.2.4 DIAGNOSIS OF ANAEMIA

Anaemia is the most common hematologic disorder. Anaemia is best defined in relation to H&H (haemoglobin (Hb) and haematocrit (HCT) levels below the normal reference range, because a patient's symptoms and physiologic consequences are the result of decreased oxygen-carrying capacity of the blood (Barros *et al.*, 2010). According to World Health Organization (WHO) criteria, anaemia is diagnosed in males when Hb is <130 g/L (13 g/dL) and HCT is <0.39 (39%); and in females, when Hb is <120 g/L (12 g/dL) and HCT is <0.36 (36%).Other terms that indicate the presence of anaemia are;

❖ Haemoglobin concentration (Hb) is an estimate of the oxygen-carrying capacity of the blood

❖ Haematocrit value or ratio (HCT) also called packed cell volume (PCV). A measure of the relative volume occupied by RBCs in capillary or venous whole blood samples (Barros *et al.,* 2010). The HCT reflects, therefore, the body's red cell mass divided by the total blood volume. The HCT is used to detect the presence or absence of anaemia and polycythemia. The HCT can be determined mechanically, by spinning the cells in a microcentrifuge, and electronically, using an automated cell counter. The spun microhaematocrit suffers from an inherent problem of plasma trapping and is often slightly higher (0.01–0.03 or 1–3%) than the automated haematocrit (Dacie*et al*., 1991). This phenomenon causes an erroneously high manual HCT in samples with deformed RBCs such as sickle cells. In normal samples, the microhaematocrit and the electronic HCT should agree within ±3% (HCT units). Although the spun microhaematocrit has limitations, it is the reference method for HCT measurements. The haematocrit on Cell-Dyn hematology analyzers is a calculated parameter, expressed as percent or L/L (SI units) derived from the following equation: HCT (L/L) = RBC (1012/L) _ MCV (fL)/1000 (Groner*et al*., 1995).

2.2.5 RED BLOOD CELLS

Red blood cells, 5 to 6 million/mm3 are biconcave diskswhich function in transporting oxygen and carbon dioxide to and from tissues. Their shape facilitates both volume and surface area. Their structure is that of a flexible membrane sack (Coin *et al.,* 1979). This feature allows erythrocytes, which have a

7µm diameter, to squeeze through capillaries as small as 3 µm wide (Branemark*et al.,* 1963). Erythrocytes contain tremendous amounts of haemoglobin, the protein that binds oxygen. In order to make room for more haemoglobin to carry more oxygen, erythrocytes lose their nucleus and other organelles as they develop in the bone marrow. Because they lack a nucleus and other cellular machinery, erythrocytes cannot repair themselves when damaged; consequently they have a limited life span of about 120 days (Williams *et al.,* 1980). The removal of old and dying erythrocytes is carried out by the spleen. Erythrocyte production must equal erythrocyte death or the cell population would decline.

2.2.6 RED BLOOD CELL COUNTS

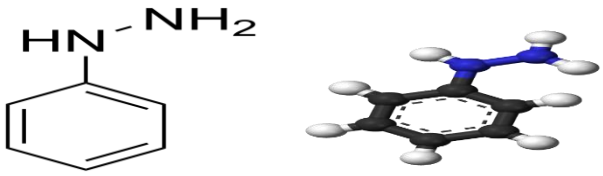
The typical red blood cell count is 4,600,000-6, 2000,000 cells per mm3 for males and 4,500,000-5,100,000 cells per mm3 for females. The number of red blood cells is a measure of the blood's oxygen-carrying capacity (Coin *et al*., 1979).

2.2.7 DESTRUCTION OF RED BLOOD CELLS

With age, red blood cells become increasingly fragile and are damaged by passing through narrow capillaries. Macrophages in the liver and spleen phagocytize damaged red blood cells. Haemoglobin from the decomposed red blood cells is converted into heme and globin. Heme is decomposed into iron and biliverdin. Iron is recycled into new haemoglobin or stored in the liver. Some biliverdin is converted into bilirubin. Biliverdin and bilirubin are excreted in bile as bile pigments (Coin *et al*., 1979).

2.2.8 PHENYLHYDRAZINE

Phenylhydrazine is the chemical compound with the formula $C_6H_5NHNH_2$. It is often abbreviated as $PhNHNH_2$. It has a molar mass of 108.14112 g and a mass Percentage of C 66.639 %; H 7.4564 %; N 25.904 %. Isomers include p-phenylenediamine, adiponitrile, o-phenylenediamine.



Phenylhydrazine molecule

2.2.9 CHEMICAL PROPERTIES

Phenylhydrazine forms monoclinic prisms that melt to an oil around room temperature which may turn yellow to dark red upon exposure to air. Phenylhydrazine is miscible with ethanol, diethyl ether, chloroform, and benzene. It is sparingly soluble in water.

2.3 HISTORY OF PHENYLHYDRAZINE

Phenylhydrazine was the first hydrazine derivative characterized, reported by Emil Fischer in 1875. (Fischer, *et al.* 1875). He prepared it by reduction of a phenyl diazonium salt using sulfite salts. Fischer used phenylhydrazine to characterize sugars via formation of hydrazones with the sugar aldehyde. He also demonstrated in this first paper many of the key properties recognized for hydrazines.

2.3.0 PHENYLHYDRAZINE IN MEDICINE

Phenylhydrazine (PHZ) is an antipyretic drug that was first characterized by Herman Emil Fisher in 1875.

This drug is well known for its ability to produce hemolysis in rats and humans (Dornfest*et al.,* 1983; Dornfest*et al.,* 1992; Ogiso*et al.,* 1989).

PHZ has been reported to cause vascular thrombosis as a side effect in clinical use (Reinhardt et al., 1981). PHZ is known to induce acute thrombosis in rat lung (Sato *et al.,* 2012).

For many years phenylhydrazine was used for experimental induction of anaemia in animals until Morawitz and Pratt suggested it as a drug for polycythemia vera (Falconer *1933*), a clonal disorder (Spivak 2002) which is known by a net increase in the total number of erythrocytes in the body.

Earlier in the last century, PHZ and phenylhydrazine hydrochloride were administered orally (usually around 100-200 mg/day) for the treatment of blood disorders (Giffin *et al., 1993*). In some cases, treatment was effective; in others, however, the outcome was fatal (Allen *et al., 1928,* Giffin *et al. 1929*). PHZ decreases haemoglobin level, red blood cell concentration, and packed cell volume, and impairs erythrocyte deformability. It induces reticulocytosis, increased osmotic resistance, free plasma haemoglobin, mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and erythropoietin levels, and extramedularhaematopoiesis in the spleen and liver (Hara *et al.,* 1975, Stern 1989). Phenylhydrazine compound can induce vascular dysfunction and haemodynamic disturbance, and also, a decrease in mean arterial pressure and hindlimb vascular resistance (Luangaram*et al.,* 2007).

2.3.1 USES OF PHENYLHYDRAZINE

Phenyl-hydrazine is used to prepare indoles via the Fischer Indole Synthesis, which are intermediates in the synthesis of various dyes and pharmaceuticals. This molecule is also used to induce acute hemolytic anemia in animal models. The phenylhydrazine-induced hemolysis is due to oxidative stress (Berger, 2007).

2.3.2 MECHANISM OF ACTION OF PHENYLHRDRAZINE

A number of hemolytic agents are known that induce nonindigenous redox processes in erythrocytes. These drugs, by reducing the life span of red blood cells below that deemed normal (about 120 days), can lower the circulating red blood cell population in susceptible individuals. Presumably, drug-mediated processes cause a large enough change in certain properties of a significant number of red blood cells, e.g., membrane deformability (Clark *et al*., 1983), that they are removed from circulation by the spleen and liver and then hemolyzed. Persons with genetic deficiencies in key enzymes involved in red cell metabolism (e.g., glucose-6-phosphate dehydrogenase, glutathione reductase, glutathione peroxidase) are particularly susceptible to induction of hemolytic anemia by these redox drugs. Such enzymes are important components of biochemical pathways that maintain hemoglobin in its functional reduced state and help avoid or repair the effects of redox processes that challenge the integrity of the erythrocyte. Phenylhydrazine (PHZ), a particularly potent redoxactive drug, can induce hemolytic anemia, even in individuals without erthrocytic enzyme deficiencies (Beutler, 1969). Indeed, the ability of PHZ to cause removal of erythrocytes from circulation. Observations in the last century indicated that PHZ could induce dramatic changes in erythrocytes in vitro, as well as in vivo. Rabbits treated with PHZ was brown in color and that addition of PHZ to suspended erythrocytes gave

them also a brown coloration; spectroscopic studies indicated that oxyhemoglobin (HbO2) had disappeared (Beaven*et al*., 1954).
Hemoglobin, whether free in solution or within erythrocytes, reacts with PHZ to yield"green hemoglobin", a form in which the heme group is modified (Beaven*et al*., 1954). Processes induced by PHZ also cause destabilization of the globin portion of hemoglobin, leading to denaturation and precipitation (Beaven*et al*., 1954).

