ANTIANAEMIC AND ANTIOXIDANT EFFECTS OF EXTRACTS OF *GNETUMAFRICANUM*LEAVES ON PHENYLHYDRAZINE-INDUCED ANAEMIC AND NORMAL ALBINO RATS

BY

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(M.Sc/Scien/6368/2011-2012)

A THESIS SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES, AHMADU BELLO UNIVERSITY, ZARIA

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD

OF A

MASTER OF SCIENCE DEGREE IN BIOCHEMISTRY

DEPARTMENT OF BIOCHEMISTRY

FACULTY OF SCIENCE

AHMADU BELLO UNIVERSITY,ZARIA

NIGERIA.

JULY, 2014

DECLARATION

The research work in this thesis entitled ANTIANAEMIC AND ANTIOXIDANT EFFECTS OF EXTRACTSOF*GNETUM AFRICANUM*LEAVES ON PHENYLHYDRAZINE-INDUCED ANAEMIC AND NORMAL ALBINO RATS has been performed by me in the Department of Biochemistry,Ahmadu Bello University, Zaria under the supervision of Mr. O. A.Owolabi and Prof. H.C Nzelibe

The information derived from the literature has been duly acknowledged in the text and the list of references provided. No part of this dissertation was previously presented for another degree or diploma at any university.

AndongmaBindaTembeng

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Name of student	Signature	Date

CERTIFICATION

This thesis entitled ANTIANAEMIC AND ANTIOXIDANT EFFECTS OF EXTRACTS OF *GNETUM AFRICANUM*LEAVES ON PHENYLHYDRAZINE-INDUCED ANAEMIC AND NORMALALBINO RATS by AndongmaBindaTembeng meets the regulations governing the award of Master of Science atAhmaduBello University, Zaria, and is approved for its contribution to knowledge and literature presentation.

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(Dean, Postgraduate school) Signature Date

DEDICATION

To God Almighty and to

My beloved Parents Mr. and Mrs. Simon Andongma

For their boundless love, understanding and care.

ACKNOWLEDGEMENT

My most sincere gratitude goes to God Almighty for keeping me alive throughout my study period in this institution and also for the gift of knowledge, wisdom and understanding leading to the completion of this research work. In high regards, my heartfelt gratitude goes to my supervisors, Mr. O.A. Owolabi and Prof. H.C Nzelibe for their enthusiasm, unlimited assistance, valuable contributions, constructive criticisms, guidance during the research and for creating sufficient time to read through my research work to completion.

I sincerely thank the Head, Departmentof Biochemistry, the Postgraduate Coordinator, Department of Biochemistry and the academic staff of Biochemistry Department for their invaluable contributions in my study. My thanks also go to the Technical and Administrative Staff of the Department for their assistance.

I express deep appreciation to my wonderful father, Mr. Simon Andongma, and my ever-loving and caring mother, Mrs. Grace Andongma. To my siblings; Awawing, Wanduku and EsackAndongma and my aunt Mrs. Alice Djamengo, I say your support and encouragements have been so marvelous. My appreciation also goes to all my family members who have affected me positively in many ways.

I acknowledge all the contributions and assistance of my friends and colleagues notably JacobHiambong, Mrs. FloreEdwige, IsaacShaibu, VictorSheneni, Blessing Gana, ToyinBabalola, WilliamsChintem, JoyUgbede, Adams Ibrahim, KenneathYatai, Mr. Ernest Penn, IdrisMuluh, HarounaDifo, HabibaKasimu, GbengaAmode, Timothy Tese, Joseph Ameh, Emmanuel Ochai, Emmanuel Ezegwu, AbubakarSaddeeq, OtaruAbdullahi, Mohammed Ghadaffi, Halima Musa, SanusiAbdullahi, SaniGombe, MichealAondofa, Mansur Maitoand endless list of students in the Department of Biochemistry, inABU Zaria and beyond. Your support and encouragements have had tremendous effects on my academic life in ABU Zaria.

Finally my appreciation to all the members of Postgraduate Fellowship, Chapel of Redemption, Sunday Schoolteachers of Chapel of Redemption and Presbyterian Church Ngomgham and to NUCs Zaria. I am indebted and most grateful to you all.

ABSTRACT

Antianaemic and antioxidant effects of extracts (aqueous, 70% methanol, 70% ethanol and 70% acetone) of *Gnetumafricanum* leaves on phenlyhydrazine (phz)-induced anaemicand normal albino rats were investigated. Phytochemical quantification (mg/g) carried out on the dry leave samples revealed Glycosides (0.97±0.07) was significantly ($P<0.05$) higher than Saponins (0.71 ± 0.03), Alkaloids (0.54 ± 0.07), Flavonoids (0.1 ± 0.01) and Tannins (0.03 ± 0.01). Total polyphenol content in mg gallic acid/g of dry sample was significantly ($P<0.05$) higher in aqueous (0.45 ± 0.06) and ethanol (0.38 ± 0.07) extracts than in methanol (0.17 ± 0.03) and acetone (0.26 ± 0.04) extracts. 1, 1-diphenyl-2-picrylhydrazyl (DPPH) activity indicates that aqueous extract had the lowest inhibitory concentration (IC_{50}) (mg/ml) of 0.355 followed by ethanol (0.357), acetone (0.565) and methanol extract (0.571). Preliminary investigation carried out to determine the extract with most potent haematopoietic potential by administration of all extracts at 200mg/Kg body weight to (phz)-induced anaemic rats for 7 days revealed methanol extract as the most potent extract for increasing the Packed Cell Volume (PCV) by 28%. Induction of anaemia significantly ($P<0.05$) decreased PCV, Haemoglobin concentration (Hb), Red Blood Cell count (RBC), Mean Corpuscular Volume (MCV), Mean Corpuscular Haemoglobin (MCH) and White Blood Cell count (WBC) of anaemic not treated rats compared to normal control rats with the exception of Mean Corpuscular Haemoglobin Concentration (MCHC) where no significant ($P>0.05$) change was recorded. In the groups treated with methanol extract at the doses of 50, 100 and 200mg/Kg, WBC was significantly ($P<0.05$) increased while there was significant ($P<0.05$) reduction in the RBC, PCV and Hb with no significant ($P>0.05$) difference in MCV, MCH and MCHC of the anaemic rats treated with 50, 100

mg/Kg of extract and Vitamin B_{12} when compared to the normal control group. However, the anaemic rats treated with 200mg/Kg recorded no significant ($P>0.05$) difference in RBC, PCV and Hb when compared with normal control group. There was no significant ($P>0.05$) difference in all assayed haematological parameters in normal animals receiving extract compared to normal control group. There was no significant ($P>0.05$) difference in Thiobarbituric Acid Reactive Substances (TBARS), Superoxide dismutase (SOD) and Catalase (CAT) of liver and kidney between the normal control group, anaemic groups receiving extracts and anaemic not treated groups. CAT recorded a significant ($P<0.05$) reduction in the kidneys of anaemic group treated with Vit B_{12}. There was a significant ($P<0.05$) increase in the level of ALT in the anaemic not treated and a significant ($P<0.05$) increase in ALP in treated groups compared to normal control. A significant ($P<0.05$) increase in total and Indirect bilirubin and significant ($P<0.05$) decrease in direct bilirubin were recorded in anaemic treated group compared to normal control. A significant ($P<0.05$) increase was recorded in albumin of normal treated animals compared to the normal control group. Evaluation of effect of extract on relative organ weights demonstrated a significant ($P<0.05$) increase in the relative liver and kidney weight of anaemic treated, compared with the normal control group. These results illustrate that the extracts of *Gnetumafricanum* leaves possess haematopoietic and antioxidant properties. A dose of 200mg/Kg of methanol extract has antianaemic potentials.

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LIST OF ABBREVIATIONS

%:	Percentage
ALB:	Albumin
ALP:	Alkaline Phosphatase
ALT:	Alanine Amino Transferase
AST:	Aspartate Amino Transferase
CAT:	Catalase
Dir. Bil.:	Direct Bilirubin
DPPH:	1,1 diphenyl-2-picrylhydrazyl
G. africanum:	*Gnetumafricanum*
Hb:	Haemoglobin
IC_{50}:	Median Inhibitory Concentration
Ind. Bil.:	Indirect Bilirubin
LD_{50}:	Median Lethal Dose
MCH:	Mean Corpuscular Haemoglobin
MCHC:	Mean Corpuscular Haemoglobin Concentration
MCV:	Mean Corpuscular Volume
NaCl:	Sodium Chloride

Na_2SO_4: Sodium sulphate

PCV: Packed Cell Volume

PHZ: Phenylhydrazine

RBCs: Red Blood Cells

ROS: Reactive Oxygen Specie

SOD: Superoxide Dismutase

TBARS: Thiobarbituric Acid Reactive Substances

Tot. Bil.: Total Bilirubin

TP: Total Protein

WBCs: White Blood Cells

WHO: World Health Organisation

CHAPTER 1

1.0 INTRODUCTION:

Anaemia is a condition characterized by a decrease in the level of circulating haemoglobin, less than 13g/dl in males and 12g/dl in females (Okochi *et al*., 2004). Anaemia is one of the clinical conditions that constitute a serious health problem in many tropical countries as a result of the prevalence of different forms of parasitic infections, including malaria (Dacie and Lewis, 1995). There are various causes and types of anaemia, these include: sickle-cell anaemia, iron deficiency anaemia, vitamin B_{12} anaemia, drug induced anaemia as side effect of drug therapy, disease induced anaemia, etc (Iwalewa *et al.*, 2009).Therefore factors contributing to anaemia may be related not only to malnutrition and poverty, but also from the free radicals due to the excessive consumption of drugs and other xenobioticsas well as viral and parasitic infections (WHO, 2000[a]; Biapa *et al.*, 2011).In the tropics, due to endemicity of malaria and other parasitic infections, between 10 to 20% of the population is reported to possess less than 10 g/dl of haemoglobin in the blood (Diallo *et al.*, 2008). Severe anaemia can be caused by microorganisms such as *Plasmodium falciparum* (Iwalewa *et al.*, 2009). Haemolytic anaemia refers to a condition in which erythrocytes have a shortened life-span (Berger, 2007).

Phenylhydrazine is used for the induction of haemolyticanaemia and the study of its mechanismin many species.Phenylhydrazine is used worldwide mainly as a chemical intermediate in the pharmaceutical, agrochemical, and chemical industries. It is toxic by single exposure through the oral route with $LD_{50}$of 80–188 mg/kg body weight and is expected to be toxic by the inhalation and dermal routes but data from these routes of

exposure are less clear (WHO, 2000[b]).Mechanism of action of phenylhydrazine at cellular level is by generating Reactive Oxygen Species (ROS) within both human and rat erythrocytes;ROS production was associated with extensive binding of oxidized and denatured haemoglobin tothe membrane cytoskeleton.This free radical haemolyses red cells rapidly and converts oxyhaemoglobin into methaemoglobin. The oxidized and denatured haemoglobin cross-links and precipitates intracellularly,forming inclusions that are identified as Heinz bodies onthe supravital stain of the peripheral smear. Heinz bodiesare removed in the spleen, leaving erythrocytes witha missing section of cytoplasm; these "bite cells" can beseen on the routine blood smear. The altered erythrocytesundergo both intravascular and extravascular destruction (Goldberg and Stern, 1997). Phenylhydrazine-induced haemolytic injury seems to be derived fromoxidative alterations to red blood cell proteins (by free radicals)rather than to membrane lipids (McMillan *et al*, 2005).

Medicinal plants are useful worldwide in the treatment of diseases. Plants have provided the basis for traditional treatment for different types of disease and still offer an enormous potential source of new chemotherapeutic agents. In a recent report released by the World Health Organization, more than 80% of the world population still relies on herbal medicines as their primary source of health care. Millions of Africans of all ages rely on herbal medicine for primary health care (Abubakar *et al.*, 2005). The medicinal use of plant has been attributed to the presence of phytochemicals. These phytochemicals include glycosides, saponins, tannins, alkaloids and flavonoids (Giral and Hidalgo, 1983). Phytochemical screening of plants provides a knowledge of the chemical constituents of plants that is desirable, for the discovery of therapeutic agents andinformation may be of

value in disclosing new sources of such compounds as precursor for the synthesis of complex chemical substance and used in folkloric remedies (Farnsworth, 1966).Antioxidantcompounds like phenolic acids, polyphenols and flavonoidsscavenge free radicals such as peroxide, hydroperoxide or lipidperoxyl and thus inhibit the oxidative mechanisms that lead todegenerative diseases. They are therefore necessary in preventing haemolytic diseases, such as anaemia.

*Gnetumafricanum*is a popular non-woody, wild dioecious vegetable that grows on trees. It has a large distribution across tropical Africa and tolerates a range of habitats and environmental conditions. The leaves are elliptic and lined with recticulate veins, similar to those of dicotyledonous angiosperms (Iweala and Osundiya, 2010).It is edible and it can be taken in raw state as salad or cooked as the major vegetable of soups.*Gnetumafricanum* is commonly known in southeastern Nigeria as "Okazi" by the Igbo, "Afang" by the Efik/Ibiobio tribes and "Eru" in Cameroon. Apart from consumption, it is used by many Nigerians for varying purposes. Due to its social, cultural, medicinal, nutritive and economic values, this forest vegetable plays a key role in the livelihood of mostrural and even urban communities of Africa (Ibeawuchi *et al.*, 2008). The medicinal and culinary applications of the leaves of *Gnetum africanum* underscore its importance as a major dietary supplement with potential biological effects(Iweala and Osundiya, 2010).

1.1 STATEMENT OF RESEARCH PROBLEM

Anaemia is one of the clinical conditions that constitute a serious health problem in many tropical countries (Dacie and Lewis,1995). Anaemia, even when mild to moderate affects the sense of well-being resulting in fatigue, stress and reduced work productivity (Buseri *et*

al., 2008). It is associated with serious consequences including growth retardation, impaired motor and cognitive development, and increased morbidity and mortality (Ramakrishnan and Semba, 2008).

Several studies have shown an association between anaemia and maternal mortality from both hospital data and community based studies. It is estimated that anaemia may be responsible for as much as 20% of all maternal deaths in sub-Saharan Africa (Buseri *et al.*, 2008).Globally, anaemia affects 1.62 billion people which correspond to 24.8% of the population (WHO,2006).The incidence of anaemiaishigher in the third world than in developed countries due to the presence of manyaggravatingfactorssuch as poor nutrition, high prevalence of blood parasites for example, plasmodium, trypanosomes and helminthes infestation(Ogbe *et al.*, 2010). The anaemiaprevalenceremains high in Africa, with an overall incidence of 64.6% in children, 55.8% amongpregnantwomen and 44.4% amongyoung girls (WHO, 2006). This indicatesthatwomen are susceptible to anaemiaduringpregnancy due to high demandfrom the developingfoetus (Ong, 1974; Orna, 1991).Nigeria is one of the countries where the prevalence of anaemia is severe, that ismore than40% of the population suffer from anaemia as a public health problems (WHO, 2006).Anaemiaisverycommon and the incidence is likely to increase in future (Duff, 2008),

1.2 JUSTIFICATION

Since time immemorial, man hasused various parts of plants in the treatment andprevention of various ailments (Tanaka *et al.*, 2002).Though a large number of medicinal plants and their purified constituents have shown beneficial therapeutic potentials (Nooman *et al.*, 2008), the body of knowledge about plants, herbs, and spices

and their respective and collective roles in promoting human health is modest. Many poor people, especially those in developing countries like Nigeriadependheavily on plants and herbal products for the treatment of diseases and anaemia because drugs are not affordable. In the treatment of diseases like malaria and anaemia, the beneficial effects of analysed drug materials especially from plants are mainly attributed to the presence of constituents (alkaloids, saponins, terpenoids, anthraquinones, essential oils, flavonoids, tannins, etc). Endogenous antioxidant defenses are inadequate to completely minimize the ongoing oxidative damage to DNA, lipids, proteins and other biological molecules. Hence exogenous sources of antioxidants may be particularly important in protection against diseases resulting from cellular damages (Anyasor *et al.*, 2009).It has been reported that the presence of compounds such as flavones, isoflavones, flavonoides, tannins, catechines coumarin, lignans, isocatechins and anthocyanines in the medicinal plants is very helpful to eradicate free radical production (Nooman *et al.*, 2008; Douglas *et al.*, 2009). The phytochemical screening of *Gnetum africanum* leavesreveals the presenceof flavonoids which are powerful antioxidant polyphenolic compounds (Iweala and Osundiya, 2010). Torel*et al. (*1986) and Faure *et al. (*1991) have shown that flavonoids inhibit peroxidation of polyunsaturated fatty acids in cell membranes.The main characteristic of an antioxidant is its ability to trap free radicals. Highly reactive free radicals and oxygen speciesare present in biological systems from a wide variety of sources.These free radicals may oxidize nucleic acids, proteins, lipidsor DNA and can initiate degenerative disease like hemolytic anaemia caused by phenylhydrazine.

Anaemia is one of the numerous ailments claimed to have been successfully treated with plant materials by traditional medicine practitioners. In China for instance, blood diseases

such as malformation of blood circulatory system, anaemia, varicose veins and haemorrhages have been treated with plant materials (Richard, 1978). The crude extract of *Fagarazanthoxylum*was reported to be effective in the treatment of sickle cell anaemia (Sofowora, 1993). It was also reported that the aqueous crude extract of *Telfairiaoccidentalis*leaves has haematinic activity (Dina *et al*., 2000). It is well established that man consumes a wide variety of local crops and vegetables, which are believed to contribute significantly to the improvement of human health in terms of disease prevention and therapy (Breazile, 1971; Burkill, 2000). *Gnetum africanum* is used in the treatment of enlarged spleen, sore throat and Carthartic (Burkill, 2000). It is also used to reduce nausea, neutralize poisons and in making preparations for child birth. It can be used externally to manage boils and warts.There are also claims by traditional medicine practitioners that *Gnetumafricanum*leavesis used for the treatment of anaemia but it appears there is little scientific research and publication to verify this claim. The presence of flavonoids and other antioxidants on the leaves of *Gnetumafricanum* indicates its antioxidant property.

To date, little is known on the effect of extracts*Gnetumafricanum*leaves on blood and liver parameters.Therefore, this study was initiated to investigate the antianaemic and antioxidant effects of extracts of*Gnetumafricanum*leaves on phenylhydrazine-induced anaemic andnormalalbino rats.

1.3 AIM AND OBJECTIVES

1.3.1 Aim

To investigate the antianaemic,antioxidant andhepatic effects of extracts of *Gnetum africanum*leaves, for the treatment of haemolytic anaemia.

1.3.2 Specific objectives

The specific objectives were to;

i. Carry out phytochemical analysis of the leaves and leaves extracts.

ii. Determine the total polyphenol content, DPPH radical scavenging activity and biological activity of the extracts

iii. Investigate the effects of the extract on some haematological parameters of phenylhydrazine-induced anaemic and normal rats

iv. Examine the effects of the extract on some liver function parameters and other biochemical parameters of phenylhydrazine-induced anaemic and normal rats

v. Study the *in vivo* antioxidant potential of the extract and effects of the extract on body weight and relative organweight of phenylhydrazine-induced anaemic and normal rats.