CHAPTER THREE

3.0MATERIALS AND METHODS

3.1 MATERIALS

- Wooden cages
- Manual weighing balance
- Electrical weighing balance
- Hand gloves
- Oral cannula

- Cotton swab
- Injection water
- Flask and beaker (250, 500mls)
- EDTA bottles
- Marker
- Water
- Feed pellets (Top Feed Grower)
- Chloroform
- Distilled water
- Water bath (Buchi, model B-480)
- Heparinsed capillary tubes
- Syringes (2,3,5mls)

3.2.1 METHODS

Experimental animal:

Forty female albino wistar rats, were sourced from Mr. Arinze's animal farm from Agbor Delta state.

3.2.2 HOUSING

The rats were housed in wooden cages and acclimatized for two weeks in the animal house of the department of physiology, Faculty of Basic Medical Science Madonna University, Elele, Rivers state where they were fed with top feed and water. The cages were cleaned regularly to prevent infection of the

animals, care and treatment were conducted in conformity with the institutional guidelines which are in compliance with the guide for the care of laboratory animals, United States National Research Council, 1996.

3.2.3 PREPARATION OF THE EXTRACT

COLLECTION AND AUTHENTICATION OF PLANT MATERIAL

Fresh stem barks of *Theobromacacao*L.were collected from Adedeji farm, at Igbajo Boluduro L.G.A. of Osun State and were authenticated by a botanist, Dr. Ekeke Chimizie (Ph.D.) of the Department of Plant Science and Biotechnology Uniport, Rivers State.

3.2.4 PREPARATION OF PLANT MATERIAL FOR EXTRACTION

Fresh stem barks of *Theobroma cacao L.*were air dried under the sun for 10 days to remove sufficient moisture. The dried sample was cut into smaller pieces and further pulverized with the aid of manual mill. The powdered plant sample was then taken for extraction.

3.2.5 EXTRACTION METHOD

290g of the pulverized stem bark was macerated in 1650ml of distilled water for 48 hours and then filtered with cheeze cloth. The marc was rewashed until all the extractable constituents were completely washed out and then refiltered. Both filtrates were concentrated at 80c in a water bath. The weight of the extract was

gotten using an analytical weighing balance and the percentage yield was calculated.

3.2.6 EXPERIMENTAL DESIGN

The rats were divided into 8 groups of 5 rats each

Group 1: the normal control group fed with normal feed and water for the period of the experiment

Group 2: positive control was fed with normal rat feed, water and induced with 60mg/kg phenylhydrazine without treatment

Group 3: was fed with normal rat feed, water and induced with 60mg/kg phenylhydrazine, and treated with 200mg /kg body weight of the extract for 21 days

Group 4: was fed with normal rat feed, water and induced with 60mg/kg phenylhydrazine, treated with 500mg /kg body weight of the extract for 21 days

Group 5: was fed with normal rat feed, water and induced with 60mg/kg phenylhydrazine, treated with 800mg /kg body weight of the extract for 21 days

Group 6: was fed with normal rat feed, water and administered with 1000mg/body weight of the extract for 21 days

Group 7: was fed with normal rat feed, water and administered with 3000mg/body weight of the extract for 21 days

Group 8: was fed with normal rat feed, water and administered with 5000mg/body weight of the extract for 21 days. (Administration time for all the groups was in the early hours of the morning)

The rats were monitored daily for any physical or behavior changes throughout the period of the experiment.

Table 3.2.7 showing the administration of aqueous extract of *T. Cacao L.* stem bark and phenylhdraine.

GROUPS	RAT NUMBER	PHZ DOSE mg/kg	Extract Dose mg/kg	Water	Feed
GROUP 1 (NORMAL CONTROL)	5	NIL	NIL	YES	YES
GROUP 2(POSITIVE CONTROL)	5	60mg/kg	NIL	YES	YES

GROUP 3	5	60mg/kg	200mg/kg	YES	YES
GROUP 4	5	60mg/kg	500mg/kg	YES	YES
GROUP 5	5	60mg/kg	800mg/kg	YES	YES
GROUP 6	5	NIL	1000mg/kg	YES	YES
GROUP 7	5	NIL	3000mg/kg	YES	YES
GROUP 8	5	NIL	5000mg/kg	YES	YES

3.2.8 PREPARATION OF STOCK SOLUTION

Stock solution was prepared by dissolving 1g ofaqueous extract of*Theobroma L. cacao* stem bark in 10mls of distilled water to yield 100mg/ml of the extract.

3.2.9 CALCULATION OF DOSES

The Doses of the extract administered to the rats were calculated using the formula;

$$\text{Dose in ml} = \frac{\text{reference dose in mg/kg x weight of rats (kg)}}{\text{Concentration of stock solution (Dose in mg/ml)}}$$

3.3ANAEMIA INDUCTION

Induction was done by modified the method of Iwalewa *et al.* (2005). Rats were injected twice intraperitoneally for two days with 60 mg/kg of phenylhydrazine hydrochloride.

3.3.0 EXTRACT ADMINISTRATION

Extract was administered using oral cannula for each group

3.3.1ANALYTICAL METHODS

HEMATOLOGY

The hematological parameters were analyzed using a machine-automated hematology analyzer Kx-21, made by Sysmex, Japan. The blood parameters analyzed were:

- Red blood cell count
- Packed cell volume
- Hemogblobin concentration
- Mean cell hemoglobin
- Mean Cell concentration
- Mean cell hemoglobin concentration
- White blood cell total and differential counts
- Platelet count

3.3.2 STATISTICAL ANALYSIS

Results were expressed as mean ±SEM. All data obtained were analyzed using anova (analysis of variance) significance between the various groups was determined using Fisher's Least significant difference (LSD) post hoc test (SPSS 16 software).

CHAPTER FOUR

4.0: RESULT

White blood cell (WBC) of group 6(normal rats with 1000mg/kg of extract), 7 and 8(normal rats with 3000mg/kg and 5000mg/kg respectively) were significantly increased at ($p<0.001$) when compared to normal control.

Neutrophil count of group 6 showed a significant decrease ($p<0.001$) when compared with the normal control. Neutrophil count of group 7 showed a significant increase ($p<0.05$) and that of group 8 showed a significant decrease ($p<0.05$) when compared with the normal control.

Monocyte of group 6 showed a significant increase ($p<0.05$) when compared with the normal control, a significant decrease ($p<0.01$); ($p<0.001$) was shown in groups 7 and 8 respectively when compared with the normal control.

RBC of group 6 when compared with normal control showed a significant increase ($p<0.05$), a significant increase ($p<0.001$) was also shown in groups 7 and 8 when compared with the normal control.

Haemoglobin concentration HGB of group 6 showed a significant increase ($p<0.001$) when compared with the normal control. There was also a significant increase ($p<0.001$) shown in group 7 compared with the normal control. HGB of group 8 showed no significant difference when compared with the normal control.

Hematocrit of groups 6 and 7 showed a significant increase ($p<0.001$) when compared with the normal control, while no significant difference was seen in group 8 when compared with the normal control.

Platelet count of group 6 and 7 when compared with the normal control showed a significant decrease ($p<0.001$), while group 8 platelet showed a significant increase ($p<0.001$) when compared with normal control.

White blood cell counts of GROUPs 3(anaemic rats treated with 200mg/kg), 4(anaemic rats treated with 500mg/kg) and 5(anaemic rats treated with 800mg/kg) showed a significant increase at ($P<0.001$) when compared with the positive control.

Neutrophil counts of group 3, 4 and 5 when compared with the positive control showed a significant decrease ($p<0.001$).

Monocytes of group 3, 4 and 5 when compared with the positive control showed a significant decrease ($p<0.001$).

Red Blood Cell counts of group 3 when compared with the positive control showed a significant increase ($p<0.01$), whereas no significant difference was shown in the RBC of group 4 and 5 when compared with the positive control.

Hemoglobin concentrations of group 3 and 5 when compared with the positive control showed a significant increase ($p<0.001$), while that of group 4 also showed a significant increase at ($p<0.05$) when compared with the positive control.

Hematocrit counts of groups 3, 4 and 5 showed a significant increase at ($p<0.001$) when compared with the positive control.

Platelet counts of group 3, 4 and 5 when compared with the positive control showed a significant decrease at ($p<0.001$).

Table 4. 1: Showing the summary of the mean±SEM of aqueous extract of *Theobroma cacao* stem bark on some of the haematological parameters.

PARAMETERS	GRP1	GRP2	GRP3	GRP4	GRP5	GRP6	GRP7	GRP8
WBC	4.05±0.06	2.62±0.16	5.67±0.01	4.32±0.01	5.17±0.00	5.29±0.11	5.75±0.01	7.83±0.06
NEU(10^3/UL)	21.40±0.40	17.40±0.30	9.46±0.65	9.94±0.17	14.06±0.26	16.62±0.22	22.64±0.02	20.30±0.30
MON(10^3/UL)	0.40±0.03	4.50±0.06	.26±0.02	.44±0.07	.32±0.04	.92±0.07	.60±0.00	.54±0.02
EOS(10^3/UL)	5.94±0.59	0.89±0.11	0.78±0.73	1.46±0.40	0.48±0.49	1.92±0.37	0.70±0.00	6.42±0.20
BAS(10^3/UL)	0.24±0.04	0.18±0.04	0.14±0.02	0.10±0.00	0.28±0.07	0.24±0.02	0.28±0.02	0.26±0.02
RBC(10^6/UL)	4.53±0.02	4.76±0.02	5.03±0.03	4.94±0.04	4.93±0.03	4.73±0.13	5.09±0.10	4.97±0.08
HGB G/ DL	9.14±0.04	9.68±0.22	10.86±0.10	10.12±0.09	10.50±0.05	9.92±0.09	10.02±0.09	9.26±0.22
HCT(%)(PG)	27.98±0.06	29.96±0.15	33.72±0.54	33.92±0.28	34.70±0.21	30.06±0.49	31.01±0.25	28.70±0.13
MCV(FL)	58.30±0.89	61.60±1.84	67.22±1.11	68.64±0.02	71.40±0.74	65.74±0.15	60.80±1.0	57.92±0.75
MCH(PG)	19.06±0.10	20.52±0.81	21.54±0.07	21.48±0.204	21.34±0.44	20.08±0.13	19.60±0.19	19.08±0.22
MCHC(G/DL)	32.67±0.29	33.56±0.35	31.96±0.60	31.14±0.28	29.92±0.57	31.64±0.26	32.30±0.05	31.88±0.17
PLT(10^3/UL)	164.80±1.28	154.40±4.63	135.40±1.39	107.00±0.45	135.40±0.25	128.00±0.45	125.00±0.55	262.60±0.68

PDW	7.52±0.14	7.36±0.28	6.96±0.28	7.14±0.17	7.48±0.05	7.16±0.14	7.44±0.18	7.66±0.19

4.2: CHARTS

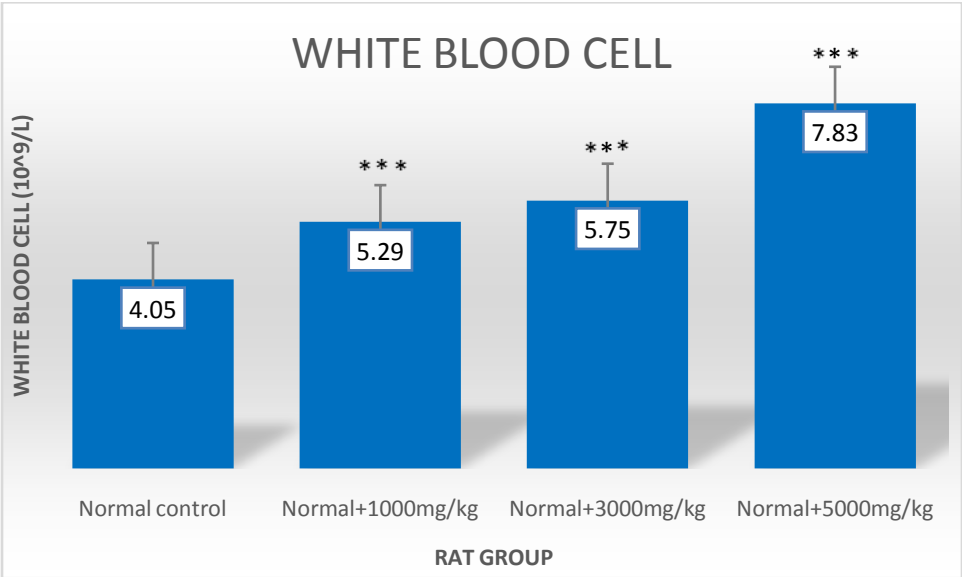


Figure 4. 1: Bar chart showing the effect of aqueous extract of *Theobroma cacao* stem bark on white blood cell count of normal female albino wistar rats.

***p<0.001vs Normal control

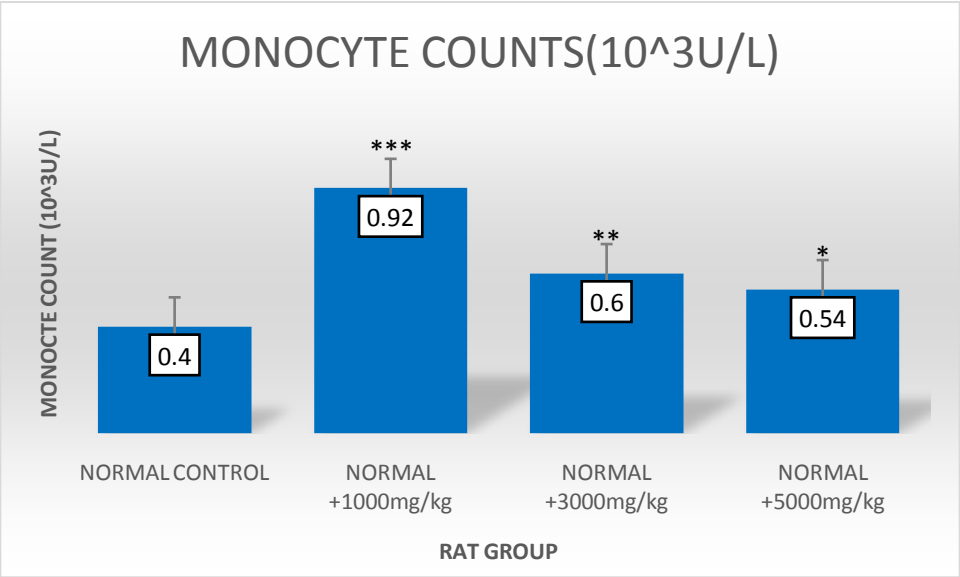


Figure 4. 1.2:Bar chart showing the effect of *Theobroma cacao* stem bark extract on the monocyte count of female normal albino wistar rats.

***p<0.001; **P<0.01;*P<0.05 vs Normal control

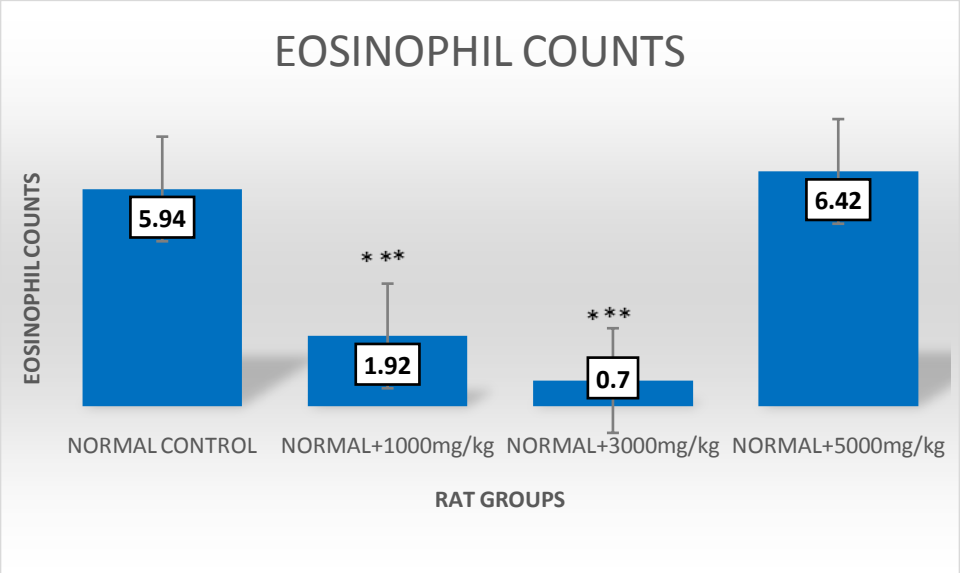


Figure 4. 1.3:Bar chart showing the effect of *Theobroma cacao* stem bark extract on the eosinophil counts of female normal albino wistar rats.

*P<0.001 vs Normal control

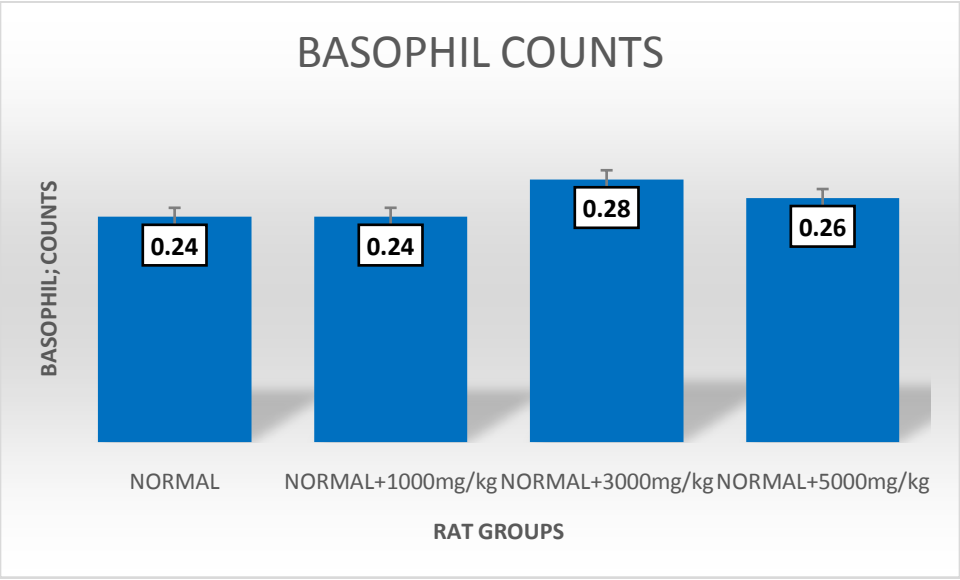


Figure 4. 1.4:Bar chart showing the effect of *Theobroma cacao* stem bark extract on the basophil counts of female normal albino wistar rats.