CHAPTER 2

2.0: LITERATURE REVIEW

2.1: *GNETUM AFRICANUM*

2.1.1: General description of the plant

Gnetum africanum is a dioecious liana up to 10 m long but sometimes longer; branches somewhat thickened at the nodes, glabrous. Leaves are decussately opposite, sometimes in whorls of 3, simple; stipules absent; petiole up to 1 cm long, canaliculate above; blade ovate-oblong to elliptical-oblong, rarely lanceolate, 5–14 cm long, 2–5 cm broad, base attenuate, apex abruptly acuminate, obtuse or minutely apiculate, entire, thick-papery, glabrous, pale green above, paler beneath, with 3–6 pairs of strongly curved lateral veins looped near the margin. Inflorescence an unbranched catkin, axillary or terminal on a short branch, solitary but male inflorescences at apex of branches often in groups of 3, up to 8 cm long, jointed, peduncle 1–1.5 cm long, with a pair of scale-like, triangular bracts; male inflorescence with slender internodes and whorls of flowers at nodes; female inflorescence with slightly turbinate internodes and 2–3 flowers at each node. Flowers are small, 2 mm long, with moniliform hairs at base and an envelope; male flowers with a tubular envelope and exserted staminal column bearing 2 anthers; female flowers with cupular envelope and naked, sessile ovule. Seed resembling a drupe, ellipsoid, 10–15 mm × 4–8 mm, apiculate, enclosed in the fleshy envelope, orange-red when ripe, with copious endosperm (Orwa *et al*., 2009).

2.1.2: Other botanical information

Gnetum comprises approximately 35 species of small trees, shrubs or most often lianas, found in tropical South and Central America (about 7 species), Africa (2 species) and Asia (about 25 species). They look much like dicotyledonous flowering plants having opposite leaves with a net venation and cherry-like seeds, although in fact they are gymnosperms. *Gnetum africanum* has leaves which are relatively thin and pale green. Its male catkins have slender internodes of equal width from the base to the tip. *Gnetum buchholzianum* has thick dark green leaves. The male catkins have thick internodes widening towards the terminal part. It was recently proposed that all *Gnetum* species be transferred to *Thoa*, except two Asiatic species, mainly based on seedcoat structure (Orwa *et al*., 2009).

2.1.3: Location of the plant

Gnetum africanum is an endangered liane normally found in humid tropical forest. It is usually found with other climbers on middle- and under-storey trees, frequently forming thickets. It can also be found in riverine forest in areas that are otherwise too dry for the species. *Gnetum africanum* is mostly found at the periphery of primary forest and in secondary forest. In terms of geographical distribution, it extends from south east Nigeria, to Congo and as far as Angola in the south (Orwa *et al*., 2009).

2.1.4: Classification of *Gnetum africanum*

The scientific classification of *Gnetum africanum* was obtained from the encyclopedia of life (EOL) website (Explorer, 2002). The classification is as follows:

Kingdom: Plantae; Phylum: Tracheophyta; Class: Gnetopsida; Order: Gnetales; Family: Gnetaceae; Genus: *Gnetum*; Specie: *africanum*

(a)  (b) 

Plate 1.1: Photograph of (a) whole stem of *Gnetum africanum* in its natural habitat (b) a closer look on the leaves.

2.1.5: Uses of *Gnetum africanum*

2.1.5.1: Food:

*Gnetum africanum*is one of the most popular green leafy vegetable in Nigeria and is gaining popularity as a delicious food leaf in other African countries such as Cameroon, Gabon, Congo and Angola (Eyo and Abel, 1983). In Nigeria, *Gnetum africanum* leaves are

widely consumed in the South Eastern region due to its palatability and taste. The leaves of this species are edible, as are those of other American and Asian *Gnetum* species. It can be eaten raw, but is generally added to meat and fish dishes after cooking time. The popularly known “afang” soup that is often listed in many continental restaurant menus is prepared from these leaves which sometimes are cooked with water leaves (*Talicum traiangulare*) to give the soup a special savour. The fruit and seeds are also edible. *G. africanum* holds an important place in the diets of many people in central Africa and hencecommerce in *Gnetum* has increased considerably with much of the relished leaves being gathered and sold all year round.

2.1.5.2 Agriculture and commerce:

Women are directly involved in vegetable production and marketing for income, food and health care for their families (Udoh, 1999). *Gnetum africanum* is an allseason vegetable, very popular and important leafy vegetable mostly sought after by the people of the southeastern zone of Nigeria (Udoh and Emem, 2011). Olasantan, (2001) and Isong *et al*.(1999) assert that the plant, *G. africanum*is still growing and harvested from the wild. However, it is cultivated by farmers for proper access, acquisition and marketing.In Akwa Ibom state of Nigeria, this improved practice of cultivation of *Gnetum africanum* on-farm and on homestead was introduced to farmers in 1995 by Akwa Ibom State Agricultural Development (AKADEP). The age-long method of harvesting *G. africanum* from the wild state is gradually giving way to both homestead and farm cultivation of *G. africanum* and therefore sustainability of the crop. This equally accounts for year round availabilityand considerable increase in commerce with much of the relished leaves being gathered and sold(Udoh and Emem, 2011).

2.1.5.3: Medicinal use:

The leaves of *G. africanum*are used to treat enlarged spleen, sore throat and also used as a cathartic (Ekpo, 2007). The plant provides an arrow poison antidote (Burkill, 2000). In West Africa chopped leaves are used as a dressing on furuncles to hasten maturation. The stem is used in making preparations to ease childbirth. The leaves have equally been used in the treatment of diabetes mellitus, and as worm expeller (Ekpo, 2007; Orwa *et al*., 2009)

Various fractions of *G. africanum* are used medicinally to treat many different illnesses (Ali *et al*, 2011). Aqueous *Gnetum africanum*(fresh and dry) has been demonstrated to have the highest antimicrobial activity against *P. aeruginosa*and against *Staphylococcus aureus*, indicating good antibacterial efficacy of this plant on the test Organisms (Enyi-Idoh *et al.*, 2013). Crude methanol extract of *Gnetum africanum* isdemonstrated to have a stimulatory effect on haemopoiesis in a dose and time dependent manner (Nubila *et al.*, 2013). The medicinal value of mast plants is attributed to the presence of phytochemicals.

2.2: COMPOSITION OF *Gnetum africanum*.

2.2.1: Phytochemical constituents

Medicinal plants are plants which contain substances that could be used for therapeutic purposes or which are precursors for the synthesis of useful drugs (Abolaji *et al*., 2007). This therefore implies that medicinal value of a plant depends on its bioactive phytochemical constituents that produce definite physiological action in the body. Some of the most important bioactive phytochemical constituents include alkaloids, flavonoids, phenolics, essential oils, tannins saponins, phytosterols, glycosides, and anthocyanidins (Krishnaiah *et al*., 2009).These phytochemicals are known to perform several general and

specific functions in plants, and may exhibit different biochemical and pharmacological actions in different species of animals when ingested (Asaolu *et al*., 2009). These actions range from cell toxicity to cell protective effects (Trease and Evans, 1996).

Phenolic compounds protect against oxidative stress and degenerative diseases (Han *et al.*, 2007). Oleanolic acid (saponin) has been reported to have hypoglycemic activity (Güçlü-Ünstündağ and Mazza, 2007). *In vivo* antidiabetic activity of several plant extracts has been correlated with their total phenol and flavonoid content (Aslan *et al.*, 2007; Rauter *et al.*, 2009). Alkaloids, glycosides, carbohydrates, and steroids have demonstrated activity consistent with their possible use in treatment of type-2 diabetes. Terpenoids have been shown to decrease blood glucose levels in animal studies (Kumar *et al*., 2009).

Iweala and Osundiya, (2010)established that the leaves of *Gnetum africanum* contains phenolic substances, flavonoids, anthocyanidins, phytosterols, tannins, saponins, alkaloids, glycosides, cyanogenic glycosides and cardiac glycosides.

2.2.2: Proximate and nutrient constituent of *Gnetum africanum* leaves.

Many studies have also shown that the chemical composition of the leaves of this plant gives it significant nutritional properties, and its high fiber, protein, and calorie content support these claims (Ali *et al.*, 2011). The nutritional value of *Gnetum africanum* leaves is high and it is a source of protein (about 30% of dry weight), (Isong *et al*., 1999). Essential amino acids (isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine), non essential amino acids (aspartic acid, serine, glutamic acid, proline, glycine, alanine, cysteine, tyrosine, histidine and arginine) are found. The fat content in

Gnetum africanum leavesis significant, up to 14.20% (Okafor *et al.*, 1996.). The potential of African species to yield a potable sap as the Asian species is worth investigation.

Ekpo, (2007) reported that both fresh domestic leaves and wild leaves of *Gnetum africanum* had high nutritive values. The percentage moisture content, total ash, crude fat, crude protein, fibre content and carbohydrate values (43.23 %, 62.12%, 43.17%, 15.02%, 12.36% and 20.17%) respectively of wild leaves of *Gnetum africana* were significantly ($p<0.05$) lower than values obtained from fresh domestic leaves of *Gnetum africanum* (87.24%, 12.40%, 26.22%, 48.19%, 38.15% and 37.27%) respectively. Mineral constituent in the leaves (i.e. macro and micro-elements) is sufficient (7.0% dry wt.). The concentrations of mineral elements phosphorous, potassium, iron, zinc, sodium, calcium and magnesium of fresh domestic leaves of *Gnetum africanum* were 2.13%, 1.72%, 4.45%, 2.14%, 1.45%, 2.05% and 2.29% respectively. While the values obtained from wild leaves of *Gnetum africanum* were 0.15%, 0.28%, 1.47%, 1.02%, 1.01%, 0.70% and 1.04% respectively. The values of mineral elements were significantly higher ($p<0.05$) in fresh domestic leaves of *Gnetum africanum* than wild leaves of *Gnetum africanum*. The values of hydrogen cyanide, phytate, oxalate and tannin in wild leaves of *Gnetum africanum* were (3.55g/100g, 5.12g/100g, 7.23g/100g and 4.10g/100g) respectively. These values were significantly higher than values obtained from fresh domestic leaves of *Gnetum africanum*.

2.3: BLOOD CELLS

Blood cells (erythrocytes, leukocytes and platelets)are formed in the bone marrow stem cells (Bain, 2008) which are immortal, undifferentiated and pluripotent(George-Gay and Parker, 2003). Plate 2.1 illustrates the different types of blood cells. Leukocytes, also

known as white blood cells, are a group of related cell types that are involved in immune function. Leukocytes include neutrophils, eosinophils, basophils, lymphocytes and monocytes.

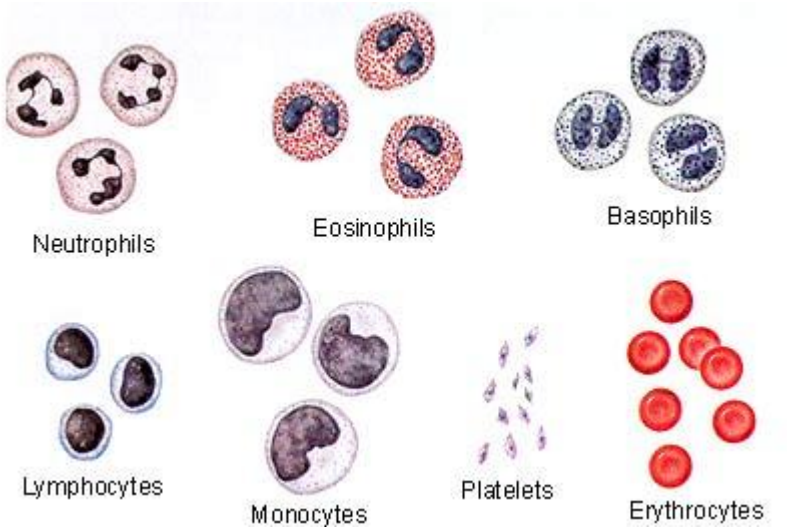
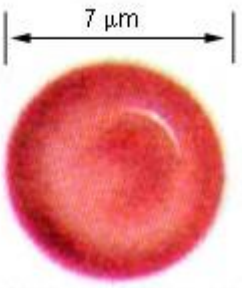


Plate 2.1: Blood cells(Adapted from www.biosbcc.net)

2.3.1: Erythrocytes (Red Blood Cells)

Erythrocytes, also known as red blood cells (RBCs), function to transport oxygen in the blood. The shape of erythrocytes is ideal for this function. Seen from the top, erythrocytes appear to be circular (Plate 2.2a), but a side view shows that they are actually biconcaved discs (Plate 2.2b). This shape increases the surface area-to-volume ratio of the cell, thus increasing the efficiency of diffusion of oxygen and carbon dioxide into and out of the cell (George-Gay and Parker, 2003). Erythrocytes also have a flexible plasma membrane. This feature allows erythrocytes, which have a 7µm diameter, to squeeze through capillaries as small as 3 µm wide (Branemark and Lindstrom, 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 loose 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 and Morris, 1980). The removal of old and dying erythrocytes is carryied out by the spleen. Erythrocyte production must equal erythrocyte death or the cell population would decline.

1) 

2) 

Plate 2.2: (a) Top (circular) view of erythrocyte; (b) Side (biconcave) view of erythrocyte.Adapted from Bain, (2008).

2.3.1.1: Erythropoiesis

The body has a way to assess the concentration of erythrocytes in the blood such that erythrocytes are produced at a rate that matches the body's needs. This implies that blood cells are constantly formed inside the body through a process called haemopoiesis, and erythropoiesis refers to the formation of erythrocytes (Goodnough *et al*., 2000). Erythropoiesis is an extremely active process that requires several metabolites for synthesis of haemoglobin, which contains globin as protein part and haem as prosthetic group (Lohar *et al*, 2009). The mechanism of erythropoesis occurs in the following way. The kidney

monitors the level of oxygen in the blood. If oxygen levels are low then the kidney secretes a hormone called erythropoetin. Erythropoetin enters the blood stream and travels throughout the body. All cells are exposed to erythropoetin, but only red bone marrow cells, which have erythropoetin receptors, respond to the hormone. Erythropoetin stimulates the production of erythrocytes in the bone marrow. These erythrocytes leave the bone marrow and move into the blood stream. As the erythrocyte population increases, the oxygen carrying capacity of the blood increases. When the kidney senses that oxygen levels are adequate, it responds by slowing the secretion of erythropoetin. This negative feedback loop ensures that the size of the erythrocyte population remains relatively constant and that the oxygen carrying capacity of the blood is always sufficient to meet the needs of the body (Jelkmann, 1992).The gene encoding erythropoetin was cloned as reported by Jacobs *et al*, (1985). Through recombinant DNA technology erythropoetin is now produced in large quantities and is available for clinical use (Winearls *et al.*, 1986).

Clinical uses of recombinant erythropoetin include:to boost erythrocyte production prior to surgery as a way to decrease the volume of transfused donor blood required and to boost erythrocyte production following chemotherapy for cancer. In untreated subjects, the most common type is the so-called anaemia of chronic disease, a condition characterised by excessive release of cytokines such as interleukin-1 and tumour necrosis factor. These peptides both inhibit erythroid marrow proliferation and blunt the normal exponential relationship between haematocrit and serum erythropoietin.Chemotherapy targets fast growing cells. Cancer cells are fast-growing and therefore die, but erythrocytes, which are also fast growing cells, suffer decline following chemotherapy (Cazzola, 2000).

2.3.1.2: Haemoglobin

Haemoglobin is an iron-containing protein found in red blood cells. It has two major parts:

■ Haem molecules – structure containing iron

■ Globin molecules – proteins that surround and protect heme

Iron is the building block needed to produce haemoglobin. Red blood cells are continually being replaced and this process reuses iron from theold red cells. Despite this recycling, our body has an ongoing need for iron from the dietsince availability of iron in a diet may modulate the regenerative response (Burkhard *et al*., 2001). Oxygen moves into the red blood cell and binds to haemoglobin, which allows it to be transported around the body. Therefore, haemoglobin's main function in the body is to carry oxygen from the lungs to the tissues and to assist in transporting carbon dioxide from the tissues to the lungs. The formation of haemoglobin takes place in the developing red cells located in bone marrow. Haemoglobin values are affected by age, sex, pregnancy, disease, and altitude. Statistical and physiological evidence indicate that haemoglobin distributions vary with smoking (Nordenberg*et al*, 1990) and altitude (Hurtado*et al*, 1945). The normal values for haemoglobin in g/100ml of blood in humans are male (14-17), female (12.5-15) and new born (17-23).

2.3.2: Leucocytes

White blood cells are an important component of the host defence system, responsible for protection against bacteria, fungi, viruses, and invading parasites. An intricate cytokine network and hierarchy of progenitor cells maintain baseline myelopoiesis and also allow

rapid adjustment in the rates of production of these cell types that occur in response to acute and chronic stress (Metcalf, 1993; Ogawa, 1993). White blood cells originate from pluripotent haemopoietic stem cells (Metcalf, 1993; Ogawa, 1993). Under the influence of various externalstimuli (cytokines, matrix proteins, and accessory cells), stem cells develop into haemopoietic progenitor cells of various lineages (Ogawa, 1993). Growth factors that regulate the development of particular populations of white blood cells have been identified (Metcalf, 1993; Ogawa, 1993). Nucleated cells are much more resistant to complement lysis than are erythrocytes (Ohanian and Schlager, 1981). Neutrophils make up more than half of all leucocytes and are the dominant class of circulating phagocytes (normal count $1{\cdot}8–7{\cdot}7X10^9$/L) (Malech and Gallin, 1987). They are the front line in defence against bacterial infections (Stock and Hoffman, 2000). Lymphocytes represent about a third of the white bloodcells in the peripheral blood ($1{\cdot}0–4{\cdot}0X10^9$/L). Most have a compact rounded or gently notched nucleus with scant agranular cytoplasm. Two thirds are T cells, which participate in cell-mediated immune responses. The remainders are B cells, which are programmed to produce antibodies (Stock and Hoffman, 2000). Mature eosinophils, which make up 5–10% of granulocytes ($0{\cdot}2X10^9$ /L), have a bilobed nucleus and numerous orange cytoplasmic granules. Eosinophils have substantial proinflammatory and cytotoxic activity and play an important part in the pathogenesis of various allergic, parasitic, and neoplastic disease processes (Sanderson, 1992). Basophils are the least common granulocytes and are distinguished from eosinophils by large metachromatic (purple-black) granules rich in histamine, serotonin, and leukotrienes (Denburg, 1992). Mast cells are related to, but distinct from, basophils. Basophils are bilobed, whereas mast cells are long-lived cells that reside in tissues rather than peripheral blood and are capable

of cell division. Both cell types are involved in immediate and cutaneous hypersensitivity reactions including asthma, urticaria, allergic rhinitis, and anaphylaxis (Stock and Hoffman, 2000).