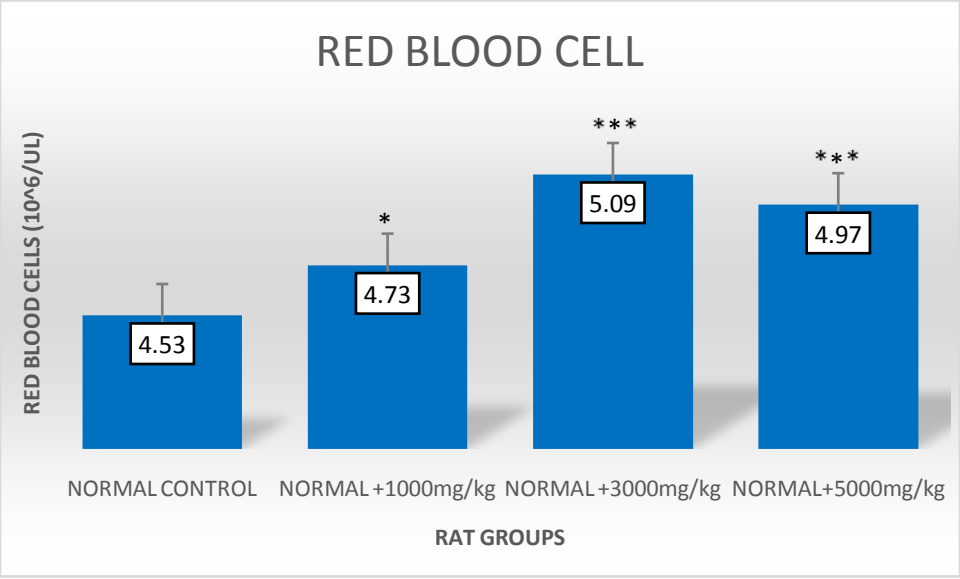


Figure 4. 1.5:Bar chart showing the effect of *Theobroma cacao* stem bark extract on the red blood cell of female normal albino wistar rats.

*** $p<0.001$;* $p<0.05$

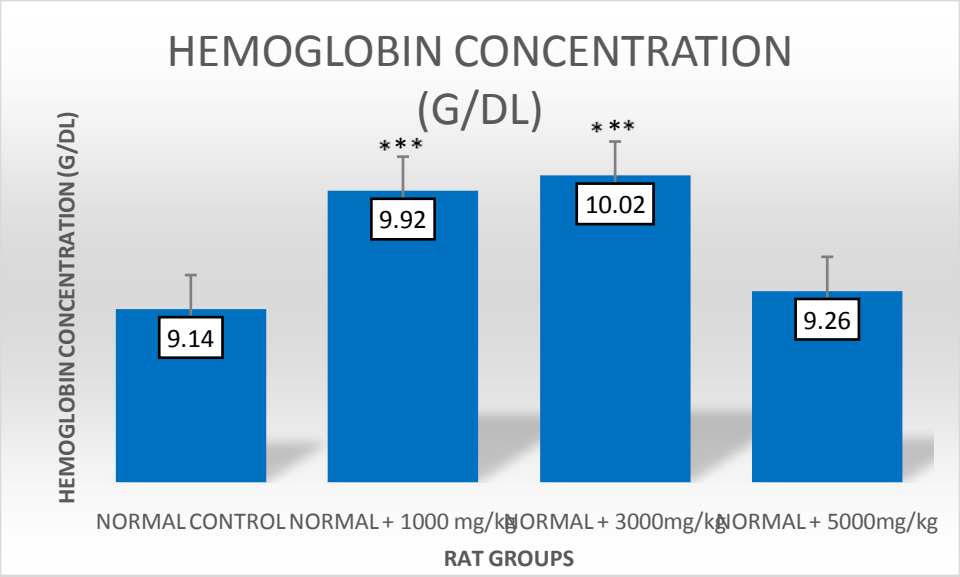


Figure 4. 1.6:Bar chart showing the effect of *Theobroma cacao* stem bark extract on the hemoglobin concentration of female normal albino wistar rats.

*** p<0.001 vs Normal control

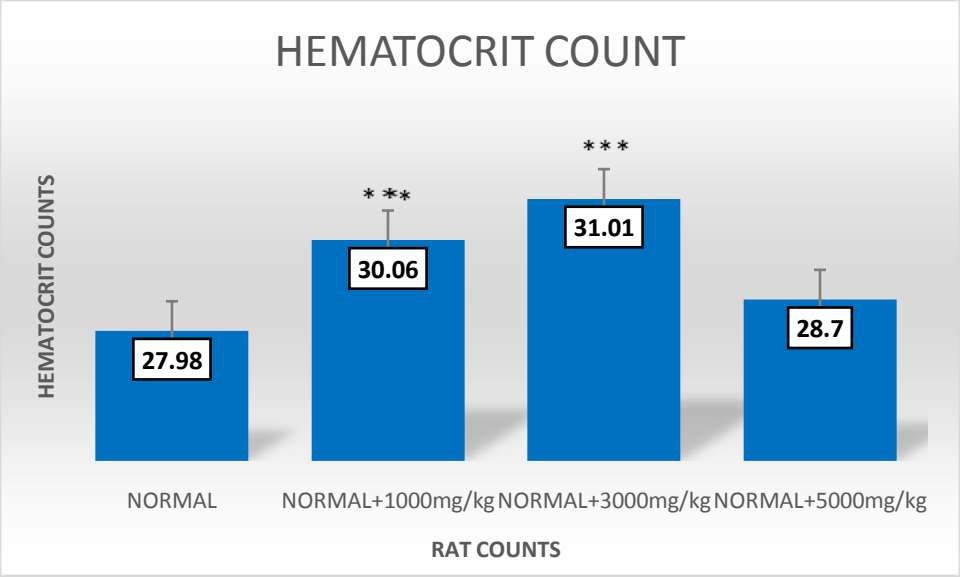


Figure 4. 1.7:Bar chart showing the effect of *Theobroma cacao* stem bark extract on the hematocrit count of female normal albino wistar rats.

***p<0.001 vs Normal control

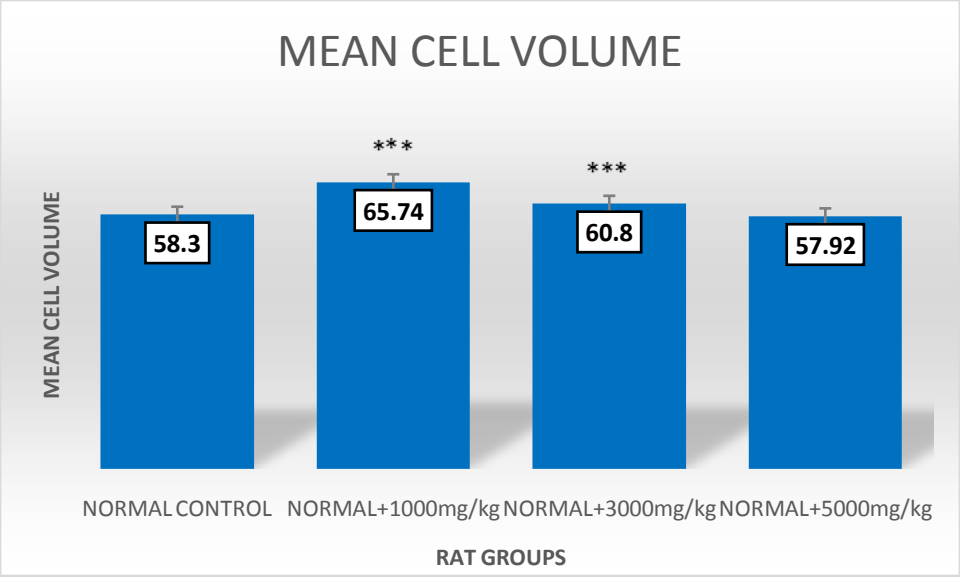


Figure 4. 1.8:Bar chart showing the effect of *Theobroma cacao* stem bark extract on the mean cell volume count of female normal albino wistar rats.

***p<0.001 vs Normal control

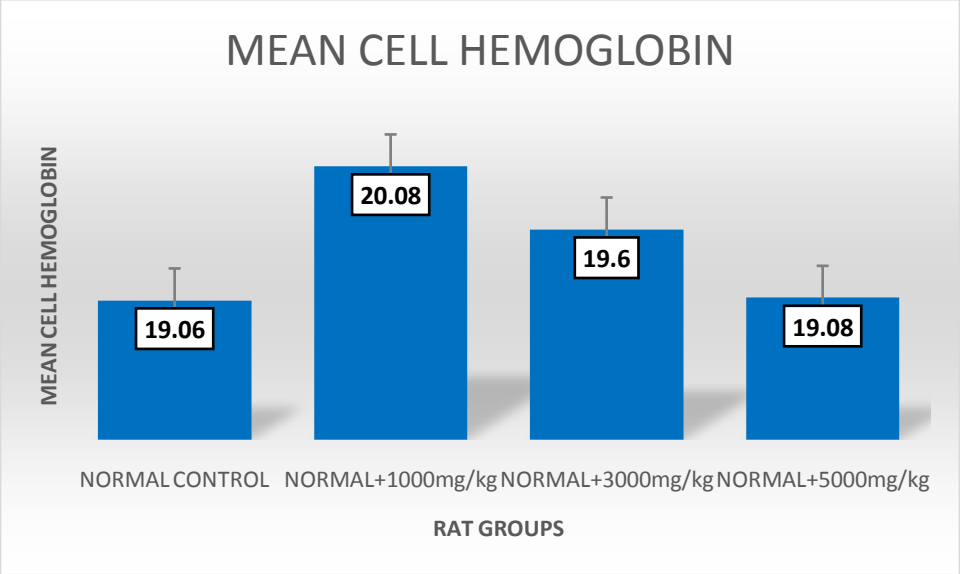


Figure 4. 1.9:Bar chart showing the effect of *Theobroma cacao* stem bark extract on the mean cell hemoglobin count of female normal albino wistar rats.