2.3.3: Platelets

Human platelets are small and discoid in shape, with dimensions of approximately 2.0–4.0 by 0.5 lm, and a mean volume of 7–11 fl (George, 2000). They are the second most numerous corpuscles in the blood normally circulating at between 150–450X10^9/l. Platelets are anucleated cells derived from megakaryocytes and typically circulate for 10 days. Their shape and small size enables the platelets to be pushed to the edge of vessels, placing them in the optimum location required to constantly survey the integrity of the vasculature. Platelets are also surprisingly multifunctional and are involved in many pathophysiological processes including haemostasis and thrombosis, clot retraction, vessel constriction and repair, inflammation including promotion of atherosclerosis, host defence and even tumour growth/metastasis. Although, any test(s) of platelets could therefore potentiallymeasure any one or more of these vital processes, the majority of available tests focus only on those function(s) involved directly in haemostasis (Harrison, 2005).

2.4: HAEMATOCRIT

Whole blood is composed of plasma (liquid), cells and platelets. If whole blood is placed into a tube and centrifuged, the cells and the plasma will separate. The erythrocytes, which are heavy, will pack into the bottom of the tube, the plasma will be at the top of the tube, and the leukocytes and platelets will form a thin layer (buffy coat) between the erythrocytes and the plasma. The haematocrit is defined as the percentage of erythrocytesin

whole blood. This value is determined by dividing the height of the erythrocytes by the total height of the blood in the tube and multiplying by 100 (Cheesbrough, 2000)

Haematocrits vary depending on sex and environmental conditions, but there is a range of values that is considered normal.It is normally about 45% for men and 40% for women (Purves *et al*.,2004).

Factors that will raise the haematocrit include:

- **Exercise**. During aerobic exercise blood oxygen levels are lowered due to rapid consumption of oxygen by active skeletal muscle. This stimulates an increase in erythropoesis, which increases haematocrit, thus increasing the oxygen carrying capacity of the blood. Thus regular aerobic exercise raises the haematocrit.
- **Living at high altitude**. The air is thinner at higher altitude; therefore fewer molecules of oxygen enter the lungs with each breath. Oxygen levels in the blood are lower when breathing such thin air. A person that moves from areas at sea level, to highlands, will experience a rise in haematocrit as compensatory response to the thin air.
- **Injection of recombinant erythropoetin**. Some endurance athletes use erythropoetin (illegally) to increase their haematocrit as a way to increase stamina (www.biosbcc.net).

2.5: ANAEMIA

Anaemia, one of the most common and widespread disorders in the world, is a public health problem in both industrialised and non-industrialised countries. According to the

World Health Organization (WHO, 2011), anaemia is a condition in which the number of red blood cells or their oxygen-carrying capacity is insufficient to meet physiologic needs, which vary by age, sex, altitude, smoking, and pregnancy status. WHO, (2001) definitions for anaemia differ by age, sex and pregnancy status as follows: children 6 months to 5 year anaemia is defined as a Hb level<11g/dl, children 5–11 years Hb < 11.5 g/dl, adult males Hb < 13 g/dl; non-pregnant women Hb <12g/dl and pregnant women Hb < 11g/dl. The primary cause of anaemia is iron deficiency, but a number of other conditions, such as malaria, parasitic infection, excessiveconsumption of drugs and other xenobiotics, other nutritional deficiencies, and haemoglobinopathies are also responsible, often in combination (Beutler, 2001; Benoist *et al*., 2008). In 2002, iron deficiency anaemia (IDA) was considered to be among the most important contributing factors to the global burden of disease (WHO, 2002).

2.5.1: High-risk groups:

Children and women of reproductive age, in part because of their physiological vulnerability, are at high risk, followed by elderly people, and men (Mclean *et al*., 2009). Adolescents are also a key group, with adolescent girls being targeted for intervention before the onset of childbearing (Micronutrient initiative, 2009). However in most settings, anaemia is a marker of socioeconomic disadvantage, with the poorest and least educated being at greatest risk of exposure to risk factors for anaemia and its sequelae (Balarajan *et al*., 2011).

2.5.2: Symptoms and Signs of Anaemia

Symptoms and signs of anaemia include: pale skin, lips, tongue and inner surface of eyelids, (conjunctiva), fatigue, irritability, weakness, shortness of breath, low blood pressure with position change from lying or sitting to standing (orthostatic hypotension), sore tongue, brittle nails, concave nails, unusual food cravings (called pica), decreased appetite (especially in children), headache – frontal, low haematocrit and haemoglobin in a RBC and low serum ferritin (serum iron) level (Unicef, 2002).

2.5.3: Types of Anaemia

There are over a dozen different types of anaemia, some due to a deficiency of either a single or several essential nutrients and others from conditions that are not related to nutrition such as infections (Unicef, 2002; WHO, 2004). The types of anaemia include, anaemia of B_{12} deficiency, anaemia of chronic disease, anaemia of folate deficiency, drug-induced immune, haemolytic anaemia,due to glucose-6-phosphate dehydrogenase deficiency, idiopathic aplastic anaemia, idiopathic autoimmune haemolytic anaemia, immune haemolytic anaemia, iron deficiency anaemia, pernicious anaemia, secondary aplastic anaemia and sickle cell anaemia (Unicef, 2002).

2.5.3.1: Haemolytic anaemia

The exposure of humans and animals to many chemicals including the administration of some drugs has been associated with red blood cell destruction (Beutler 2001), and haemolytic anaemia is a part of the clinical syndrome associated with intoxication. Chemicals can cause haemolysis by interacting with sulfhydryl groups, the inhibition of various enzymes, immune mechanisms, and the fragmentation of erythrocytes as they pass

through the platelet-fibrin mesh or by unknown or poorly defined mechanisms (Berger, 2007). In haemolytic anaemia, erythrocytes have a shortened life-span (Beutler, 1969).

2.5.4: Tests for anaemia

According to Okonkwo *et al*.(2004), accurate laboratory determination of blood parameters remains the only sensitive and reliable foundation for ethical and rational research, diagnosis, treatment and prevention of anaemia.

2.6: PHENYLHYDRAZINE

Phenylhydrazine (PHZ) (CAS No. 100-63-0) was the first hydrazine derivative characterized by Hermann Emil Fischer in 1875. PHZ, ($C_6H_8N_2$)has a molecular weight 108; it exists as yellow to pale brown crystals or as a yellowish oily liquid, with a freezing point of 19.6°C and a boiling point of 243.4°C (WHO, 2000). It is sparingly soluble in water and is miscible with other organic solvents (Brooke, 1997). The limited data on toxicokinetics indicate that Phenylhydrazine is well absorbed by inhalation, oral, and dermal routes. Metabolism seems to occur through ring hydroxylation and conjugation, probably with glucuronic acid. Excretion is primarily through the urine (WHO, 2000[b]).

Phenylhydrazine (PHZ) is a strong oxidant agent, which is extensively used in industry, laboratory and therapeutic settings. It is mutagenic *in vitro*, and there is some evidence to indicate that it may express genotoxic activity *in vivo*(WHO, 2000[b]). Phenylhydrazine has potential for skin and eye irritation and there is evidence that it has skin-sensitizing properties in humans. The substance is clearly carcinogenic in mice following oral dosing, inducing tumours of the vascular system (Berger, 2007). A variety of toxic effects of PHZ have been described, including haemolytic anaemia, hypoxia, inflammation, alterations in

the liver, kidney, central nervous system, autoimmune disturbances and cancer (Goldberg and Stern, 1977; Parodi *et al*., 1981; Nässberger *et al*., 1991; Brugnara and de Franceschi, 1993; Nicolas *et al*., 2002). PHZ is known to shorten life-span of red blood cells.

PHZ-induced anaemia can be used as a model for the evaluation of its influence on therapeutic effectiveness, e.g., of antitumour therapy (Golab *et al*., 2002) or as a model of reticulocyte research or erythrocyte senescence under abnormal physiological conditions (Xie *et al*., 2002). PHZ-induced anaemia is a model for the study of haematinic effects (Agbor *et al*., 2005; Biswas *et al*., 2005). In 1987, Berger discovered that upon induction of anaemia using phenylhydrazine in rats of different ages, the deepest anaemia was found in haemoglobin level, red blood cell counts and packed cell volume and reticulocytosis in old rats, while the highest increase in mean corpuscular volume (MCV) and MCH was found in young adult animals. This could be due to the lower PHZ-resistance of mature red blood cells in elder animals. Thus indicating that intensity of induced anaemia depends on age (Berger 1987, Naeshiro *et al*. 1998).

2.6.1: Mechanism of action of phyenylhydrazine at cellular level

PHZ increases reactive oxygen species (ROS) and lipid peroxidation, and decreases glutathione (GSH); these effects are reversed by N-acetyl cysteine, a known ROS scavenger (Hill and Thornalley, 1983; Clemens *et al*., 1984; Amer *et al*., 2004). Haemolytic anaemia has long been known to be caused by uptake of erythrocytes by macrophages in the spleen and translocation of phosphatidylserine from the inner to the outer of the plasma membrane has been identified as a signal for phagocytosis of cells under programmed death by macrophages. PHZ generates ROS within both human and rat

erythrocytes; no evidence for lipid peroxidation or phosphatidyl serine externalisation was detected (deJong *et al*., 1997; McMillan *et al*., 2005).

ROS production was associated with extensive binding of oxidized and denatured haemoglobin to the membrane cytoskeleton. Thus, PHZ-induced haemolytic injury seems to be derived from oxidative alterations to red blood cell proteins rather than to membrane lipids (McMillan *et al*., 2005).

Vitamins C and E contribute to the decrease in oxidative stress caused by PHZ *in vitro* (Claro *et al*., 2006). They inhibited Heinz bodies and methemoglobin formation but they did not protect against GSH depletion by PHZ. Quercetin, an antioxidant bioflavanoid compound, also suppresses reactive oxygen and nitrogen species, and it partially protects reduced glutathione (GSH), malondialdehyde levels (Luangaram *et al*., 2007). Melatonin as a free radical scavenger protects against phenylhydrazine-induced oxidative damage to cellular membranes (Karbownik *et al*. 2001).

PHZ induces Heinz body formation and oxidative degradation of spectrin without any cross-linking of membrane proteins; both these findings impair erythrocyte deformability (Miyazaki*et al*., 1993). Formation of phenyl radicals and the replacement of haem with phenyl-substituted protoporphyrins, causes the destabilisation of haemoglobins to induce Heinz bodies and haemolytic anaemia with PHZ.(Nakanishi *et al*.,2007).

PHZ treatment increases the transport rates in Na-K pump, Na-H exchange, Na-Li exchange, and K-Cl cotransport*in vivo*; while a decrease in Na-K pump, Na-H exchange, Na-Li exchange and increase K-Cl contransport were found in rabbit red cells (Brugnara and DeFranceschi, 1993).

2.7: ROLE OF MEDICINAL PLANTS IN THE TREATMENT OF ANAEMIA

The major concern of the scientific communities with regard to medicinal plants and haematological studies focuses on the measures that can maintain a normal haematological state of being and reverse any negative haematological status associated with various anemic conditions (Alexander and Griffiths, 1993).

CHAPTER 3

3.0 MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Sample Collection and Identification.

The matured leaves of the plant were harvested in January 2013 from the South West Region of Cameroon. The plant was identified and authenticated at the Herbarium section of the Department of Biological Science, Ahmadu Bello University (ABU) Zaria where a voucher specimen number (1259) was deposited.

3.1.2Chemicals and Reagents:

All chemicals and reagents used were of analytical grade. The phz (CAS Number : 59-88-1 Product Number : P0184) was purchased from TCI, America.Other chemicals were purchased from Okey pharmaceutical in Kaduna, Kaduna State-Nigeria.

3.1.3 Animals

A total ofseventy-nine (79) Wistar rats of both sexes weighing between 150-250g were obtained and kept in well aerated laboratory cages in the Animal house, Department of Pharmacology, Faculty of Pharmaceutical Sciences, ABU, Zaria. The animals were allowed to acclimatise to the laboratory environment for a period of two weeks before the commencement of the experiment. They were fed with water and grower mash from Vital Feed Company, Zaria*ad libitum* during the stabilisation period.

3.2 METHODS

3.2.1 Extraction of Leaves Material and Determination of Percentage Yield.

3.2.1.1 Preparation of Leaves sample

The leaves were washed and air dried at room temperature for three weeks. After three weeks, dry samples were ground at the Institute of Agricultural Research (IAR), Ahmadu Bello Univerrsity, Zaria using the Thomas-Wiley Laboratory mill, Model 4. The powder was sieved with the sieve of mesh size 1mm and then stored in polythene bags for further use.

3.2.1.2 Preparation of Extracts

Four extracts;aqueous, 70 % methanol, 70% ethanoland 70% acetone wereprepared. One litre of the various solvents (distilled water, methanol, ethanol and acetone) wasused to dissolve 100g of the powder of the leaves. The prepared solutions wereallowed to stand for 48 hours. Each extracted solution was filtered off using a Whatman® filter paper (Cat no 1001 125) of pore size 125mm. The filtrate was concentrated by distilling off the solvent and then evaporated to dryness on a water bath at 45°C. The samples were then stored in refrigerator for subsequent usage.

3.2.1.3 Determination of Percentage Yield:

The four extracts obtained (without the solvent) were weighed. The percentage yield per extract was calculated in terms of air dried weight of the leaf material as:

$$\text{Percentage yield} = \frac{\text{Amount of extract obtained}}{\text{Amount of initial sample}} \text{ X } 100$$

3.2.2Phytochemical Analyses

3.2.2.1 Phytochemical screening

This chemical test was carried out on all four extractsusing a standard procedure for identification of phytochemistry constituents as described by Harborne, (1973); Trease and Evans, (1983) and Sofowora, (1993). Extract solution was prepared by dissolving 10g of extract in distilled water to make up 100ml solution. The extract solution was then used for phytochemical screening.

Test for Carbohydrates.

Molisch's Test: Few drops of Molisch reagent was added to 5ml of extract solution in a test tube and a small quantity of concentrated sulphuric acid was allowed to run down the side of the test tube. A purple to violet colour at the interface indicates the presence of carbohydrates (Trease and Evans, 1983).

Test for Free Anthraquinones

Bornirager's Test: Extract solution (5 ml) was shaken with 10ml of benzene and filtered. Then 10% of ammonia solution (5 ml) was added to the filtrate and stirred. The production of a pink-red or violet colour indicates the presence of free anthraquinones.(Trease and Evans, 1983).

Test for Cardiac Glycosides.

Kella Killiani Test: Extract (1 g)was dissolved in 5ml of glacial acetic acid containing traces of ferric chloride. The test tube was held at an angle of 45 degree, 1ml of

concentrated sulphuric acid was added down the side.Purple ring colour at the interface indicate cardiac glycosides (Trease and Evans, 1983).

Test for Saponins.

Frothing test: Extract solution(5 ml) was shaken vigorously for 30 seconds and was allowed to stand for 30 minutes. A honey comb formed for more than 30 minutes indicates saponins.

Test for Steroids and Triterpenes

Liberman-Burchards Test:Equal volume of acetic anhydride was added to about 5ml of the extract solution. Concentrated sulphuric acid (1 ml) was added down side of the tube. The colour change was observed immediately and later. Red, pink or purple colour indicates the presence of triterpenes while blue or blue-green colour indicatethe presence of steroids (Trease and Evans, 1983).

Test for Flavonoids.

Sodium Hydroxide Test: Few drops of aqueous NaOH were added to 5ml of extract solution, a yellow colouration shows the presence of flavonoids (Trease and Evans, 1983).

Test for Tannins.

Ferric Chloride Test:Extract solution (0.5 ml) was dissolved in 10ml of distilled water, and then filtered. Few drops of ferric chloride solution were added to the filterate. Formation of

a blue-black precipitate indicates hydrolysable tannins and green precipitates indicate the presence of condensed tannins.

Test for Alkaloids.

Meyers Test:Few drops of Meyer'sreagent were added to 5ml of the extract solution in a test tube Green precipitate indicates the presence of alkaloids.

Dragendoff's Test: Few drops of Dragendoff's reagent were added to 5ml of the extract solution. Rose-red precipitate indicates the presence of alkaloids.

Wagners Test: Drops of Wagner's were added to 5ml of the extract solution. Whitish precipitate indicates the presence of alkaloids.

3.2.2.2 Quantitative Phytochemical Determination

Determination of Saponins

A gravimetric method of AOAC, (1984) employing the use of a soxhlet extractor and two different organic solvents was used. The first solvent (acetone) extracts lipids and interfering pigments while the second solvent (methanol) extracts saponins. Two grams each of the G*netum afrianum* were weighed into a thimble and put in a soxhlet extractor with a condenser fitted on top. Extraction was done with acetone in a 250ml round bottom flask for 3hrs, after which the other weighed 250ml round bottom flask containing methanol was fitted to the same extractor continued for another 3hrs. At the end of second extraction, the methanol was recovered by distillation and the flask oven-dried to remove

the remaining solvent in the flask. The flask was allowed to cool in a dessicator and weighed.

Calculation:

%Saponin = (A- B) / W × 100

Where A= weight of flask and extract (saponin), B = weight of empty flask

W = weight of sample.

Determination of Flavonoids

Flavonoid determination was done using the method of Boham and Kocipal-Abyazan, (1974). Ten grams of the plant samples were extracted with 100ml of 80% aqueous methanol at room temperature. The whole solution was filtered through whatman filter paper no 42 (125mm), the filtrate was later transferred into a crucible and was evaporated to dryness in a water bath and weighed as flavonoid.

Calculation

% Flavonoid = weight of flavonoids/ weight of sample x 100/1

Determination of Tannins

Concentration of tannins was determined using the standard method described by AOAC, (1984). Two grams of the dried sample was boiled with 300ml of distilled water. This was diluted in a standard volumetric flask and filtered through a non-absorbent cotton wool. A volume of 25ml of the infusion was measured into a 2 litre porcelain dish and titrated with 0.1N potassium permanganate (0.1N potassium permanganate was standardized against

0.1N Oxalic acid) until the blue solution changed green, Then few drops of 0.1N potassium permanganate was added at a time until the solution turns golden yellow.