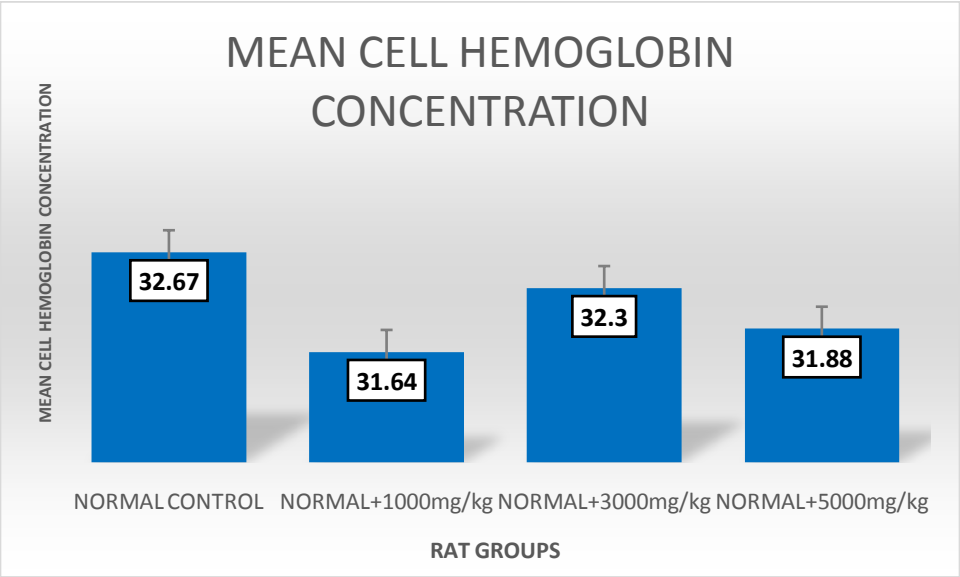


Figure 4. 2:Bar chart showing the effect of *Theobroma cacao* stem bark extract on the mean cell hemoglobin concentration count of female normal albino wistar rats.

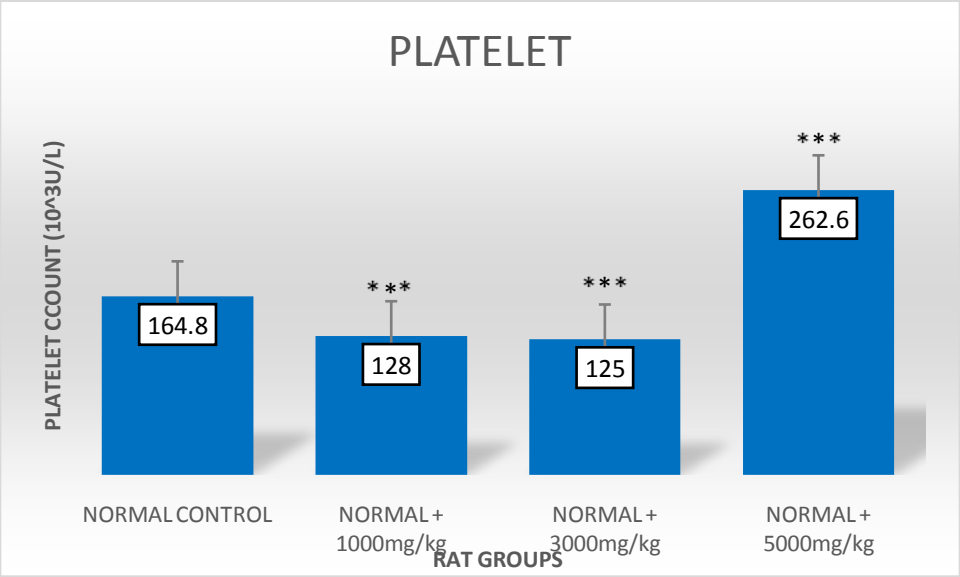


Figure 4. 2.1:Bar chart showing the effect of *Theobroma cacao* stem bark extract on the platelet count of female normal albino wistar rats.

***p<0.001 vs Normal control

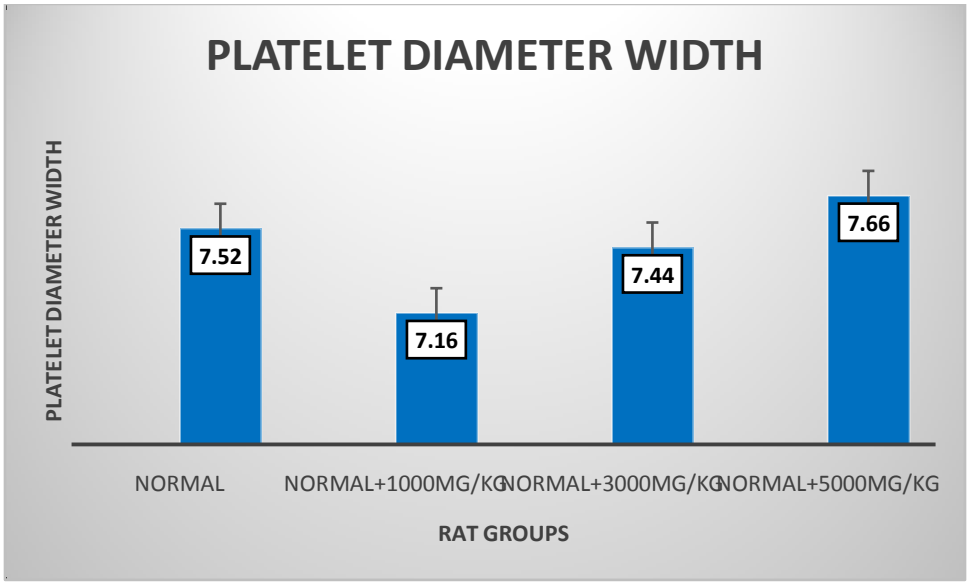


Figure 4. 2.2:Bar chart showing the effect of *Theobroma cacao* stem bark extract on the platelet diameter width of female normal albino wistar rats.

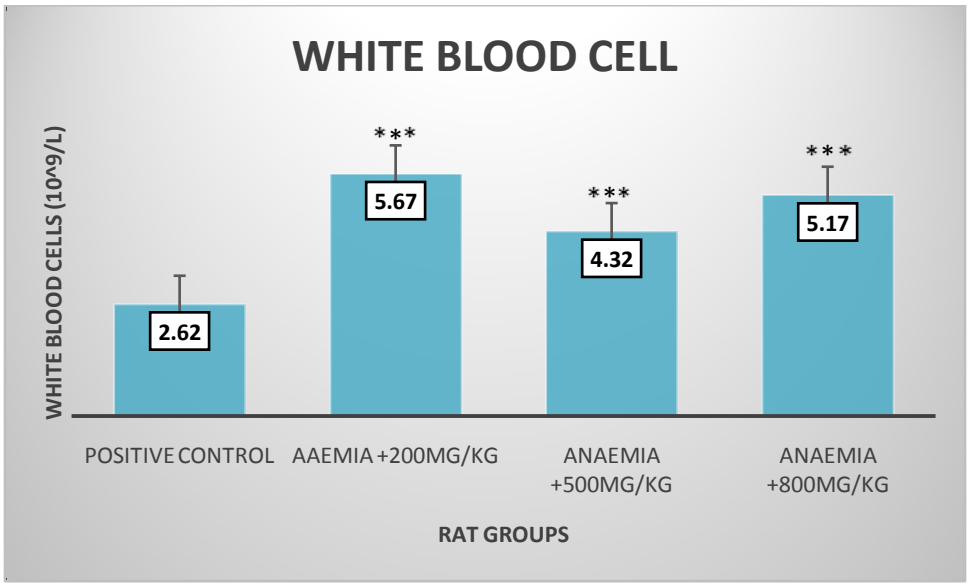


Figure 4. 2.3: Bar chart showing the effect of *Theobroma cacao* stem bark extract on the white blood cell count of female anaemic albino wistar rats.