The tannin content in the sample was calculated by multiplying the volume of 0.1N potassium permanganate used (titre value) by 0.0066235g. Using the equation below;

1ml of 0.1N potassium permanganate (titre value) = 0.0066235g Tannins
Therefore Tannins content in the sample = titre value x 0.0066235g

Determination of Glycosides

The procedure of AOAC, (1984) was used in determination of cyanogenic glycosides. Five grams of sample was placed in a 200ml conical flask; 50ml of distilled water was added. The content of the flask was stirred and allowed to stand for 4hrs. The filtrate was steam distilled into 20ml of 2.5% NaOH. About 75ml of the distillate was collected. This was titrated with 0.02N $AgNO_3$ (silver nitrate) after the addition of 8ml of 6N ammonium hydroxide and 2ml of 2% potassium iodide. Permanent turbidity indicated end-point. The cyanogenic glycosides was calculated by multiplying the volume of 0.02N $AgNO_3$ used (titre value) by 1.08mg, using the equation below;

1ml 0.02N $AgN0_3$= 1.08mg HCN

Therefore cyanogenic glycosides content in the sample = titre value x 1.08mg

Determination of Alkaloids

This was done using themethod of Harborne, (1998)

Method:

Sample (5g) was weighed into a 250 ml beaker and 200 ml of 10% acetic acid in ethanol was added and covered and allowed to stand for 4 h. This was filtered and the extract was concentrated on a water bath to one-quarter of the original volume. Concentrated ammonium hydroxide was added dropwise to the extract until the precipitation was complete. The whole solution was allowed to settle and the precipitate was collected and washed with dilute ammonium hydroxide and then filtered. The residue is the alkaloid, which was dried and weighed.

Determination of Total Phenolic Content (TPC)

The total phenolic content was determined on the four extracts using the Folin-Ciocalteu method adopted by Amin *et al*.(2004).

Principle:The reaction is based on the reduction of phosphor-wolframate-phosphomolybdate complex by phenolics to a blue reaction product with a maximum absorptionat 765nm which can be measured spectrophotometrically (Amin *et al*., 2004).

Procedure:The Folin-Ciocalteu reagent was diluted 10 times (2.5 ml) and mixed with 2 ml of saturated sodium carbonate (75 g/l) and 6050 µl of sample (supernatant) and homogenized for 10 seconds and heated for 30 minutes at 45^0C. The absorbance was measured at 765 nm after cooling at room temperature. The data obtained was calculated by comparison between a standard curve (µg Gallic acid/ml) and the absorbance of each sample. The data obtained were expressed as mg Gallic acid equivalents per gram of dry matter.

3.2.3DPPH Radical Scavenging Activity of the Extracts

The DPPH radical scavenging activity of the plant extracts were assayed by the DPPH radical scavenging method of Chan *et al.* (2007).

Principle: 1,1-Diphenyl-2-picrylhydrazyl (DPPH) contains an odd electron in its structure. Its purple colour is reduced to yellow-coloured diphenylpicryhydrazine when it reacts with an antioxidant, which can donate a hydrogen atom or an electron to it. The change in colour was measured spectrophotometrically at 520 nm using a UV/Visible light spectrophotometer.

Procedure: DPPH solution was prepared by dissolving 6 mg of DPPH in 100 ml of methanol. To 1ml of various concentrations of the extracts (0.020, 0.040, 0.060, 0.080, 0.100 mg/ml), 2 ml of DPPH solution (0.1mM) was added. An equal amount of methanol and DPPH served as control. The mixture was shaken vigorously and was left to stand in dark for 30min. The absorbance of the resulting solution was measured spectrophotometrically at 520 nm. The experiments were performed in triplicate and the percentage scavenging activity of each extract on DPPH radical was calculated using the following formula:

$$\text{Scavenging activity (\%)} = \left[1-\left(\frac{Ae}{Ab}\right)\right]x100$$

Where Ab is absorbance of the blank sample, Ae is the absorbance of the extract.

DPPH radical scavenging activities of the extracts were expressed as IC_{50} values. IC_{50}, the effective concentration of the extract required for 50% scavenging of DPPH radical was

calculated from the graph of scavenging activity plotted against sample concentration using Microsoft Excel software. The IC_{50} of Vitamin C was determined and compared to that of the extracts.

3.2.4Induction of Anaemia

Anaemia was induced by modified method of Iwalewa*et al*.(2009). Rats were injected intraperitonially twice (40 mg/kg of phenyl hydrazine hydrochloride). The second induction was within 24 hours from the first.Anaemia was allowed to establish in 24 hour after the second induction.Anaemia was confirmed when Packed Cell volume (PCV) was less than35%. On the day of establishment of anaemia (day 1), the weight, PCV, Hb were taken in all the groups. Animals were treated with the extracts and with vitamin B_{12} (100 µg/kg), just after establishment of anaemia in the animals.

3.2.5 Determination of Extract with the Most Potent Antianaemic Potential (Biological Activity of Extracts)

This preliminary test was carried out for 7 days to determine the extract with the most potent antianaemic effect amongst four prepared extracts (Aqueous, Methanol, Ethanol and Acetone). This assessment was carried out using 12 rats. The rats were divided into 6 groups with 2 rats each. The grouping were as follows

Group 1 (NRC): Normal Rats fed with normal feed and water *ad libitum*

Group 2 (ARC): Anaemic Rats fed with normal feed and water *ad libitum*

Group 3 (AQ): Anaemic rats fed with normal feed and water *ad libitum* and 200mg/Kg of aqueous extract

Group 4 (ME): Anaemic rats fed with normal feed and water *ad libitum* and 200mg//kg of methanol extract

Group 5 (ETH): Anaemic rats fed with normal feed and water *ad libitum* and 200mg/kg of ethanol extract

Group 6 (ACE): Anaemic rats fed with normal feed and water *ad libitum* and 200mg/kg of acetone extract

PCV of the animals were determined on Day 0,1, 4 and 7, while animals were observed daily for 7 days for signs of toxicity. The extract which generated the greatest increment in PCV and as well exhibited the least toxic effects within the 7 days was used as the most potent. Subsequent *in vivo*work were focused on the most potent extract.

3.2.6Determination of Median Lethal Dose (LD_{50})

This was done to obtain a safe dose for the evaluation ofeffects of the most potent extract. The Median Lethal Dose (LD_{50}) for the extract was determined by method of Lorke, (1983) using thirteen (13) rats.

In the first phase of the investigation, three dose groups consisting of 3 rats each were administered the extract of *Gnetum africanum* at 10, 100 and 1000 mg/kg orally. Animals in each group were observed for any immediate signs of toxicity and mortality within 24 h. The second phase of the acutetoxicity testing 4 rats were divided into 4 groups, followed by administration of different doses of extracts orally based on the result obtained from phase one (the doses administered were 1200, 1600, 2900 and 5000 mg/kg body weight. They were also observed for another 24 h. The LD_{50}wascalculated based on mortality as

square root of the product of the lowest and highiest non-lethal dose, i,e the geometric mean of the consecutive doses which 0 and 100% survival rates was recorded.

3.2.7Animal Grouping for Sub Acute Study.

The animals were randomly divided into nine (9) groups with six rats each.Methanol extract of *Gnetum africanum* leaves was administered to rats in six (6) groups for twenty-one (21) days. The grouping was as follows:

Group 1 (NRC): Normal rats received normal chow and water *ad libitum*

Group 2 (ARC): Anaemic rats were given normal chow and water*ad libitum*

Group 3 (NR + 50mg/Kg): Normal rats were fed on normal chow and water *ad libitum* and 50mg/Kg of extract

Group 4 (NR + 100mg/Kg): Normal rats were fed on normal chow and water*ad libitum*and100mg/Kg of extract.

Group 5 (NR + 200mg/Kg): Normal rats were fed on normal chow and water*ad libitum* and 200mg/Kg of extract.

Group 6 (AR + 50mg/Kg): Anaemic ratswere fed on normal chow andwater*ad libitum*,and50mg/Kg of extract.

Group 7 (AR + 100mg/Kg): Anaemic ratswere fed on normal chow andwater *ad libitum*and 100mg/Kg of extract.

Group 8 (AR + 200mg/Kg): Anaemic rats were fed on normal chow and water*ad libitum*,and200mg/Kg of extract.

Group (AR + Vit B_{12}): Anaemic rats were fed on normal chow and water*ad libitum*and standard drug (100µg/Kg of Vitamin B_{12}) was also administered.

3.2.8Haematological Analyses

Haematologic parameters were determined by standard methods. Blood sample were collected through the tail vein. Packed Cell Volume and haemoglobin concentrations were determined on day 0(before induction of anaemia), day 1 (day of anaemia induction)and on days 14 and 21. These analyses served as markers for the antianaemic activity of the plant before the animals were sacrificed.

3.2.8.1 Determination of Packed cell volume

The PCV is the volume of red blood cells (RBCs) expressed as a fraction of the total volume of the blood. The microhaematocrit method was used (Cheesbrough, 2000).

Principle: The red blood cells are heavier than plasma with specific gravity of 1090 and 1030 respectively. When blood is placed in a capillary tube and centrifuged, they settle and become packed because of the centrifugal force acting on them. The volume occupied by the cells is measured and its ratio with that of the volume of the whole blood is calculated.

Procedure: Blood from EDTA bottle was allowed by capillary action to flow through the capillary tube and one end of the tube was sealed by flaming. It was then centrifuged at a speed of 3000rpm for 10 minutes. The PCV was estimated using a microheamatocrit reader and expressed as percentage erythrocytes the blood contain.

3.2.8.2 Determination of haemoglobin concentration

Principle:Blood is mixed with dilute hydrochloric acid. This process haemolyses the red cells, disrupting the integrity of the red cells' membrane and causing the release of haemoglobin, which, in turn, is converted to a brownish-colored solution of acid haematin. The acid haematin solution is then compared with a color standard (Jain, 1986).

Procedure:

Sample solutions and standard solutions were prepared as follows.

Random sample: 5000 µl of Drabkin reagent was mixed with 20 µl of distilled water

Standard: 5000 µl of Drabkin reagent was mixed with 20 µl of sample solution of haemoglobin

Target sample: 5000 µl of Drabkin reagent + 20 µl of blood

The concentration of haemoglobin was marked with Drabkin's method, with the use of a spectrophotometer, at 540nm wavelength. Once Drabkin reagent was mixed with the blood, the solution was incubated at room temperature for the duration of 5 mins and absorbance was measured. The spectrophotometer was set to zero using distilled water.

Measurement formula: Haemoglobin and its derivatives affected by Drabkin reagent transform into cyanmethemoglobin, whose optical density may be measured photometrically.

The concentration of hemoglobin was calculated according to the following formula:

$$\text{Hb concentration (g/dl)} = \frac{\text{absorbance of tested sample}}{\text{absorbance of standard}} \text{ X concentration of standard in g/dl}$$

3.2.8.3 Determination of White Blood Cells and Red Blood Cells Count

Improved Neubeur haemocytometer (counting chamber) was used to count both white blood cells (WBCs) and red blood cells (RBCs) under the light microscope (Blaxhall and Daisley, 1973).

Principle: Blood is diluted with a fluid containing glacial acetic acid that lyses the red cells but not the white cells and a dye (gentian violet) which stains the nucleus of the white cells to facilitate counting.

Procedure: The white blood cell and red cell pipettes were used to draw blood and fill to 0.5 mark of both WBC and RBC pipettes. WBC dilution fluid (2% acetic acid and 1% gentian violet in distilled water) and RBC diluting fluid (Hayem's fluid made up of 1g NaCl, 5g Na_2SO_4, 0.5g mercuric chlorised per 200ml solution) were drawn to 11 and 101 marks respectively. The fluid and the blood were then mixed gently and transferred into the counting chamber. It was allowed to settle for 2mins and the chamber placed on the stage of the microscope for counting. The white blood count was done using the x10 objective while the red blood cell counts were done using the x40 objective.

Calculation: Number of cell counted divided by volume of one square ($0.1mm^3$) multiplying number of squares counted and multiply by dilution factor (20 for WBC count and 200 for RBC).

3.2.8.4 Mean Cell Volume: The mean cell volume (MCV) was determined as

$$MCV = 10 X \frac{\text{Hamatocrit}}{\text{Red Blood Cell Count}}$$

*3.2.8.5Mean Cell Haemoglobin:*Mean Corpuscular Haemoglobin (MCH) was calculated as follows

$$MCH = 10 X \frac{\text{Haemoglobin}}{\text{Red Blood Cell Count}}$$

3.2.8.6Mean Corpuscular Haemoglobin Concentration (MCHC): It was calculated as follows

$$MCHC = \frac{\text{Haemoglobin}}{\text{Hamatocrit}} X 100$$

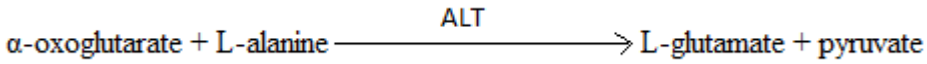
3.2.9Determination of Biochemical Parameters

3.2.9.1 Alanine Aminotransferase (ALT) and Aspartate Aminotransferase (AST)

Activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were assayed for by the method of Reitman and Frankel 1957 using Randox assay kits.

Alanine Aminotransferase (ALT)

Principle



Alanine Aminotransferase is measured by monitoring the concentration of pyruvate hydrazone formed with 2, 4-dinitrophenylhydrazine at 540 nm and 37^0C.

Reagent Composition

The initial chemical composition of the reagent was as follows

- Reagent (R1): This was buffer solution. It was made up of phosphate buffer (100mmol/l, pH 7.4), L-alanine (200mmol/l), α-oxoglutarate (2mmol/l).
- Reagent (R2). This was 2, 4-dinitrophenylhydrazine (2mmol/l)

Procedure: Exactly, 0.5ml of reagent 1 which is made up of phosphate buffer, L-alanine and α-Oxoglutarate was added into two clean test tubes, one containing 0.1 ml of serum and the other containing 0.1ml distilled water (blank). The content in each test tube was mixed, incubated for exactly 30 minutes at 37^0C. 0.5 ml of reagent 2 which is made up of 2, 4-dinitrophenylhydrazine was added to each of the test tubes, mixed and allowed to stand for exactly 20 minutes at 20-25^0C. Then 0.5ml of sodium hydroxide solution was added to each of the test tubes, the content in each of test tubes was mixed and absorbance was read against the blank at 540nm after 5 minutes. The ALT activity (U/I) was determined from the standard calibration table provided in the manual of Randox Lab. Ltd, UK Reagent Kit.

Aspartate Aminotransferase (AST)

Principle

$$\alpha\text{-oxoglutarate} + \text{L-aspartate} \xrightarrow{\text{AST}} \text{L-glutamate} + \text{Oxaloacetate}$$

AST was measured by monitoring the concentration of oxaloacetate hydrazone formed with 2, 4-dinitrophenylhydrazine at 540 nm and 37^0C.

Reagent Composition

The initial chemical composition of the reagent was as follows

- Reagent (R1) was Buffer. It was made up of phosphate buffer (100mmol/l, pH 7.4), l-aspartate (100mmol/l) and α-oxoglutarate 2mmol/l)
- Reagent (R2) was 2, 4-dinitrophenylhydrazine (2 mmol/l)

Procedure: Exactly, 0.5ml of reagent 1 which is made up of phosphate buffer, L-aspartate and α-Oxoglutarate was added into two clean test tubes labelled as test sample and reagent blank, containing 0.1 ml of serum and 0.1 ml of distilled water, the content in each test tubes was mixed and incubated for exactly 30 minutes at 37^0C. 0.5 ml of reagent 2 which is made up of 2, 4-dinitrophenylhydrazine was added to each of the test tubes, the content of each of the test tubes was mixed and allowed to stand for exactly 20 minutes at 20-25^0C. To each of the test tubes, 0.5ml of sodium hydroxide solution was added, mixed and absorbance was read against the blank at 540nm after 5 minutes. The AST activity (U/l)

was determined from the standard calibration table provided in the manual of Randox Lab. Ltd, UK Reagent Kit.

3.2.9.2 Alkaline Phosphatase (ALP)

The serum level of alkaline phosphatase was quantified by optimized standard method described by Haussament, (1977) using Randox assay kits.

Principle

P-nitrophenylphosphate + H_2O $\xrightarrow{ALP}$ phosphate + p-nitrophenol

Alkaline phosphatase is measured by monitoring the concentration of phosphate hydrazone formed with 2, 4-dinitrophenylhydrazine at 405 nm and 37^0C.

Reagent Composition

The chemical composition of the Reagent were as follows

- Reagent (R1a): This was a buffer. Its initial composition was Diethanolamine buffer (1mol/l, pH 9.8), $MgCl_2$ (0.5mmol/l)
- Reagent (R1b): This was the substrate. Its initial chemical composition was p-nitrophenylphosphate (10mmol/l)

Preparation of Reagents

One vial of substrate R l b was reconstituted with the 20ml of Buffer R l a

Procedure: Exactly 1ml of reagent 1 containing Diethanolamine buffer, magnesium chloride and substrate (P-nitrophenylphosphate) was added into a clean test tube containing 0.02ml of serum. This was mixed, initial absorbance was read and timer was started simultaneously, absorbance was read again after 1, 2 and 3 min at 405nm.

Calculation

The ALP activity was calculated using the following formulae:

U/l = 2760 x Change in absorbance (405 nm/ min).

Change in absorbance(405 nm/ min) = change in absorbance at 405nm per min

3.2.9.3 Total Protein (TP)

Total protein was determined colorimetrically according to the method described by Fine, (1935) using Randox assay kits.

Principle: The protein assay (using the Randox laboratories assay kit) is based on the reaction of protein with an alkaline copper tartarate solution and Folin reagent. Two steps lead to colour development. First is the reaction between protein and copper at alkaline pH, and subsequently, the reduction of Folin reagent by the copper-treated protein. Colour formation is due to amino acids that reduce the Folin reagent, yielding reduced species that imparts a characteristic blue colour. Bovine serum albumin is used as standard.

Reagent Composition

The initial reagent solution was made up of the following

- ❖ Reagent (R1): This was Biuret reagent. It was made up of Sodium hydroxide (100mmol/l), Na-K tartrate (16mmol/l), Potassium iodide (15mmol/l), cupric sulphate (6mmol/l)
- ❖ Reagent (R2): This was made up of sodium hydroxide (100mmol/l), Na-K tartrate (16mmol/l)
- ❖ Calibration solution: Protein (58.48g/l)

Preparation of Reagent

- ❖ Reagent (R1): Biuret reagent*:* The content of bottle R1 was diluted with 400 ml of double distilled water, rinsing the bottle thoroughly.

Reagent(R2):Blank reagent: The content of bottle R2 was diluted with 400 ml of double distilled water, rinsing the bottle thoroughly.

Procedure:Exactly 1ml of biuret reagent was added to three clean test tubes labelled as sample, standard and blank containing 0.02ml of serum, 0.02ml of standard and 0.02ml of distilled water respectively, the content in each of the test tube was mixed, incubated for 10 minutes under room temperature. The absorbance was read at 540nm. The total protein concentration was calculated using the formula below

$$\text{Total protein concentration} = \frac{\text{Change in absorbance for sample}}{\text{Change in absorbance for standard}} \text{ X Concentration of the standard}$$

3.2.9.4 Albumin (ALB)

The serum albumin was determined by the method of Doumas *et al.*(1971) using randox assay kit.

Principle: The measurement of serum albumin is based on its quantitative binding to the indicator 3, 3 ’5, 5’ – tetrabromo-m cresol sulphonephthalein (bromocresol green, BCG). The absorbance of the blue green colour of albumin-BCG-complex at 630nm is directly proportional to the concentration of albumin in the sample.