***p<0.001 vs Positive control

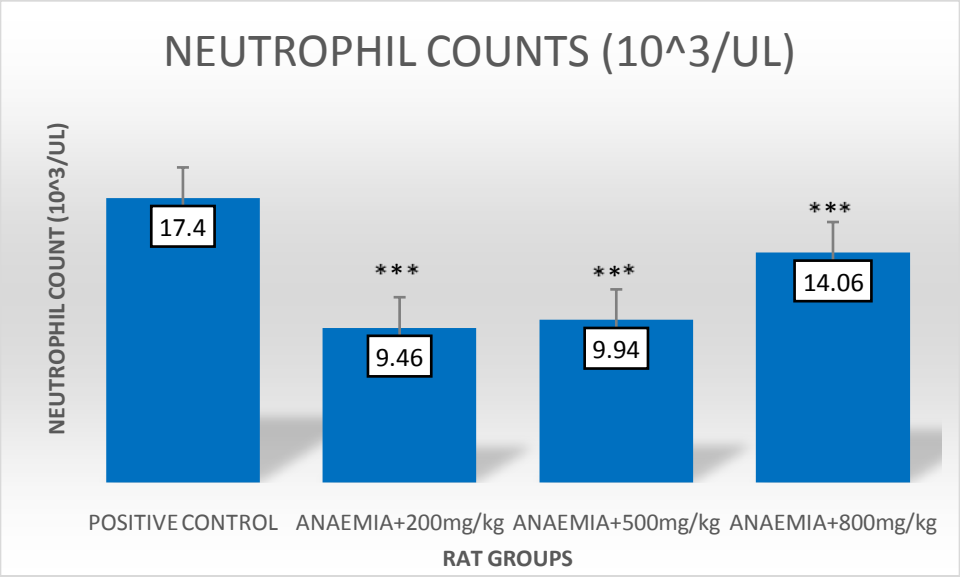


Figure 4. 2.4:Bar chart showing the effect of *Theobroma cacao* stem bark extract on the neutrophil count of female anaemic albino wistar rats.

***p<0.001 vs Positive control

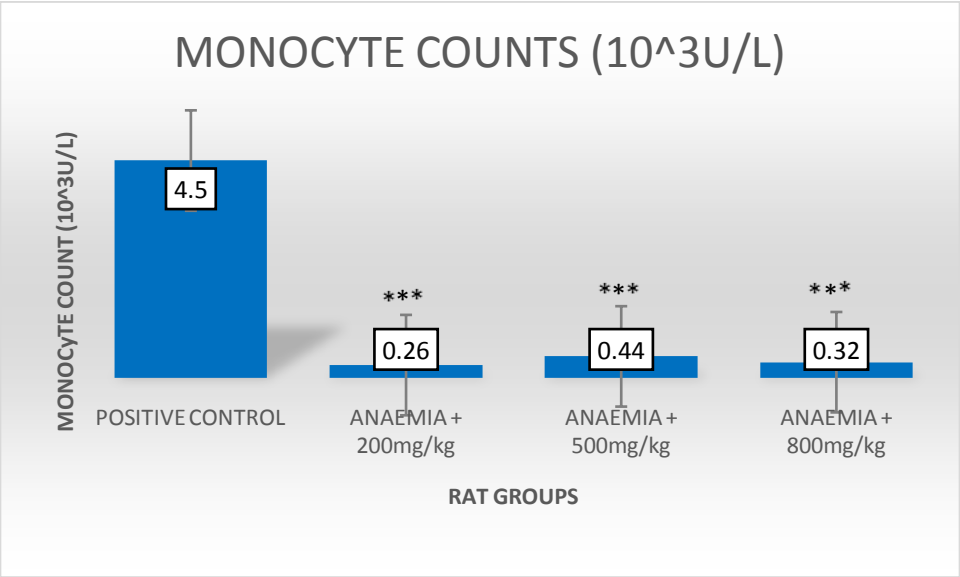


Figure 4. 2.5:Bar chart showing the effect of *Theobroma cacao* stem bark extract on the monocyte count of female anaemic albino wistar rats.

*** p<0.001 vs Positive control

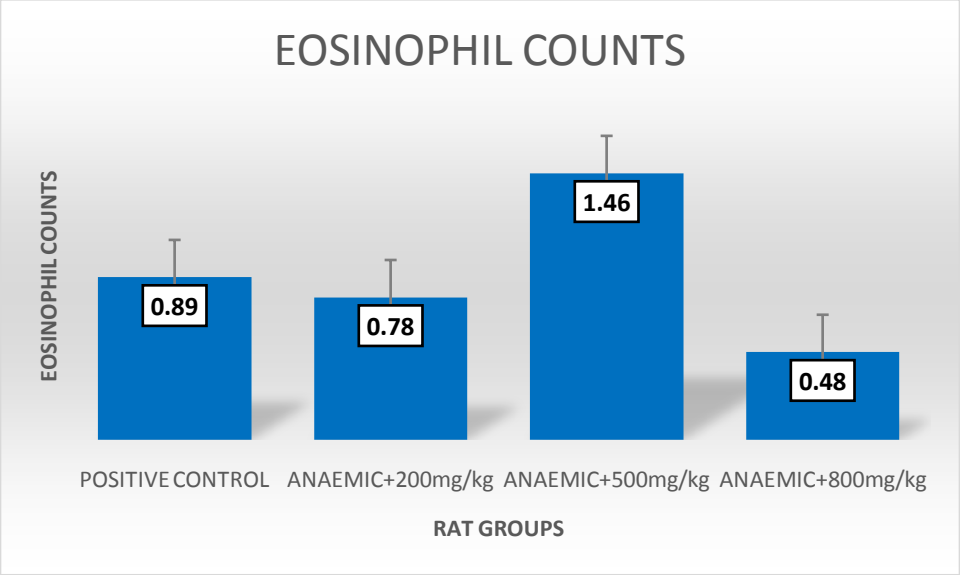


Figure 4. 2.6:Bar chart showing the effect of *Theobroma cacao* stem bark extract on the eosinophil counts of female anaemic albino wistar rats.

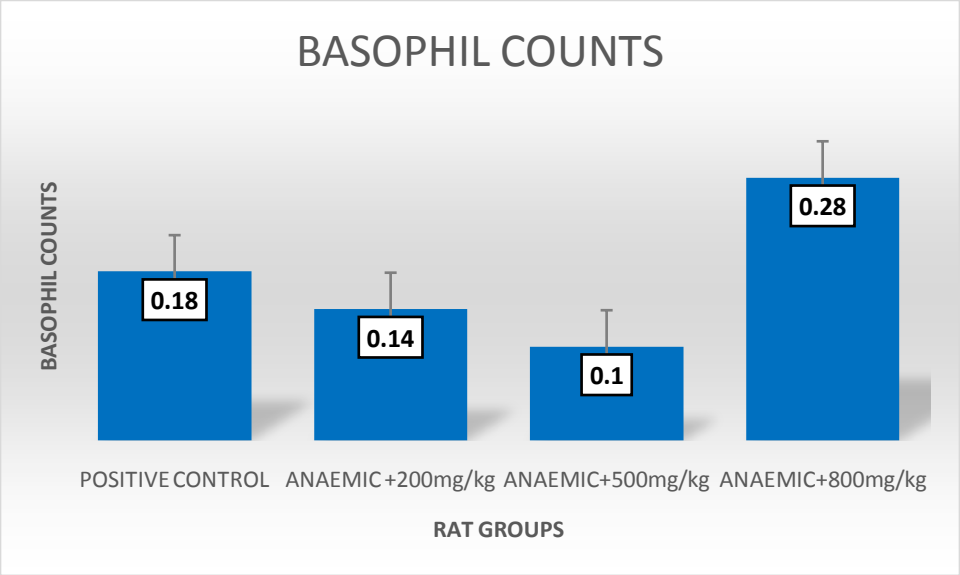


Figure 4. 2.7:Bar chart showing the effect of *Theobroma cacao* stem bark extract on the basophil counts of female anaemic albino wistar rats.

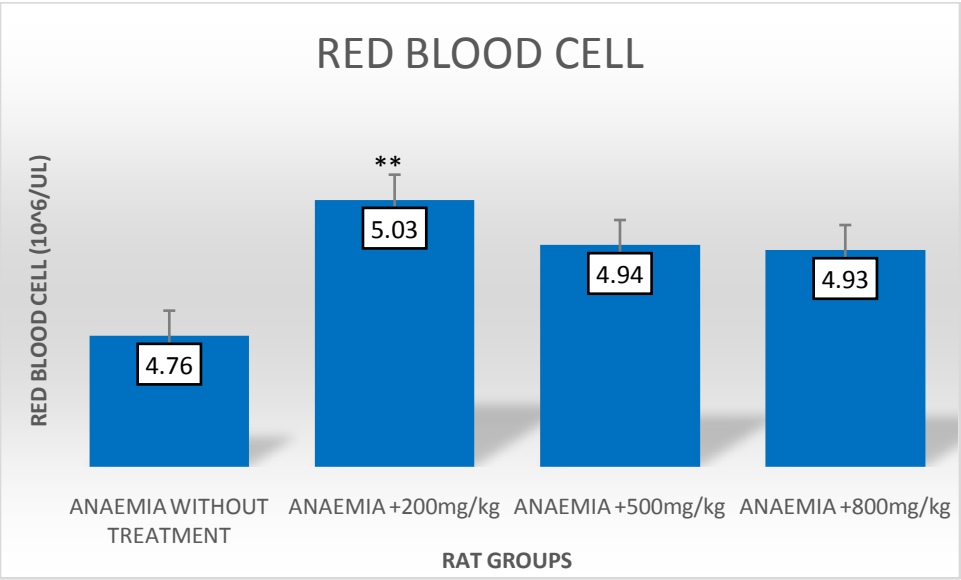


Figure 4. 2.8:Bar chart showing the effect of *Theobroma cacao* stem bark extract on the red blood cell of female anaemic albino wistar rats.

**p<0.01 vs Positive control

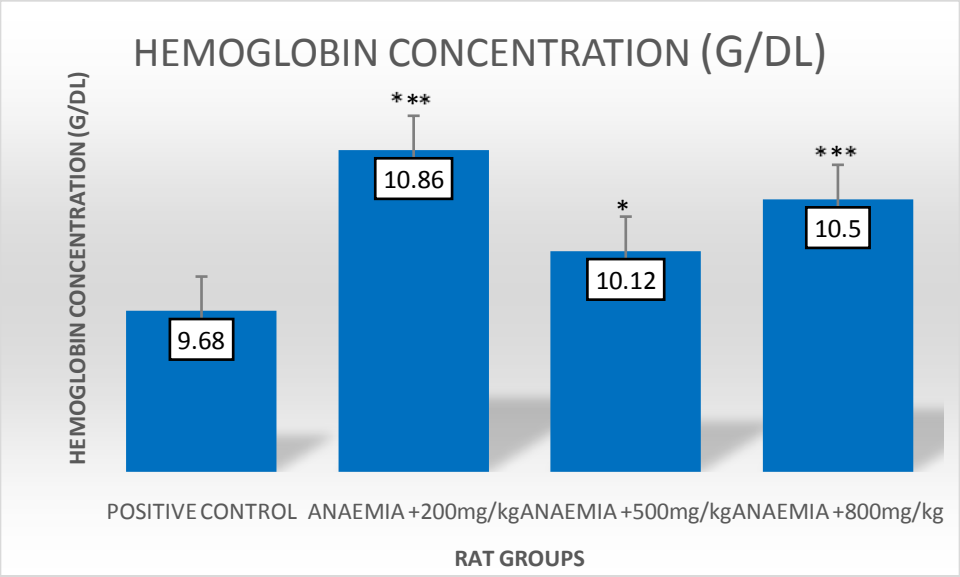


Figure 4. 2.9:Bar chart showing the effect of *Theobroma cacao* stem bark extract on the hemoglobin concentration of female anaemic albino wistar rats.

***p<0.001;*p<0.05 vs Positive control

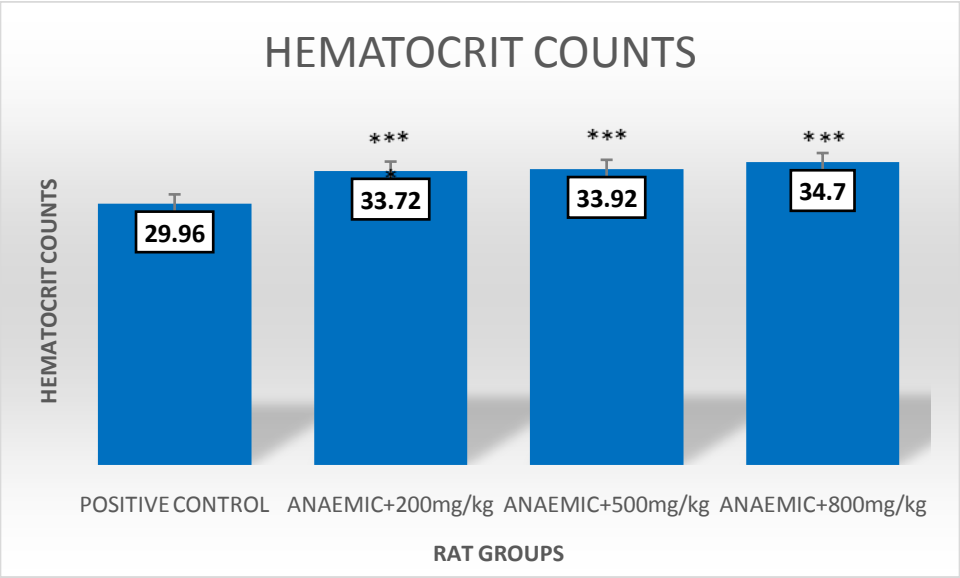


Figure 4. 3:Bar chart showing the effect of *Theobroma cacao* stem bark extract on the hematocrit counts of female anaemic albino wistar rats.

***p<0.001 vs Positive control

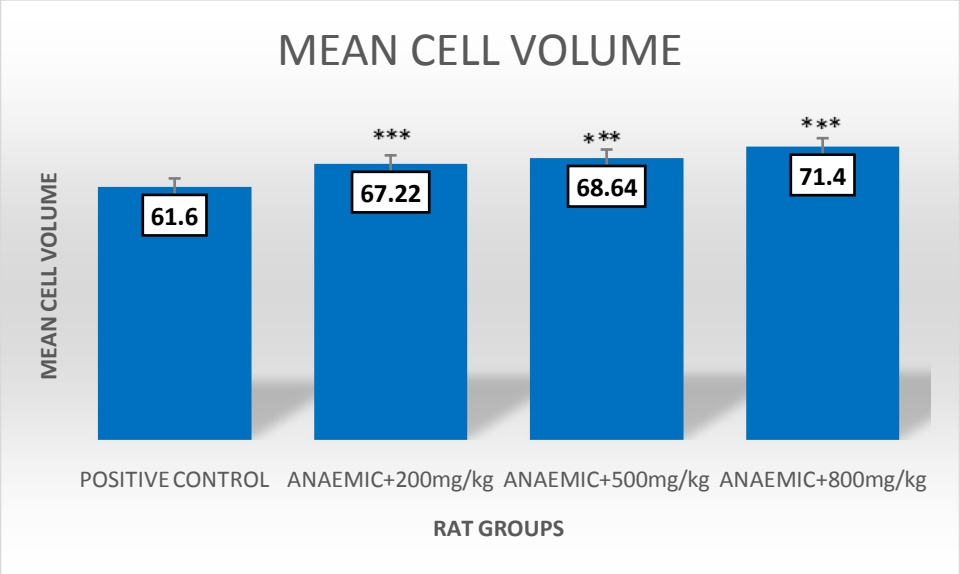


Figure 4. 3.1: Bar chart showing the effect of *Theobroma cacao* stem bark extract on the mean cell volume of female anaemic albino wistar rats.

***p<0.001 vs Positive control

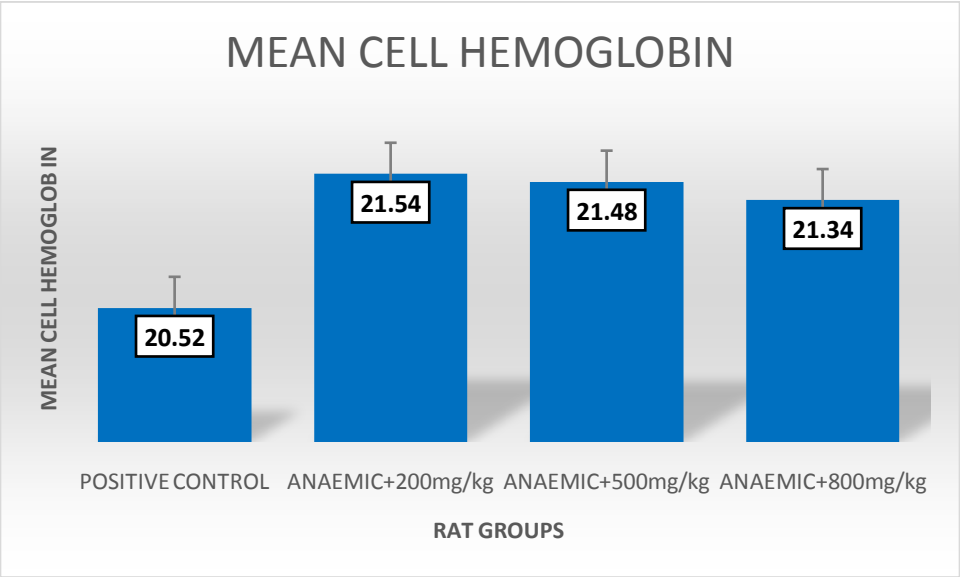


Figure 4. 3.2:Bar chart showing the effect of *Theobroma cacao* stem bark extract on the mean cell hemoglobin concentration count of female normal albino wistar rats.

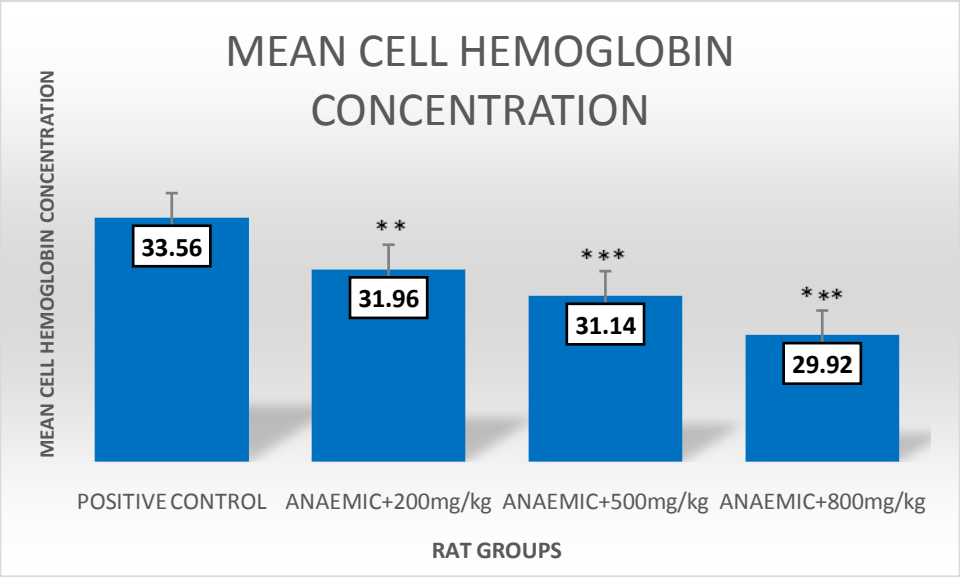


Figure 4. 3.3: Bar chart showing the effect of *Theobroma cacao* stem bark extract on the mean cell volume of female anaemic albino wistar rats.