Reagent Composition

The initial chemical composition of the reagents was as follows

- Reagent (R1): This solution was BCG concentrate. It was made up of Succinate buffer (75mmol/l: pH 4.2), Bromocresol green (46.75g/l)
- Calibration standard (46.75g/l)

Preparation of Reagent

R1. BCG Concentrate

The content of a bottle R1 was diluted with 87 ml of distilled water.

Procedure:Exactly1ml of Bromocresol green reagent (R1) was added to three clean test tubes labelled test sample, standard and reagent blank containing 0.02ml of serum sample, 0.02ml of standard and 0.02ml distilled water respectively. The mixtures in each of the test tubes were incubated for 10min under room temperature. The absorbance of the serum sample (**A** sample) and of the standard (A standard) was read at630nm against the reagent blank

Calculation: The albumin concentration was calculated from the following formula in g/l:

$$\text{Albumin concentration (g/l)} = \frac{\text{Change in absorbance of sample}}{\text{Change in absorbance of standard}} \quad X \quad \text{Concentration of the standard}$$

3.2.9.5 Determination of Serum Total Bilirubin (TB) Concentration

The serum TB concentration was determined using Randox Kit (Randox laboratories limited UK) based on the method described by Jendrassik and Grof, (1938) and Sherlock, (1951).

Principle:Total bilirubin is determined in the presence of caffeine, which releases albumin bound bilirubin, by the reaction which diazotized sulphanillic acid.

Procedure:Exactly, 200ml of reagent 1 (sulphanillic acid) was dispensed each into two different test tubes labeled 'sample blank' and 'sample' followed by the addition of 1 drop (50µl) of reagent 2 (nitrite) and 1000 µl of reagent 3 (caffeine). 200 µl of the test serum was the dispensed into each of the test tubes and the mixtures incubated in a water bath for 10 minutes at 25^0C. This was followed by the addition of 100 µl of reagent 4 (tartrate) and the mixture incubated again at 25^0C for 10 minutes. The absorbance of the sample (A_{TB}) was then read against the sample blank using a colorimeter at 578nm wavelength. The total bilirubin concentration was then calculated using the formula provided by the manufacturers viz:

Total bilirubin (mg/dl) = $10.8 \times A_{TB}$ (578nm)

Where A_{TB} = absorbance of total bilirubin

3.2.9.6 Determination of Serum Direct/Indirect Bilirubin (DB/IB) Concentration

The serum DB/IB concentration was determined using the Randox Kit (Randox laboratories limited UK) based on the method described by Jendrassik and Grof, (1938) and Sherlock, (1951).

Principle:Direct/indirect bilirubins react with diazotized sulphanillic acid in alkaline medium to form a blue coloured complex**.**

Procedure:Exactly, 200ml of reagent 1 (sulphanillic acid) was dispensed each into two different test tubes labeled 'sample blank' and 'sample' followed by the addition of 1 drop (50µl) of reagent 2 (nitrite) and 2000 µl of 0.9 % NaCl. 200 µl of the test serum was the dispensed into each of the test tubes and the mixtures incubated in a water bath for 10 minutes at 25^0C. The absorbance of the sample (A_{TB}) was then read against the sample blank using a colorimeter at 546 nm wavelength. The direct bilirubin concentration was then calculated using the formula

Direct bilirubin (mg/dl) = $14.4 \times A_{DB}$ (546nm)

Where A_{DB} = absorbance of direct bilirubin

Indirect Bilirubin = Total bilirubin – Direct bilirubin

3.2.9.7 Determination of Serum Iron Concentration

Serum Iron was determined by the method of Makino *et al.* (1988) using the Nitro-PAPS (monoreagent) kit.

Principle: Iron (Fe^{3+}) ions are dissociated from its carrier protein, transferrin in an acid medium and simultaneously reduced to the ferrous form. The ferrous ion reacts with the chromogen Nitro-PAPS to a colour-complex highlyspecific. The resulting absorbance is directly proportional to the Iron contents.

Transferrin-Fe-complex $\longrightarrow$ Apotransferrin + Fe^{3+}

$Fe^{3+} \xrightarrow{\text{ascobate}} Fe^{2+}$

Nitro PAPS + Fe^{2+} $\longrightarrow$ Coloured complex

Procedure

Exactly 100µl of reagent were pipetted into separate testtubes. 50µl of standard was added into a test tube and the same volume of sample was added into other testtubes, with exception of the tube designated for reagent blank (RBL) where 50µl of distilled water was added.The resulting solution was mixed and incubated at 25^{o}C for 10 minutes. The mixture was transferred to a cuvette and the absorbance of the sample $A_{(sample)}$ and standard $A_{(standard)}$ were measured against the blank $A_{(RBL)}$ at 578nm.

$\delta A_{(sample)} = A_{(sample)} - A_{(RBL)}$

$\delta A_{(standard)} = A_{(standard)} - A_{(RBL)}$

Concentration of Iron = $30 \times \frac{\delta A_{(s)}}{\delta A_{(STD)}}$ (µmol/l)

3.2.10 *In vivo* Antioxidant Studies

3.2.10.1 Superoxide Dismutase (SOD) activity

Superoxide dismutase (SOD) was determined by a method described by Fridovich, (1989). Principle: The ability of superoxide dismutase (SOD) to inhibit auto oxidation of adrenaline at pH10.2 forms the basis of this assay.

Reagents

Carbonate buffer (0.05M): 14.3g of Na_2CO_3 and 4.2g of $NaHCO_3$ were dissolved in distilled water and made up of 100ml mark in a volumetric flask. The buffer was adjusted to pH 10.2.

Adrenaline (0.3mM): 0.01g of adrenaline was dissolved in 17ml of distilled water. The solution was prepared fresh.

Procedure:

Tissue homogenate (0.1ml) was diluted in 0.9ml of distilled water to make 1:10 dilution of microsome. An aliquant mixture of 0.20ml of the diluted microsome was added to 2.5ml of 0.05M carbonate buffer. The reaction was started with the addition of 0.3ml of 0.3mM Adrenaline. The reference mixture contains 2.5ml of 0.05M carbonate buffer, 0.3ml of 0.3mM Adrenaline and 0.20ml of distilled water. Absorbance was measured over 30s up to 150s at 480nm in a colorimeter.

Calculations

Increase in absorbance per minute $= \frac{(A_5 - A_1)}{2.5}$

% inhibition = $100 - \frac{\uparrow A_s}{\uparrow A_x} x100$

Where $\uparrow A_s$ is increase in absorbance of substrate and $\uparrow A_b$ is increase in absorbance of the blank

1 unit of SOD activity is the quantity of SOD necessary to elicit 50% inhibition of the oxidation of adrenaline to adenochrome in 1 minute.

$$\text{SOD activity} = \frac{\text{Percentage Inhibition}}{\text{50 X weight of organ}}$$

3.2.10.2. Catalase (CAT) Activity

Catalase activity was determined using the method described by Sinha, (1972).

Principle: The method is based on the reduction of dichromate in acetic acid to chromic acetate, when heated in the presence of hydrogen peroxide with the formation of perchromic acid as unstable intermediate. Chromic acetate so produced is measured colorimetrically using spectrophotometer at 570nm. This is because dichromate has no absorbance at 570nm and does not interfere with the determination of catalase.

Reagents

0.01M Phosphate buffer (pH 7.0): 5% potassium heptaoxochromate (V1), $K_2Cr_2O_7$: 5g of $K_2Cr_2O_7$ was dissolved in little quantity of distilled water and made up to 100ml.

0.2M H_2O_2: 0.6ml of $H_2O_2$was dissolved in little quantity of distilled water and made up to 100ml. It was stored at 4^0C.

Dichromate / Acetic acid solution: 5% potassium heptaoxochromate (V1), $K_2Cr_2O_7$, was mixed with glacial acetic acid in the ratio 1:3, and was stored in brown bottle at room temperature.

Procedure: Exactly 1ml of phosphate buffer, 0.2ml of tissue homogenate and 0.4ml of H_2O_2 were added into two separate test tubes each, 1ml of potassium dichromate/glacial acetic acid was added immediately to one of the test tubes immediately after the addition of H_2O_2 (T0), while 1ml of potassium dichromate was also added to the second test tube 10 minutes after the addition of H_2O_2 (T1). The mixture in the test tubes was heated in water bath for 10 minutes at 80^0C. Absorbance was read at 570nm, in a colorimeter.

Calculations

The quantity of H_2O_2 was calculated using the formula

$$\frac{C_T}{C_S} = \frac{A_T}{A_s}$$

where

C_T = Concentration of the test

C_S = Standard concentration = 0.2M

A_T = Absorbance of test (T0 – T1)

A_S = Absorbance of blank (T0)

$$\text{Catalase activity} = \frac{C_T X \text{ total volume of homogenate}}{10 \text{ minutes X volume of homogenate used X wt of tissue}}$$

Catalase activity was expressed in moles of H_2O_2/min/g tissue.

3.2.10.3 Thiobarbituric Acid Reactive Substances (TBARS) Determination

Lipid peroxidation of LDL was assessed by Thiobarbituric acid reactive substances Determination (TBARS) formation (Ohkawa*et al*., 1979).

Principle: Malondialdehyde (MDA) is a product of lipid peroxidation and is used as an indicator of tissue damage. The MDA forms a 1:2 adduct with thiobarbituric acid (TBA) and produce a pink coloured product which has an absorption maximum of 532nm.

Working reagents

Thiobarbituric acid (TBA): 100mg of TBA was dissolved in 30ml distilled water and 30ml of acetic acid

Trichloroacetic (TCA) (10%): 10g of TCA was dissolved in distilled water and made up to 100ml with distilled water.

Normal saline solution (0.9%): 0.9g of NaCl was dissolved in distilled water and made up to 100ml with distilled water.

Procedure: Normal saline (0.2ml) was pipetted into test tubes labelled as; sample test and sample blank, 0.2ml of tissue homogenate was pipetted into the sample test tube only, 0.5ml of TCA solution was added to each of the sample test and sample blank tubes followed by addition of 0.1ml of TBA solution, 0.2ml of distilled water was added into sample blank tube in place of tissue homogenate. The mixture in each test tube was heated for 60 minutes in a water bath at 95^{o}C. After cooling to room temperature on ice bath, 3ml of n-butanol was added to the content in each test tube and then mixed vigorously, the butanol phase was mixed by centrifugation at 1000 X g for 5 minutes and the absorbance of the sample test was read against the absorbance of the sample blank at 532nm

Calculations

The concentration of TBARS is expressed in terms of Malondialdehyde (MDA) in mol/ml.

Molar extinction of MDA 1.56 x $10^{5}cm^{-1}m^{-1}$

$$\text{MDA Concentration} = \frac{\text{Absorbance}}{1.56 \times 10^{5}cm^{-1}m^{-1}}$$

$$\text{TBARS} = \frac{\text{Volume of homogenate X MDA concentration}}{\text{weight of organ}}$$

3.2.11 Determination of Body and Relative Organ Weights

The weight of the rats was determined using My Weigh 7001DX Multi-Purpose Digital Scale on day 0 (before induction of anaemia)and at the end of every week. The net change in body weight (difference between final body weight and initial body weight) was determined for all the animals.On day 22, the organisms were sacrificed after an overnight

fast. The liver and kidneys wereexcised and their weights determined. The relative liver and kidney weights were derermined using the formular

$$\text{Relative organ weight} = \frac{\text{Organ weight}}{\text{Body weight}} \text{X}\,100$$

3.3STATISTICAL ANALYSIS

Data was analysed using statistical package for the social sciences (SPSS), version 20. The results were expressedas mean ± standard deviation (SD) except where otherwise stated.The data was analysed by analysis of variance (ANOVA). The difference between the various extracts and animal groups were compared using the Duncan Multiple Range Test. P values less than 0.05 ($P<0.05$) were taken as significant.

CHAPTER 4

4.0: RESULTS

4.1. PERCENTAGE YIELD OF EXTRACTS OF *Gnetum africanum*LEAVES.

The percentage yield of aqueous,70% methanol, 70%ethanol and 70% acetone extracts of *Gnetum africanum* is represented in Table 4.1. The percentage yield of aqueous extract (13.1±0.06) was significantly (P<0.05) higherthan yield of ethanol extract (11.3±1.39).Ethanol extract in turn recorded a significantly (P<0.05) higher percentage yield than methanol (0.87±0.06) and acetone extract (0.87±0.05).

4.2 PHYTOCHEMICAL CONTENT

The quantitative phytochemical content in the leaves of *G. africanum* recorded in mg/g is summarised in Table 4.3. The results indicate that glycosides (0.95±0.03) were the most abundant phytochemical as its level was significantly (P<0.05) higher than those of other phytochemicals. Thoughsaponin content (0.71±0.03) was significantly (P<0.05) higher than alkaloids (0.54±0.07), the two phytochemicals (saponins and alkaloids) alongside glycosides were significantly (P<0.05) higher than flavonoids (0.10 ±0.014) and tannins (0.03 ±0.01).The qualitative determination of some phytochemicals in the aqueous, methanol, ethanol, and acetone extracts of *Gnetum africanum* is presented in Table 4.2. The result shows the presence of carbohydrates, saponins, steroids, tannins, flavonoids and alkaloids in all four extracts. Cardiac glycosides were absent only in acetone extract while triterprenes were present only in the methanol extract.

Table 4.1: Percentage Yield of Aqueous, Methanol, Ethanoland Acetone Extracts of *Gnetum africanum*Leaves.

Plant extract	Weight of dry samples (g)	Weight of extracts (g)	Percentage (%) yield
Aqueous	10	1.31 ± 0.06^{c}	13.1 ± 0.61^{c}
Methanoilc	10	0.87 ± 0.06^{a}	8.7 ± 0.61^{a}
Ethanol	10	1.13 ± 0.14^{b}	11.3 ± 1.39^{b}
Acetone	10	0.87 ± 0.05^{a}	8.7 ± 0.53^{a}

Values are means ± SD of three extractions. Values with different superscript down the column are significantly ($P<0.05$) different

Table 4.2: Qualitative Phytochemical Constituents in the Aqueous, Methanol, Ethanol and Acetone Extracts of *Gnetum africanum*Leaves.

Phytochemicals	Aqueous	Methanol	Ethanol	Acetone
Carbohydrates	+	+	+	+
Anthraquinone	-	-	-	-
Cardiac glycosides	±	+	+	_
Saponins	+	++	+	+
Steroids	+	+	+	+
Triterprenes	-	+	-	_
Flavonoids	+	±	±	±
Tannins	+	±	+	++
Alkaloids				
Mayer's	-	-	-	-
Dragendoff's	+	-	+	+
Wagner's	+	+	+	+

++ = present in high amount, + = present in moderate amount, ± = present in trace amount, - = absent,

Table 4.3: Quantitative Determinationof Phytochemical in Leaves of *Gnetum africanum*

Phytochemicals	Amount (mg/g)
Saponins	0.71 ± 0.03^{c}
Flavonoids	0.10 ± 0.01^{a}
Tannins	0.03 ± 0.01^{a}
Glycosides	0.95 ± 0.07^{d}
Alkaloids	0.54 ± 0.07^{b}

Values are means ± SD of three determinations. Values with different superscript down the column are significantly ($P<0.05$) different

4.3 TOTAL POLYPHENOL CONTENTOF AQUEOUS, METHANOL, ETHANOL AND ACETONE EXTRACTS OF *Gnetum africanum* LEAVES

The Total Polyphenol Content of aqueous, methanol, ethanol and acetone extracts of *Gnetum africanum*(recorded in mg gallic acid/g of dry sample) is summarised in Table 4.4. The aqueous (0.45±0.05) and ethanol (0.38±0.07) extracts recorded a significantly ($P<0.05$) higher quantity of total polyhpenols than acetone (0.26±0.04) and methanol (0.17±0.03) extracts

4.4 FREE RADICAL SCAVENGING ACTIVITY OF AQUEOUS, METHANOL, ETHANOL AND ACETONE EXTRACTS OF *Gnetum africanum*LEAVES ON DPPH

Figure 4.1 presents the percentage (%) inhibition of different concentrations (0.02, 0.04, 0.06, 0.08 and 0.1 mg/ml) of methanol, ethanol, acetone and aqueous extracts of *Gnetum africanum* as well as Vitamin C, a standard antioxidant on DPPH. This plotdepicts the antioxidant activity of the extracts of *Gnetum africanum* based on their scavenging ability on DPPH which is a stable purple-coloured radical and in the process reducing it into yellow-coloured diphenylhyrazine. From the result, the % inhibition of all extracts is generally lower than that of Vitamin C at all concentrations.

Antioxidant potential is inversely proportional to inhibitory concentration (IC_{50}) value, which was calculated from the linear regression of the % inhibition versus extracts concentrations. The IC_{50} values are found in Table 4.5. IC_{50} of extracts in increasing order of magnitude areaqueous extract (0.355mg/ml), ethanol extract (0.357mg/ml), acetone

extract (0.565mg/ml) and methanol extract (0.571mg/ml). The IC_{50} of extract were greater than that of Vitamin C (0.077mg/ml).

Table 4.4: Total polyphenol Contentof Aqueous, Methanol, Ethanol and Acetone extracts of *Gnetum africanum*Leaves.