p<0.01;*p<0.001 vs positive control

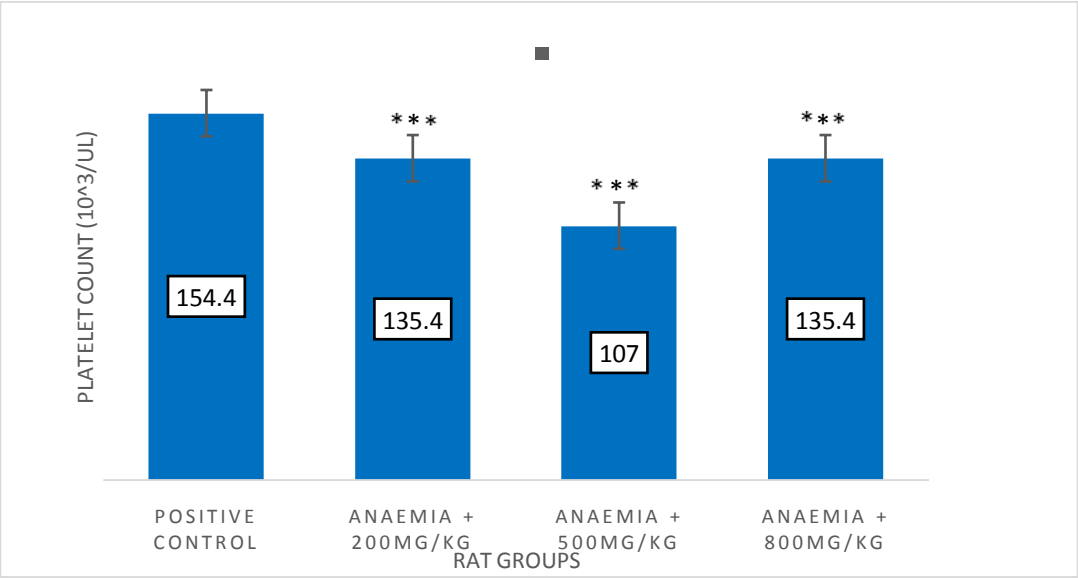


Figure 4. 3.4:Bar chart showing the effect of *Theobroma cacao* stem bark extract on the platelet count of female normal albino wistar rats.

***p<0.001 vs Positive control

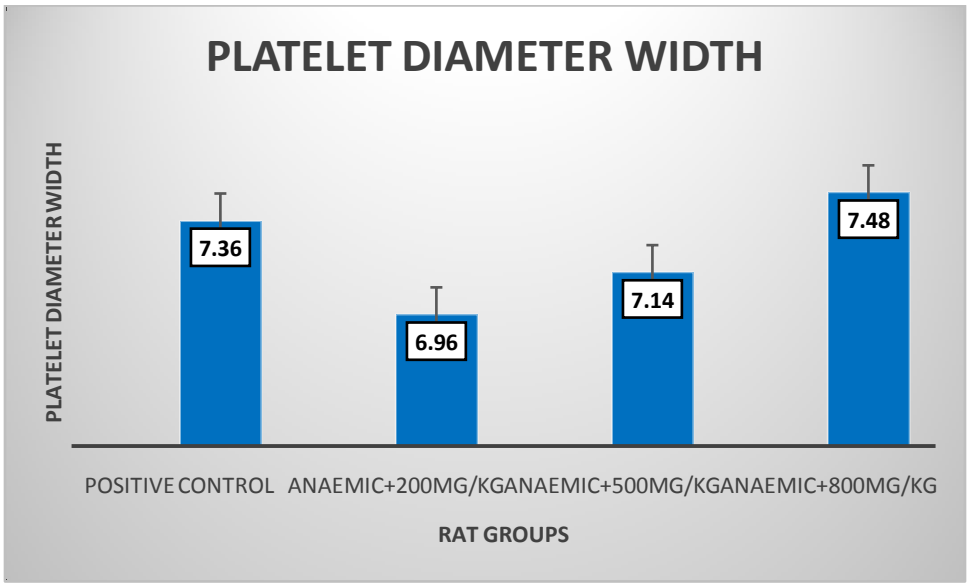


Figure 4. 3.5:Bar chart showing the effect of *Theobroma cacao* stem bark extract on the platelet diameter width of female normal albino wistar rats.

CHAPTER 5

5.0: DISCUSSION

Medicinal plants are of great importance to the health of individuals and communities and their medicinal values lie in some chemical substances that produce definite physiological actions on the human body (Edeoga*et al*., 2005). Comparison between cocoa stem bark, leaves, husk and seed is the presence of flavonoids, saponins, tannins, alkaloids, glycosides and terpens, which provide health benefit through cell signaling pathways and antioxidant effects (Hamburger *et al*., 1991).

The preliminary phytochemical analysis showed the presence of alkaloids, tannins, steroids, flavanoids, fats and oils, carbohydrates, terpenoids, glycosides, saponins, and proteins in the stem bark.

The most important of these phytochemicals are alkaloids, tannins, flavonoids and phenolic compounds (Hill, 1952). Most alkaloids have been showed to exert biological and pharmacological activities when ingested by animals affecting the nervous system, particularly the action of neurotransmitters, e.g. acetylcholine, adrenaline and dopamine (Schmeller*et al.,* 1998). The results indicate that the

Theobromacacao L. stem bark possesses some biologically active compounds which could serve as potential sources of drugs.

Previous studies showed that the aqueous extracts of *Theobroma cacao* stem barkcontain reasonable amounts of vitamins and other mineral constituents necessary for erythropoiesis such as iron. Deficiency of folic acid has been reported to cause macrocytic, megaloblastic and pernicious anaemia (Rang *et al*, 2007). These hematinic agents may have contributed to the significant increases at ($p<0.05$); ($p<0.001$) in the red blood cell counts of groups 6, 7 and 8 noticed when rats were fed aqueous extracts of *Theobroma cacao L.*stem bark(1000mg/kg body weight of extract),(3000mg/kg body weight of extract) and (5000mg/kg body weight of extract) respectively within the 21 days of treatment when compared with the normal control.

The aqueous extracts of the *Theobroma cacao L.* stem bark of these plants at these concentrations could possibly stimulate erythropoietin release into the kidney. Erythropoietin, which is the humoral regulator of RBC production increases the number of erythropoietin-sensitive committed stem cells in the bone marrow that are converted to red blood cell and subsequently to mature erythrocytes (Polenakovic*et al*., 1996: SanchezElsner*et al*., 2004; Ganong, 2005). Iron is an essential constituent of the haem-moiety of hemoglobin, thus deficiency of iron in humans and animals often leads to iron deficiency anaemia. The increase in the hemoglobin concentration could be attributed to the presence of these amino acids and high iron content. Iron is an important component of hemoglobin and functions in the transport of oxygen to cells and tissues. The increase may also be as a result of enhanced iron bioavailability as recorded by

(Hamlin *et al*., 2011). The extracts had a negative effect on the platelet counts of the rats. This could be as a result of the inhibition of the release of thrombopoietin, a regulator of thrombopoiesis or as a result of the inhibition of vitamin k, which is an important factor in the blood coagulation process. Reduction in platelet count could reduce the ability of the blood to clot and could lead to death from excessive bleeding. *Theobroma cacao* stem bark which is reported to reduce platelet count may be as a result bioactive constituent saponins (Tohti *et al.*, 2006), polyphenols in cocoa are also known to inhibit the activation of platelets (Murphy*et al.,* 2003).

The significant increase effect on the WBC may imply that there was change in the rat's body's ability to respond to infection. Positive control rats showed a significant decrease ($P<0.05$) in the values for Hb, RBC, MCV and MCHC counts when compared with groups 3, 4 and 5 treated with *T. cacao* stem bark extract by day 21.However, alkaloids and flavonoids protect cells as powerful antioxidants which prevent or repair damage done to red cells by free radicals or highly reactive oxygen species (Ogbe*et al*., 2010).

5.1:CONCLUSION

The results of this study indicate that the aqueous extracts of *Theobroma cacao L.* stem barkand at (200, 500, 800, 1000, 3000 and 5000mg/kg body weight respectively may possibly serve as an acceptable blood booster in an anaemic condition orused for prophylactic purposes without any significant toxic effects in rats as observed during the experiment.

Although the specific mechanism(s) through which the extracts enhance blood volume was not ascertained in this study, it is suggested that the extracts may have a direct effect on the body system that produces blood cells and contains constituent(s) that can interact and stimulate the formation and secretion of erythropoietin, hematopoietic growth factors/committed stem cells. This suggests that the aqueous extracts of the cocoa stem bark possesses haematinic properties. This haematopoietic effect may be due to the high content of different minerals in the stem bark of cocoa. Although the aqueous extracts could

be helpful substitutes in cases of blood shortage or other conditions which places high demand on the blood forming system of the mammalian body.

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