Extract	Total polyphenol content (mg gallic acid/g of dry sample)
Aqueous	0.45 ± 0.05^{b}
Methanol	0.17 ± 0.03^{a}
Ethanol	0.38 ± 0.07^{b}
Acetone	0.26 ± 0.04^{a}

Values are means ± SD of three determinations. Values with different superscript down the column are significantly ($P<0.05$) different

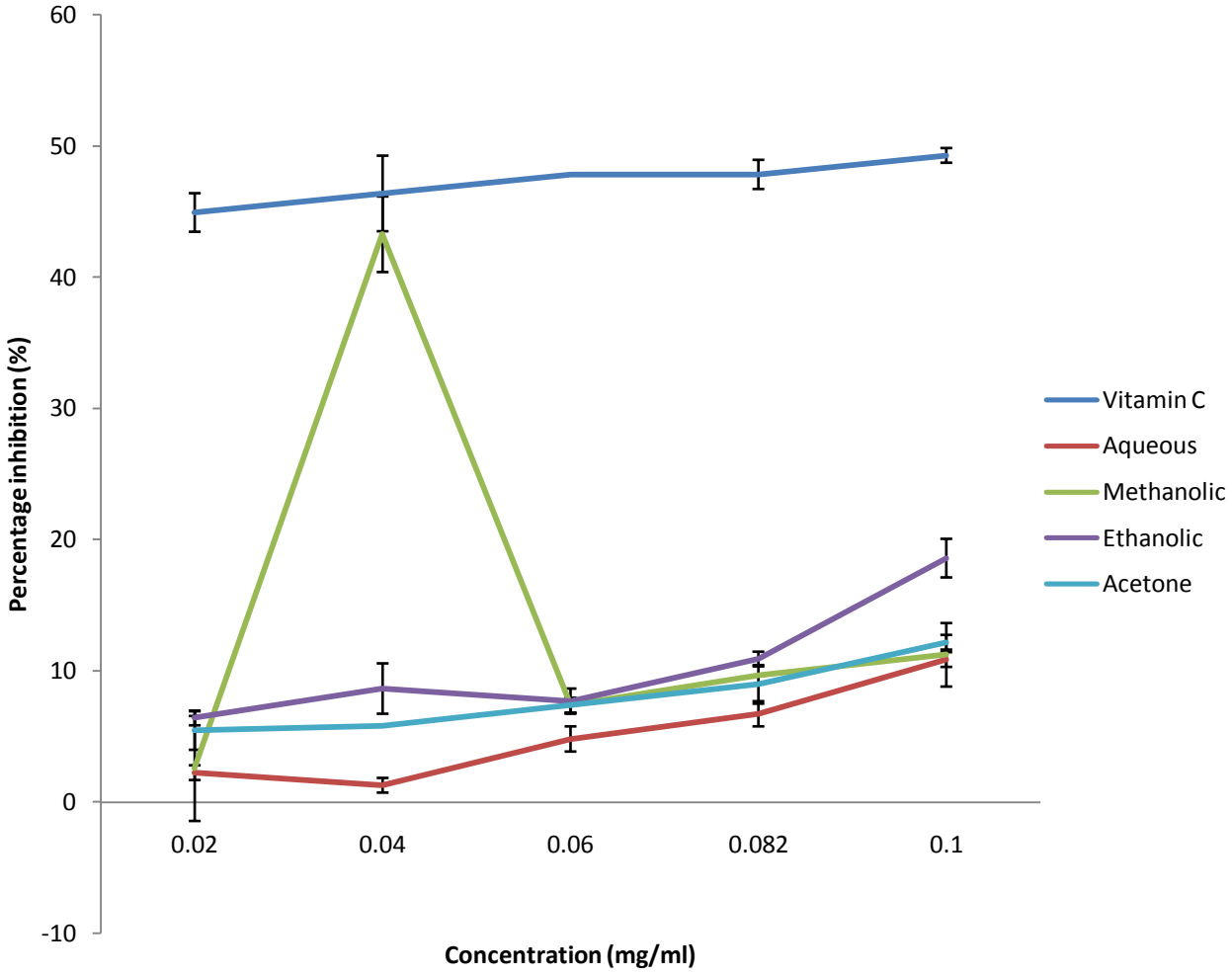


Figure 4.1: Percentage Inhibition of different concentrations of methanol, ethanol acetone and aqueous extracts of *Gnetum africanum* leaves and Vitamin C on DPPH

Table 4.5: Inhibitory Concentration (IC_{50})of Aqueous, Methanol, Ethanoland Acetone Extracts of *Gnetum africanum* leaves.

Extracts	IC_{50} values (mg/ml)
Aqueous	0.355
Methanol	0.571
Ethanol	0.357
Acetone	0.565
Vitamin C	0.077

IC_{50} values were calculated based on triplicate analysis of each plant extract and represented as mg/ml.

4.5 EXTRACT WITH THE MOST POTENT ANTIANAEMIC POTENTIALS

The effects of aqueous, methanol, ethanoland acetone extracts of *Gnetum africanum* leaves on Packed Cell Volume of phenylhydrazine-induced anaemic rats after one week of administration of 200mg/Kg of each extract is summarised in Table 4.6.This experiment was carried out to determine the extract with most potent antianaemic potentials. The fall in PCV values of phz-induced animals on Day 1compared to PCV values of Day 0 values and also to PCV values of the normal control group, confirmed induction of anaemia using phz. Increase in PCV values of all phz-induced treated groups on day 4 indicates antianaemic potential of all the extracts.The antianaemic potential of the extracts was in decreasing order: acetone, methanol, ethanol and aqueous extracts. PCV values on Day 7 generates some concerns about the safety of acetone and aqueous extract as one death was recorded in both groups, though order of antianaemic potential of the groups seemed unaffected. At the end of the experiment, methanol extractwhich had substantialhaematopoietic potential generated 28% increament in PCV andwas relatively safe for administration compared to other extracts. Methanolextract was therefore used for in vivo studies.

4.6MEDIAN LETHAL DOSE (LD_{50}) OF METHANOL EXTRACT of *Gnetum africanum*LEAVES.

Table 4.7 shows the results of the lethal dose (LD_{50}) determination of methanol extract of *G. africanum* leaves in albino rats. There was no mortality withinthe first 24 hours after oral administration of 10, 100 and 1000 mg/kg body weight of the extract to the rats in the first phase. Oral administration of 1200, 1600, 2900 and 5000 mg/kg body weight of the

extracts in the second phase, equally recorded no mortality within the next 24 hours. Therefore, the $LD_{50}$of methanol extract of *Gnetum africanum*leaves is greater than 5000mg/kg body weight.

Table 4.6: Effectof Aqueous, Methanol, Ethanoland Acetone Extracts of *Gnetum africanum*Leaves on Packed Cell Volume of Phenylhydrazine-Induced Anaemic Rats.

Group	Initial PCV (%) (Day 0)	PCV after induction (%) (Day 1)	PCV on Day 4 (%)	PCV on Day 7 (%)	Net increment in PCV (%) (Day7-Day1)	Mean Net Increase in PCV (%)
NRC	35	35	40	38	+3	+3.5
NRC	36	34	37	38	+4	
ARC	47	22	28	32	+10	+10
ARC	40	XXX	XXX	XXX	-	
AQ	42	28	29	41	+13	-
AQ	43	24	31	XXX	-	
ME	43	20	30	43	+23	+28
ME	46	21	31	54	+33	
ETH	53	36	40	53	+17	+19.5
ETH	45	25	35	47	+22	
ACE	40	21	32	56	+35	-
ACE	43	22	31	XXX	-	

NRC: Normal control animals; ARC: Anaemic control animals; ME: Anaemic animal +200mg/kg of methanol extract; ETH: Anaemic animals + 200mg/kg of ethanol extract; ACE: Anaemic rats + 200mg/kg of acetone extract; AQ: Anaemic rats + 200mg/kg of aqueous extract. XXX: dead rat

TABLE 4.7: Median Lethal Dose (LD_{50}) For MethanolExtract of *Gnetum africanum*Leaves.

Phase	Dose (mg/kg of body weight)	Number of animals	Number of deaths after 24 hrs
I	10	3	0
	100	3	0
	1000	3	0
II	1200	1	0
	1600	1	0
	2900	1	0
	5000	1	0

LD_{50}>5000mg/kg body weight

4.7HAEMATOLOGICAL STUDIES

4.7.1 Effect of Methanol Extract of *Gnetum africanum*Leaves on Packed Cell Volume and Haemoglobin Concentration

The effect of sub-acute administration of methanol extract of *Gnetum africanum* leaves and vitamin B_{12} on Packed Cell Volume and haemoglobin concentration of phenylhydrazine-induced anaemic and normal rats is presented on Figure4.2 and 4.3 respectively. There was a significant ($P<0.05$) decrease in PCV and Hb of the anaemic not treated groupcompared to the normal control group. There was a significant ($P<0.05$) decrease in PCV and Hb of anaemic treated groups compared to the normal control group with the exception of PCV and Hb ofgroup receiving 200mg/Kg (day 14 and 21) and group receiving Vit B_{12} (Day 14). Whenever PCV and HB were determined (Day 0, 1, 14 and 21), there was no significant ($P>0.05$) difference in the PCV and Hb values of the normal rats administered with the extracts compared with the normal control group.

4.7.2: Effects ofMethanol Extract of*Gnetum africanum* Leaves on Other Haematological Parameters.

The effects of daily oral administration of methanol extract of *Gnetum africanum* leaves on red blood cell (RBC) count, white blood cell (WBC) count, mean corpuscularvolume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) of phz-induced anaemic and normal rats is represented on Table 4.8. There was a significant ($P<0.05$) decrease in WBC, RBC, MCV, and MCH and no significant ($P>0.05$) change in MCHC of anaemiccontrol group compared to the normal control group. There was a significant ($P<0.05$) increase in WBC and no significant

(P>0.05) change in MCV, MCH and MCHC of anaemic treated groups compared to the normal control group. The RBC of anaemicrats treated with Vitamin B_{12} and anaemicgroups treated with extract (50 and 100mg/Kg) were significantly (P<0.05) lower compared to the normal control group. There was no significant (P>0.05) difference in RBC of anaemic group treated with 200mg/Kg, compared with the normal control group.

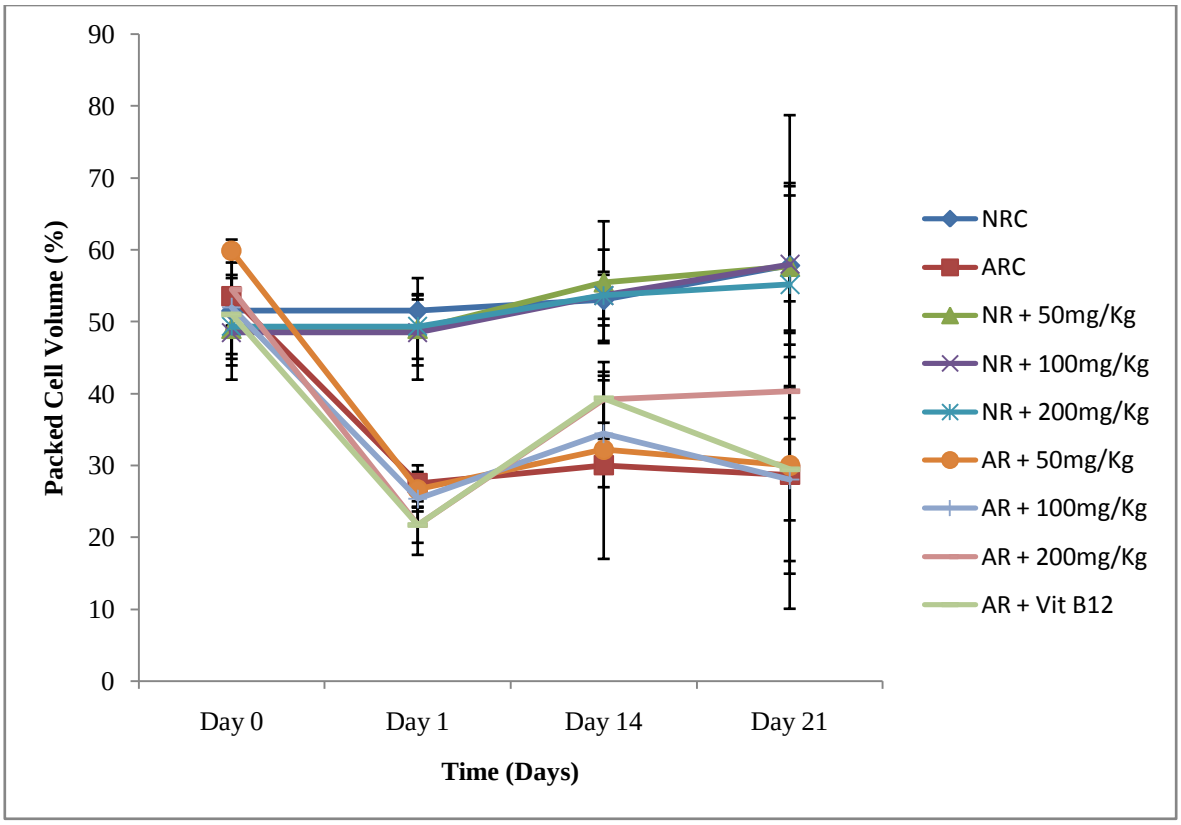


Figure 4.2: Effect of Methanol Extract of *Gnetum africanum*Leaves on Packed Cell Volume (PCV) of Phenylhydrazine-Induced Anaemic and NormalAlbino Rats.

Values are means ± SD. NRC: Normal Rats Control, NR+50mg/Kg: Normal rats + Extract (50mg/kg), NR+100mg/Kg: Normal rats + Extract (100mg/kg), NR+200mg/Kg: Normal rats + Extract (200mg/kg), ARC: Anaemic Rats Control, AR+50mg/Kg: Anaemic rats + Extract (50mg/kg), AR+100mg/Kg: Anaemic rats + Extract (100mg/kg), AR+200mg/Kg: Anaemic rats + Extract (200mg/kg), AR+Vit B12: Anaemic rats + Standard drug (Vitamin B_{12}).

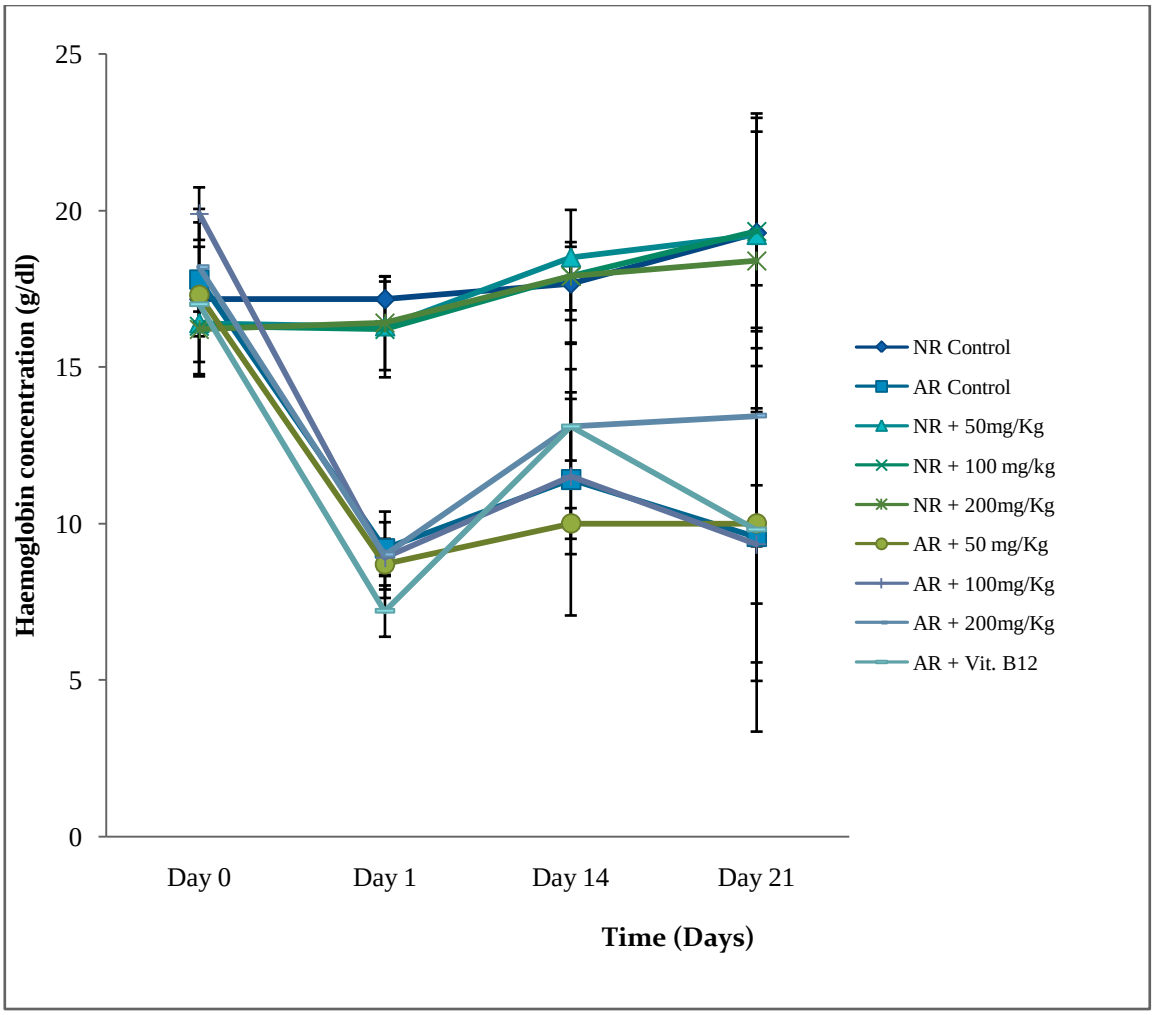


Figure 4.3: Effect of methanol Extract of *Gnetum africanum*Leaves on Haemoglobin concentration of Phenylhydrazine-induced anaemic and normalAlbino Rats.

Values are means ± SD. NRC: Normal Rats Control, NR+50mg/Kg: Normal rats + Extract (50mg/kg), NR+100mg/Kg: Normal rats + Extract (100mg/kg), NR+200mg/Kg: Normal rats + Extract (200mg/kg), ARC: Anaemic Rats Control, AR+50mg/Kg: Anaemic rats + Extract (50mg/kg), AR+100mg/Kg: Anaemic rats + Extract (100mg/kg), AR+200mg/Kg: Anaemic rats + Extract (200mg/kg), AR+Vit B12: Anaemic rats + Standard drug (Vitamin B_{12}).

Table 4.8: Effect of Methanol Extract of *Gnetum africanum* Leaves on Some Haematological Parameters of Phenylhydrazine-induced anaemic and normal Albino Rats.

Groups (n=6)	RBC count ($x10^6$µl)	WBC count ($x10^3$ µl)	HB conc. (g/dl)	PCV (%)	MCV (fl)	MCH (Pg)	MCHC (g/dl)
NRC	7.22 ± 0.40^{c}	7.14 ± 0.16^{b}	19.28 ± 3.68^{b}	57.83 ± 11.03^{b}	80.05 ± 14.44^{bc}	26.67 ± 4.79^{bc}	33.32 ± 0.05^{a}
ARC	6.15 ± 0.78^{ab}	3.38 ± 0.77^{a}	9.56 ± 4.00^{a}	28.68 ± 12.01^{a}	41.99 ± 17.77^{a}	14.03 ± 5.96^{a}	33.37 ± 0.15^{a}
NR+50mg/Kg	6.92 ± 0.41^{bc}	7.09 ± 0.25^{b}	19.22 ± 7.03^{b}	57.67 ± 21.08^{b}	82.63 ± 28.34^{c}	27.56 ± 9.44^{c}	33.37 ± 0.08^{a}
NR+100mg/Kg	7.25 ± 0.56^{c}	7.32 ± 0.86^{b}	19.33 ± 3.19^{b}	58.00 ± 9.57^{b}	80.58 ± 15.27^{bc}	26.84 ± 5.10^{bc}	33.30 ± 0.52^{a}
NR+200mg/Kg	7.32 ± 0.73^{c}	7.18 ± 0.26^{b}	18.39 ± 4.71^{b}	55.17 ± 14.13^{b}	76.05 ± 20.82^{bc}	25.34 ± 6.93^{bc}	33.37 ± 0.06^{a}
AR+50mg/Kg	6.13 ± 0.58^{ab}	8.30 ± 0.60^{c}	10.00 ± 5.02^{a}	30.00 ± 15.08^{a}	49.69 ± 24.63^{ab}	16.56 ± 8.20^{ab}	33.33 ± 0.17^{a}
AR+100mg/Kg	6.08 ± 0.64^{a}	8.50 ± 0.90^{c}	9.33 ± 1.89^{a}	28.00 ± 5.66^{a}	52.18 ± 6.86^{abc}	17.38 ± 2.27^{abc}	33.28 ± 0.11^{a}
AR+200mg/Kg	6.58 ± 0.76^{abc}	8.50 ± 0.71^{c}	13.44 ± 4.17^{ab}	40.33 ± 12.5^{ab}	66.51 ± 21.93^{abc}	22.15 ± 7.35^{abc}	33.29 ± 0.10^{a}
AR+Vit B_{12}	6.15 ± 0.26^{ab}	8.90 ± 0.42^{c}	9.80 ± 6.45^{a}	29.4 ± 19.36^{a}	48.14 ± 31.29^{ab}	16.05 ± 10.40^{ab}	33.36 ± 0.14^{a}

Values are means ± SD. Values with different superscript down the column are significantly ($P<0.05$) different. NRC: Normal Rats Control, NR+50mg/Kg: Normal rats + Extract (50mg/kg), NR+100mg/Kg: Normal rats + Extract (100mg/kg), NR+200mg/Kg: Normal rats + Extract (200mg/kg), ARC: Anaemic Rats Control, AR+50mg/Kg: Anaemic rats + Extract (50mg/kg), AR+100mg/Kg: Anaemic rats + Extract (100mg/kg), AR+200mg/Kg: Anaemic rats + Extract (200mg/kg), AR+Vit B12: Anaemic rats + Standard drug (Vitamin B_{12}).

RBC: Red Blood Cell, WBC: White Blood Cell, MCV: Mean Corpuscular Volume, MCH: Mean Corpuscular Haemoglobin, MCHC: Mean Corpuscular Haemoglobin Concentration

4.8 BIOCHEMICAL PARAMETERS.

4.8.1: Effects of Methanol Extract of *Gnetum africanum* Leaves on Liver Function Parameters

Effect of 21 days daily administration of methanol extract of *Gnetum africanum*leaveson liver function parameters (alanine amino transferase (ALT), aspartate amino transferase (AST), alkaline phosphatase (ALP), total protein (TP) and albumin (ALB) is presented in Table 4.9. There was a siginfiant ($P<0.05$) increase in ALT and no significant ($P>0.05$) change in the AST and ALP of anaemic control group compared to the normal control group. There was no significant ($P<0.05$) change in ALT, AST and ALP of all treated groups compared to the normal control group, with the exception of ALP for normal animals receiving 200mg/Kg and that of anaemic rats receiving 50mg/Kg which recorded a significant ($P<0.05$) increase. There was no significant ($P>0.05$) change in TP and ALB of the anaemic not treated group when compared to the anaemic treated and normal control groups. However, a significant ($P<0.05$) increase in ALB was recorded in the normal group receiving 100mg/Kg when compared with the normal control group.

4.8.2: Effects of Methanol Extract of *Gnetum africanum* Leaves on Serum Iron and Serum Bilirubins

The effect of daily administration of methanol extract of *Gnetum africanum* leaves on of serum iron and the bilirubins (total bilirubin, direct bilirubin and indirect bilirubin) in phenylhydrazine-induced anaemic and normal albino rats is summarised on Table 4.10. There was no significant ($P<0.05$) change in serum iron of anaemic not treated group compared to normal control rats and all treated groups. There was no significant ($P>0.05$)

change in the level of direct bilirubin and a significant ($P<0.05$) increase in total and indirect bilirubin of the anaemic not treated group compared to the normal control rats. Total bilirubin recorded no significant ($P>0.05$) change in anaemic treated groups compared to normal control rats, with the exception of anaemic rats receiving 50mg/Kg of extract where there was a significant ($P<0.05$) increase in total bilirubin. There was a significant ($P<0.05$) decrease in direct bilirubin of anaemic rats receiving 200mg/Kg and Vitamin B_{12} while no significant ($P>0.05$) change in direct bilirubin was recorded in the other anaemic treated groups when compared with the normal control rats. A significant ($P<0.05$) increase in indirect bilirubin of anaemic treated groups was recorded when compared with the normal control group, with the exception of anaemic group receiving 100mg/Kg where there was no significant ($P>0.05$) change in indirect bilirubin. Administration of extract did not significantly ($P>0.05$) change the level of total, direct, and indirect bilirubin in the normal rats compared to the normal control rats, with an exception in Direct bilirubin level of normal rats receiving 200mg/Kg which was significantly ($P<0.05$) increased.

Table 4.9: Effect of Methanol Extract of *Gnetum africanum* Leaves on Liver Function Parameters of Phenylhydrazine-Induced Anaemic and NormalAlbino Rats.

Group (n=6)	ALT (U/l)	AST(U/l)	ALP(U/l)	TP (mg/dl)	ALB (mg/dl)
NRC	57.44±13.90^{a}	108.70±21.47^{a}	468.05±63.18^{a}	9.84±1.36^{ab}	4.19±2.05^{a}
ARC	89.29±20.04^{b}	134.78±46.67^{a}	538.20±47.09^{ab}	14.50±2.10^{ab}	6.27±1.25^{ab}
NR+50 mg/Kg	66.07±32.06^{ab}	121.74±21.65^{a}	555.68±51.81^{ab}	8.17±1.52^{a}	4.15±0.94^{a}
NR+100 mg/Kg	65.18±35.37^{ab}	113.04±25.05^{a}	517.50±41.99^{ab}	9.50±0.57^{ab}	7.51±4.23^{b}
NR+200 mg/g	65.18±9.70^{ab}	94.21±18.78^{a}	663.09±133.82^{b}	10.48±3.05^{ab}	7.27±2.27^{b}
AR+50 mg/Kg	77.14±27.62^{ab}	126.09±14.02^{a}	650.67±170.35^{b}	8.94±11.27^{ab}	4.26±0.09^{a}
AR+100 mg/Kg	80.71±24.63^{ab}	130.43±37.40^{a}	518.65±40.95^{ab}	11.76±6.46^{ab}	5.05±1.08^{ab}
AR+200 mg/Kg	72.12±5.00^{ab}	123.91±38.77^{a}	535.67±71.46^{ab}	11.02±6.44^{ab}	5.11±0.80^{ab}
AR+Vit B_{12}	63.21±10.68^{ab}	117.39±26.96^{a}	439.53±17.94^{a}	16.13±2.98^{ab}	4.25±0.80^{ab}

Values are means ± SD. Values with different superscript down the column are significantly (P<0.05) different

NRC: Normal Rats Control, NR+50mg/Kg: Normal rats + Extract (50mg/kg), NR+100mg/Kg: Normal rats + Extract (100mg/kg), NR+200mg/Kg: Normal rats + Extract (200mg/kg), ARC: Anaemic Rats Control, AR+50mg/Kg: Anaemic rats + Extract (50mg/kg), AR+100mg/Kg: Anaemic rats + Extract (100mg/kg), AR+200mg/Kg: Anaemic rats + Extract (200mg/kg), AR+Vit B12: Anaemic rats + Standard drug (Vitamin B_{12}).

ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, ALP: Alkaline phosphatase, TP: Total protein, ALB:Album

Table 4.10: Effect of Methanol Extract of *Gnetum africanum* Leaves on Serum Iron and Bilirubins of Phenylhydrazine-Indced Anaemic and Normal Albino Rats.

Group (n=6)	Serum Iron (µmol/l)	Total Bilirubin (mg/ml)	Direct Bilirubin (mg/ml)	Indirect Bilirubin (mg/ml)
NRC	7.74 ± 9.44^{ab}	2.29 ± 0.07^{ab}	0.56 ± 0.29^{c}	1.73 ± 0.27^{a}
ARC	9.53 ± 1.60^{ab}	3.85 ± 2.08^{c}	0.52 ± 0.11^{c}	3.33 ± 2.08^{b}
NR+50mg/Kg	13.42 ± 15.91^{ab}	2.49 ± 0.43^{ab}	0.37 ± 0.10^{bc}	2.15 ± 0.56^{ab}
NR+100mg/Kg	3.56 ± 3.35^{ab}	2.16 ± 0.32^{a}	0.38 ± 0.39^{bc}	1.78 ± 0.50^{a}
NR+200mg/g	13.01 ± 11.06^{ab}	3.09 ± 0.48^{abc}	0.82 ± 0.04^{d}	2.26 ± 0.49^{ab}
AR+50mg/Kg	5.01 ± 2.14^{ab}	3.68 ± 0.97^{c}	0.54 ± 0.10^{c}	3.27 ± 1.08^{b}
AR+100mg/Kg	1.81 ± 1.41^{a}	3.31 ± 0.32^{abc}	0.51 ± 0.11^{c}	2.80 ± 0.32^{ab}
AR+200mg/Kg	11.00 ± 14.56^{b}	3.39 ± 0.48^{bc}	0.08 ± 0.04^{a}	3.19 ± 0.55^{b}
AR+Vit B_{12}	6.45 ± 7.91^{ab}	3.37 ± 0.76^{bc}	0.20 ± 0.06^{bc}	3.17 ± 0.78^{b}

Values are means ± SD. Values with different superscript down the column are significantly ($P<0.05$) different

NRC: Normal Rats Control, NR+50mg/Kg: Normal rats + Extract (50mg/kg), NR+100mg/Kg: Normal rats + Extract (100mg/kg), NR+200mg/Kg: Normal rats + Extract (200mg/kg), ARC: Anaemic Rats Control, AR+50mg/Kg: Anaemic rats + Extract (50mg/kg), AR+100mg/Kg: Anaemic rats + Extract (100mg/kg), AR+200mg/Kg: Anaemic rats + Extract (200mg/kg), AR+Vit B12: Anaemic rats + Standard drug (Vitamin B_{12}).

4.9: IN VIVO ANTIOXIDANT POTENTIALS OF METHANOL EXTRACT OF *Gnetum africanum*LEAVES

The effect of methanol extract of *Gnetum africanum* leaves and Vitamin B_{12} on lipid peroxidation and endogenous antioxidant enzymes of the liver and kidney of phenylhydrazine-induced anaemic and normal albino rats is presented in Table 4.11. In the liver, there was no significant ($P>0.05$) change in TBARS, SOD and CAT values in the liver of the anaemic control group compared to the anaemic treated and normal control groups. There was equally no significant ($P>0.05$) change in the levels of TBARS, SOD and CAT in the liver of normal group receiving the extract compared to the normal control group with the exception of SOD value for group receiving 50mg/Kg where there was a significant ($P<0.05$) decrease.

In the kidney, there was no significant ($P>0.05$) change in the TBARS,SOD and CAT of anaemic control group compared to the normal control group and anaemic treated groups. There was a significant ($P<0.05$) decrease in CAT of anaemic group receiving standard drug and no significant ($P>0.05$) change in CAT of anaemic groups treated with extractscompared to the normal control group. There was no significant ($P>0.05$) change in the levels of TBARS, SOD and CAT of groups of normal rats receiving extract compared to the normal control group.

4.10: EFFECTS OF METHANOL EXTRACT OF *Gnetum africanum*LEAVES ON BODY AND RELATIVE ORGAN WEIGHTS

The effect of daily administration of methanol extract of *Gnetum africanum*leaveson body weight changes and relative organ weights of phz-induced anaemic and normalalbino rats is summarised in Table 4.12. There was no significant change in percentage relative body weight of anaemic control group compared to the normal control and anaemic treated animals. However there was a non significant ($P>0.05$) decrease in body weight of normal animals receiving 50mg/kg compared to the normal control group.

The anaemic control group recorded no significant ($P>0.05$) increase in percentage relative weight of liver and kidneys when compared to the normal control group. There was a significant ($P<0.05$) increase in percentage relative weight of liver of anaemic animals receiving 100mg/Kg and in the weight of kidneys of anaemic rats receiving Vitamin B_{12} and anaemic rats receiving 200mg/Kg when compared with normal control group.

Table 4.11: Effect of Methanol Extract of *Gnetum africanum* Leaves on Lipid Peroxidation and Endogenous Antioxidant Enzymes in the Liver and Kidney of Phenylhydrazine-Induced Anaemic and NormalAlbino Rats.

Group (n=6)	Liver			Kidney		
	TBARS (mmol/g of tissue)	SOD (mmol/min/g of tissue)	CATALASE (moles of H_2O_2/min/g of tissue)	TBARS (mmol/g of tissue)	SOD (mmol/min/g of tissue)	CATALASE (moles of H_2O_2/min/g of tissue)
NRC	0.19 ± 0.04^{ab}	1.75 ± 0.15^{b}	0.13 ± 0.04^{a}	0.84 ± 0.42^{ab}	1.81 ± 0.14^{ab}	0.14 ± 0.03^{bc}
ARC	0.21 ± 0.11^{ab}	1.66 ± 0.25^{ab}	0.15 ± 0.02^{a}	0.69 ± 0.17^{a}	1.74 ± 0.04^{ab}	0.13 ± 0.01^{abc}
NR+50mg/Kg	0.26 ± 0.09^{b}	1.27 ± 0.05^{a}	0.17 ± 0.03^{a}	1.05 ± 0.05^{b}	1.70 ± 0.02^{ab}	0.15 ± 0.02^{bc}
NR+100mg/Kg	0.20 ± 0.06^{ab}	1.76 ± 0.28^{b}	0.18 ± 0.05^{a}	0.60 ± 0.64^{a}	1.78 ± 0.02^{ab}	0.14 ± 0.04^{abc}
NR+200mg/g	0.24 ± 0.08^{ab}	1.47 ± 0.63^{ab}	0.19 ± 0.04^{a}	1.10 ± 0.46^{ab}	1.64 ± 0.37^{ab}	0.16 ± 0.02^{c}
AR+50mg/Kg	0.19 ± 0.08^{ab}	1.77 ± 0.11^{b}	0.13 ± 0.03^{a}	0.83 ± 0.38^{ab}	1.99 ± 0.48^{b}	0.11 ± 0.01^{ab}
AR+100mg/Kg	0.14 ± 0.05^{ab}	1.80 ± 0.12^{b}	0.14 ± 0.05^{a}	0.54 ± 0.47^{a}	1.97 ± 0.50^{ab}	0.15 ± 0.03^{bc}
AR+200mg/Kg	0.11 ± 0.02^{a}	1.74 ± 0.13^{b}	0.15 ± 0.05^{a}	0.59 ± 0.30^{a}	1.78 ± 0.23^{ab}	0.13 ± 0.03^{abc}
AR+Vit B_{12}	0.27 ± 0.13^{b}	1.51 ± 0.07^{ab}	0.16 ± 0.03^{a}	1.03 ± 0.51^{ab}	1.59 ± 0.23^{ab}	0.09 ± 0.16^{a}

Values are means ± SD. Values with different superscript down the column are significantly (P<0.05) different

NRC: Normal Rats Control, NR+50mg/Kg: Normal rats + Extract (50mg/kg), NR+100mg/Kg: Normal rats + Extract (100mg/kg), NR+200mg/Kg: Normal rats + Extract (200mg/kg), ARC: Anaemic Rats Control, AR+50mg/Kg: Anaemic rats + Extract (50mg/kg), AR+100mg/Kg: Anaemic rats + Extract (100mg/kg), AR+200mg/Kg: Anaemic rats + Extract (200mg/kg), AR+Vit B12: Anaemic rats + Standard drug (Vitamin B_{12}).

TBARS: Thiobarbeturic Acid Reactive Substances, SOD: Super Oxide Dismutase

Table 4.12: Effect of Methanol Extract of *Gnetum africanum*Leaves on Body and Organ Weights of of Phenylhydrazine-Induced Anaemic and NormalAlbino Rats.

Group (n=6)	Mean Final Body weight (g)	Mean initial Body weight (g)	% Relative change in body weight	% Relative liver weight	% Relative Kidney weight
NRC	152.33±30.00^{a}	136.67±8.59^{a}	11.64±10.10^{ab}	2.80±0.54^{a}	0.53±0.12^{a}
ARC	228.00±52.18^{b}	214.40±49.60^{b}	6.51±4.53^{ab}	3.04±1.01^{ab}	0.61±0.19^{ab}
NR+50mg/Kg	127.50±19.95^{a}	134.67±19.27^{a}	-5.28±5.82^{a}	2.94±0.16^{a}	0.59±0.02^{ab}
NR+100mg/Kg	135.83±17.31^{a}	125.33±11.66^{a}	8.13±4.72^{ab}	3.16±0.46^{ab}	0.68±0.16^{ab}
NR+200mg/g	136.50±19.20^{a}	126.50±21.89^{a}	8.40±3.92^{ab}	2.72±0.20^{a}	0.63±0.08^{ab}
AR+50mg/Kg	128.60±16.44^{a}	115.80±13.14^{a}	10.96±4.18^{ab}	3.30±0.32^{ab}	0.68±0.08^{ab}
AR+100mg/Kg	142.40±28.31^{a}	123.40±22.96^{a}	18.35±32.29^{b}	3.69±0.54^{b}	0.68±0.08^{ab}
AR+200mg/Kg	123.00±17.32^{a}	116.00±2.00^{a}	5.91±13.51^{ab}	3.29±0.40^{ab}	0.71±0.05^{b}
AR+Vit B_{12}	127.00±16.37^{a}	114.60±15.08^{a}	11.03±7.11^{ab}	3.22±0.23^{ab}	0.72±0.11^{b}

Values are means ± SD. Values with different superscript down the column are significantly ($P<0.05$) different

NRC: Normal Rats Control, NR+50mg/Kg: Normal rats + Extract (50mg/kg), NR+100mg/Kg: Normal rats + Extract (100mg/kg), NR+200mg/Kg: Normal rats + Extract (200mg/kg), ARC: Anaemic Rats Control, AR+50mg/Kg: Anaemic rats + Extract (50mg/kg), AR+100mg/Kg: Anaemic rats + Extract (100mg/kg), AR+200mg/Kg: Anaemic rats + Extract (200mg/kg), AR+Vit B12: Anaemic rats + Standard drug (Vitamin B_{12}).

CHAPTER 5

5.0 DISCUSSION

Herbs are readily available to humans and have been explored to the maximum for their medicinal properties. Various parts of plants like roots, leaves, bark, exudates etc are used as per medicinal properties (Perumal and Gupala, 2007). This is primarily because of the general belief that herbal drugs are without any side effects, besides being cheap and locally available (Achinewu andAniena, 1995). Phytochemicals are a large group of plant-derived compounds hypothesised to be responsible for much of the disease protection conferred from diets high in fruits, vegetables, beans, cereals, and plant-based beverages such as tea and wine (Arts and Hollman, 2005). Phytochemicals in plants include carbohydrates, steroids, triterpenes, cardiac glycosides, saponins, flavonoids, tannins, free anthraquinones, alkaloids, etc.

The qualitative phytochemical analysis of aqueous, methanol, ethanol and acetone extracts of *Gnetum africanum* revealed the presence of carbohydrates, saponins, steroids, tannins, flavonoids and alkaloids in all four extracts, with flavonoids, tannins and steroids existing in trace amount in some extracts. However, cardiac glycosides was absent in acetone extract and present in the rest while triterpenes were present in only the methanol extract (Table 4.3). This result indicates the presence of a variety of phytochemicals in the extracts. Each of these phytochemicals is known for various protective and therapeutic effects (Asaolu *et al*., 2009). Quantitative analysis of some phytochemical in leaves of *Gnetum africanum* (Table 4.2) revealed glycosides to be the most abundant phytochemical while the phenolics, tannins and flavonoids were least abundant.Flavonoids are known to

possess antibacterial, anti-inflammatory, anti-allergic, antiviral and anti neoplastic activities (Alli, 2009). They have anti-oxidation effects in animals (Enwere, 1998). Stereoidal compounds are of importance due to their relationship with some compounds such as sex hormones (Okwu, 2001). Steroids, glycosides, terpenoids and alkaloids have been reported to exert inhibiting activity against most bacteria (Camacho-Corona *et al*., 2008; Al-Bayati and Sulaiman, 2009). Terpenoids and steroids possess antibacterial and antineoplastic properties (Oduro *et al*., 2009). Saponin has expectorant action, which is very useful in the management of upper respiratory tract inflammation. Saponin is also reported to have antidiabetic properties (Abdulrahman *et al*, 2010). The presence of these secondary metabolites in the leaves is important as these compounds confer biological activities to the plants (Corthout and Kotra, 1995). These phytochemicals add up to to the high nutritive value of the plant to probably account for the medicinal value of these edible vegetable leaves.

The best health and nutrition results can be achieved not only from the consumption of fruits and vegetables with high antioxidant capacities, but also from medicinal herbs and plants (Jastrzebski *et al*., 2007). Phenolic compounds have been reported to be a major contributor to the antioxidant activity of medicinal plants (Dragland *et al*., 2003; Cai *et al*., 2004). Phenolic compounds are plant metabolites characterized by the presence of several phenol groups, some of them are very reactive in neutralizing free radicals by donating a hydrogen atom or an electron, chelating metal ions in aqueous solutions (Petti and Scully, 2009). The existence of several hydroxyl groups bonded to an aromatic ring provides the polyphenol molecule with the ability of donating a proton to a radical, so acting as a possible chain-breaking molecule or antioxidant upon secondary

oxidation (Franco *et al*., 2008). Nevertheless, low levels of the phenolics, also reflected in low levels of flavonoids and tannins in the leaves and in most extracts are indicators of possible low activity of these extracts against oxidative stress. Solubility of polyphenols depends mainly on the hydroxyl groups and the molecular size and the length of hydrocarbon (Franco*et al*., 2008). The extraction yield and the antioxidant activity of the extracts from plants highly depend on the solvent polarity, which determines both qualitatively and quantitatively the extracted antioxidant compounds. The highest yields are usually achieved with ethanol and methanol and their mixtures with water, although other solvents have been widely used in the extraction of polyphenols from plants, as ethyl acetate or acetone (Franco *et al*., 2008).

1,1-diphenyl 2-picryl hydrazyl (DPPH) radical scavenging activity is considered to be primary antioxidant activity (Norshazila *et al.*, 2010), because it is one of the most effective methods for evaluating the concentration of radical-scavenging materials actively by a chain-breaking mechanism (Maisuthisakul *et al*., 2005) and is a model for a lipophilic radical. DPPH is a stable free radical at room temperature and accepts an electron or hydrogen radical to become a stable diamagnetic molecule (Soares *et al.*, 1997). The reduction capability of DPPH was determined by the decrease in its absorbance at 520 nm, which is induced by antioxidants. Results were reported as IC_{50}, which is defined as the amount of antioxidant required to inhibit 50% of DPPH free radicals under the experimental conditions (Antolovich *et al*., 2002; Nanasombat and Teckchuen, 2009). The extract that required the lowest concentration to positive DPPH test suggests that the sample was a free radical scavenger. A higher DPPH radical-scavenging activity is associated with a lower IC_{50} value (Pattanayak *et al.*, 2012).

Therefore, the aqueous extract had the least DPPH IC_{50}, followed closely by ethanol, then acetone and lastly, methanol extract had the greatest IC_{50} (Table 4.5). Methanol extract equally had the least level of total polyphenol while the aqueous extract had the greatest level (Table 4.4). The difference in IC_{50} values of extracts maybe due to differences in polyphenol content in the extracts (Table 4.4) and low quantity of flavonoids in the leaves of *Gnetum africanum* (Table 4.4). This result supports the claim that the phytochemicals which might be responsible for the scavenging activity in these extracts are phenolics, notably flavonoid constituent(Rohman *et al*., 2010; Masoumeh *et al*., 2011). Flavonoids are one of the classes of plant polyphenol with structural requirements of free radical scavengers (Jayathilakan *et al*., 2007). Plant phenolics constitute one of the major groups of compounds acting as primary antioxidants or free radical terminators (El-Sayed *et al*., 2012). Low levels of flavonoids in the leaves could therefore be responsible for poor radical scavenging activities of the extracts, with IC_{50} values ranging between 0.355 for aqueous and 0.571 for methanol (Table 4.5).

In anaemia there is decreased level of circulating haemoglobin, less than 13 g/dl in male and 12 g/dl in females (Okochi *et al*., 2004). Phz decreases haemoglobin level, red blood cell concentration, and packed cell volume, and impairs erythrocyte deformability. It induces reticulocytosis, alters osmotic resistance, free plasma haemoglobin, mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) and Erythropoietin levels, and extramedular haematopoiesis in the spleen and liver (Berger, 2007). The determination of haematological indices provides physiological information on a proper blood assessment. According to Okonkwo *et al. (*2004), accurate laboratory determination of blood parameters remains the only sensitive

and reliable foundation for ethical and rational research, diagnosis, treatment and prevention of anaemia. Researchers have shown that ingestion of medicinal compounds or drugs can alter the normal range of haematological parameters (Ajagbonna *et al*., 2001). The major concern of the scientific communities with regard to medicinal plants and haematological studies focuses on the measures that can maintain a normal haematological state of being and reverse any negative haematological status associated with various anaemic conditions (Alexander and Griffiths, 1993).

Intraperitoneal administration of phz generated a significant ($P<0.05$) decrease in PCV, Hb, WBC, RBC, MCV and MCH. This is in line with the findings of Turaskar*et al.* (2013), who carried out a research in which PHZ altered the function of RBC by haemolysis characterized by 74.06% decrease in RBC, 48.17% decrease in Hb concentration and 41.68% decrease in PCV. It has been demonstrated that intraperitoneal administration of phenylhydrazine decreased Hb concentration, Red Blood Cells number and haematocrit (O'Riordan *et al*., 1995; Criswell *et al*., 2000). Also Agbor *et al*. (2005) demonstrated that oral administration of 10 mg/kg phenylhydrazine for 8 days reduced haematological indices by 50%. The significant ($P<0.05$) decrease in RBC of anaemic treated animals compared to the normal control after the 21st day of administration may be an indication of an imbalance between the rate of production (erythropoiesis) and destruction of the blood corpuscles in favour of the destruction caused by phz. This significant ($P<0.05$) reduction in PCV, Hb and RBC confirms induction of haemolytic anaemic using phz. Decrease in PCV, Hb and RBC causes a corresponding decrease in MCV and MCH but not MCHC. Orally administered extracts (aqueous, 70% methanol, 70% ethanol and 70% acetone) to anaemic animals demonstrated haematopoietic potentials of all extracts within 7 days as

PCV of treated animals were raised beyond the threshold for anaemia (36%) whereas the PCV of the anaemic rat control group was below this threshold. Methanol extract which generated a 28% increment in PCV and possessed little observable toxic effects was used for *in vivo* studies in which dose-dependent effect of the extract on haematological parameters was investigated. Haematopoietic effect of methanol extract of *Gnetum africanum* leaves and Vitamin $B_{12}$was demonstrated by significant ($P<0.05$) increase in PCV and Hb concentration of phz-induced anaemic rats receiving 200mg/kg and Vitamin B_{12} compared to the anaemic control group on day 14 of administration of extract.A 28% increament in PCV after administration of 200mg/Kg of the extract to anaemic ratsfor 7 days also illustrates haematopoietic effect of the extract (Table 4.6). The non significant ($P>0.05$) change in MCV and MCH in anaemic treated groups coupled with significant ($P<0.05$) decrease in anaemic control rats compared to the normal control rats after 21 days of administration of the extract further demonstrates the haematopoietic effects of the extract.Nubila *et al.* (2013) documented that haematopoietic effect of the extract may be attributed to the mineral and vitamin content of *G. africanum* which include haematinic factors such as iron.Iron plays an important role in the synthesis of haemoglobin. These well-known haemopoietic factors have direct influence on the production of blood in the bone marrow (Akah *et al.*, 2008). Phytochemicals in the extracts demonstrated haematopoietic potentials. Reinduction of animals (on day 14 and 19) resulted in non-significant ($P>0.05$) elevation of PCV, Hb and RBC of the anaemic treated rats compared to the normal control rats after 21 days administration of the extract. This decrease in PCV, Hb and RBC concentration is probably a consequence of poor antioxidant potential of the extract at doses less than 200mg/Kg body weight. Antioxidant phytochemicals are known

to protect erythrocytes and reduce their destruction (Duthie *et al.*, 1996). Since phz induces haemolysis through free radical mechanism, its action on erythrocytes will be halted by antioxidant phytochemicals. The low levels of polyphenols, notably flavonoids and tannins in the leaves probably spawned a low antioxidant potential of the extract which probably accounted for inability of the extract to prevent re-induction of anaemia using phz after day 14 in anaemic groups receiving 50mg/Kg and 100mg/kg and group receiving Vitamin B_{12}. Most phytochemical constituents of plant foods affect the immune system and other haematological parameters (Kubena and McMurray, 1996). In this study, the significant ($P<0.05$) decrease in WBC count of anaemic control group compared to the normal control animals has been reported (Turaskar*et al.*, 2013). Oral administration of 10 mg/kg phenylhydrazine for 8 days caused a 55.24% decrease in WBC, an index for immune function. The significant increase ($P<0.05$) in WBC in the anaemic treated groups demonstrates the ability of the extract in fortification of immune system thereby qualifying the antimicrobial activity of the plant (Ali *et al*., 2011; Enyi-Idoh *et al*., 2013). This findings are similar to those of Iweala and Osundiya, (2010) in which the level of Hb and WBC are significantly ($P<0.05$) increased with the consumption of *Gnetum africanum*.

Studies with medicinal plant extracts have shown the varying effects of xenobiotics like phytochemicals on serum and liver enzyme activities. There are many enzymes found in the serum that did not originally originate from the serum, during tissue damage, some of these enzymes find their way into the serum, probably by leakage (Will's, 1985). Serum enzyme measurements are therefore a valuable tool in clinical diagnosis, providing information on the effect and nature of pathological damage to any tissue. Generally, hepatic injury is often associated with alterations in the serum and liver activities of some

enzymes notably alanine amino transferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase(ALP) (Antai *et al*, 2009) because hepatic damage leads to the leakage of the enzymes from the tissues to the serum (Akanji and Yakubu, 2000 ; Appidi *et al*., 2009).Alanine and aspartate amino transaminases are considered to be sensitive indicators of hepatocellular damage and within limit can provide a quantitative evaluation of the degree of damage to the liver (Al-Shoaibi *et al.*, 2002). ALT and AST have been established as markers of hepatocellular injury while ALP is a marker of cholestasis (John *et al*., 2012).

The significant ($P<0.05$) increase in ALT activity and non significant ($P>0.05$) increase in AST in serum of anaemic control animals compared to the normal control may be due to leakage from hepatocytes through peroxidative damage of their membranes (Iniaghe*et al*., 2008) leading to increased membrane permeability. The rise in levels of ALT is alwaysaccompanied by elevation in the level of AST, which play a role in the conversion of amino acid to keto acid (James *et al*., 2010). The significant increase in ALT provides a biochemical evidence of significant liver damage, caused by Phz (John et al, 2012).

Serum albumin and total protein are some of the liver function parameters while albumin transports bilirubin and other substances in blood (Vasudevan and Sreekumari, 2007). The total proteins include albumin and globulins. Test for total protein in blood is often done to diagnose nutritional problems, kidney disease or liver disease. Albumin which is produced only in the liver, is the major plasma protein that circulates in the blood stream. It helps move many small molecules through the blood, including bilirubin, calcium, progesterone, and medications. ALB also plays an important role in keeping the fluid from the blood from leaking out into the tissues. Increased albumin content in the normal animals

receiving 100mg/Kg compared to the normal control animal may be due to high protein content of their diet and/or extract. The significant (P>0.05) increase in the level of albumin may indicate that the synthetic function of the liver has not been significantly affected yet, since albumin is synthesized only in the liver.

Bilirubin is the main bile pigment that is formed from the breakdown of haem in the red blood cells. It is transported to the liver where it is secreted by the liver into the bile. Conjugation of bilirubin is a prerequisite for its excretion into the bile (Cox *et al*, 2005). The bilirubin formed from breakdown of red blood cells in the reticulo endothelial cells are transported in plasma bound to albumin (Vasudevan and Sreekumari, 2007), so the increase in bilirubin level of phz-induced animals, may be as a result of increase in the oxidative break down of red blood cells by phenylhrdrazine.

Nutritional oxidative stress describes an inbalance between the pro-oxidant load and the antioxidant defense system in the body (Sies *et al*., 2005). In the state of oxidative stress, there is a characteristic depression of the free radical defense mechanism including alterations in the activities of antioxidant enzymes, essential polyunsaturated fatty acids, vitamins and minerals elements are compromised, exposing the body's own antioxidant defense system to damage by ROS (Omoregie and Osagie, 2011). Malondialdehyde is the major oxidation product of peroxidized PUFAs and increased MDA level is an important indicator of lipid peroxidation. Catalase on the other hand, is an enzymatic antioxidant widely distributed in all animal tissues including the red blood cell and liver. Catalase decomposes H_2O_2 and helps protect the tissues from highly reactive hydroxyl radicals. SOD, another antioxidant enzyme, removes superoxide radical by converting it to H_2O_2(Krishnaraju *et al*., 2009). Natural polyphenols from plant vegetables have been

found to exert their beneficial effect by removing free radicals, chelating metal catalyst, activating antioxidant enzymes, etc (Atrooz *et al*., 2009; Oboh *et al*., 2009).

Phz had no signs of lipid peroxidation, neither does it have effects on endogenous antioxidant enzymes of liver and kidneys of rats. This is demonstrated by non-significant ($P>0.05$) changes in TBARS, SOD and CAT of anaemic control group compared to the normal control group (Table 4.11). Phenylhydrazine may however mask effects in the liver and kidney just as in human and rats erythrocytes where PHZ generates ROS within; where no evidence for lipid peroxidation or phosphatidyl serine externalisation was detected (deJong *et al*., 1997; McMillan *et al*., 2005). Neither the extract nor vitamin B_{12} had a significant ($P<0.05$) effect on antioxidant enzymes in the liver and kidneys. This is illustrated by a non-significant ($P<0.05$) changes in TBARS, SOD and CAT in extract treated groups compared to the normal control group (Tables 4.11).

Bodyweight is normally investigated as a sensitive indicator of chemically induced changes to organs. The comparison between the ratiometric differences of organ to body weight of the control group and the treated groups has been used toevaluate the toxic effect of the test substance (Peters and Boyd, 1966).

There was no significant ($P>0.05$) change of body weight and relative liver and kidney weightsof normal treated animals compared to the normal control group. This may be because the dose has not accumulatedsignificantly to manifest any significant change (Peters and Boyd, 1966). The non significant ($P>0.05$) difference in the changes in body weight of the all anaemic groups compared to the normal control group possibly indicates the body's ability to reverse the effects of haemolytic anaemia on body weight.

CHAPTER 6

6.0 SUMMARY, CONCLUSION AND RECOMMENDATIONS

6. 1: SUMMARY

From the present study, glycosides (0.95±0.03) were the most abundant phytochemical on the leaves of *Gnetum africanum* as its level was significantly ($P<0.05$) higher than those of other phytochemicals. Thoughsaponin content (0.71±0.03) was significantly ($P<0.05$) higher than alkaloids (0.54±0.07), the two phytochemicals (saponins and alkaloids) alongside glycosides were significantly ($P<0.05$) higher than flavonoids (0.095 ±0.014) and tannins (0.03 ±0.008).

Total polyphenol content (measured in mg gallic acid/g of dry sample) of aqueous (0.45±0.05) and ethanol (0.38±0.07) extracts was significantly ($P<0.05$) higher than in methanol (0.17±0.03) and acetone (0.26±0.04) extracts

1,1-Diphenyl 2-picrylhydrazyl (DPPH) radical scavenging activity indicates that the aqueous extract had the lowest IC_{50} (0.355mg/ml), then ethanol (0.357mg/ml), acetone (0.565mg/ml) and methanol had the highiest (0.571mg/ml).

Methanol extract had the highest haematopoietic potential of all extract recording mean 28% increament in PCV within 7 days after administration of 200ml/Kg body weight with no death of rats recorded. This extract was used for in vivo assays.

Haematopoietic effect of methanol extract was demonstrated as a dose of 200mg/Kg of extract generated a non significant ($P>0.05$) change in PCV, Hb, RBC, MCV and MCH and MCHC and a significant ($P<0.05$) increase in WBC of anaemic rats compared to the normal control group.

There was a significant ($P<0.05$) incrase in the activity of ALT and a non significant increase in AST in the anaemic control animals compard to the normal control group. Treatment with methanol extract generated no significant ($P<0.05$) difference in AST, ALT and total protein of treated groups compared to the normal control. However, significant ($P<0.05$) increase was recorded in ALP activity of normal and anaemic rats receiving 200mg/kg and 50mg/Kg respectively and in ALB concentration of normal animals receiving 100 and 200mg/Kg of extract, when compared to the normal control group.This shows that the extract was relative safe to the liver.

The methanol extract did not significantly ($P>0.05$) change concentration of TBARS and activity of endogenous antioxidant enzymes (SOD and CAT) in the liver and kidneys of anaemic rats.

Administration of the methanol extract did not significant ($P<0.05$) change the body weight though relative liver and kidneys weight of anaemic rats were significantly ($P<0.05$) increased.

6.2 CONCLUSION

The aqueous, methanol, ethanol and acetone extracts of *G. africanum* possess *in-vitro* antioxidant potentials. The 70% methanol extract, which had the most potent haematological potentials, appears to have a stimulatory effect on haematopoiesis in a dose and time dependent pattern. The extract does not significantly ($P<0.05$) alter biochemical parameters as well as antioxidant system in the liver and kidneys of normal and anaemic albino rats. The extract is therefore relatively safe for consumption, and has positive effects on haematinic parameters.

6.3 RECOMMENDATIONS

1. A bioactivity-guided fractionation, isolation and identification of the constituents of the extracts responsible for the observed pharmacological activities in the extracts should be studied.

2. Chronic toxicity studies of this plant should be done so as to ascertain the safety of usage on various body organs.

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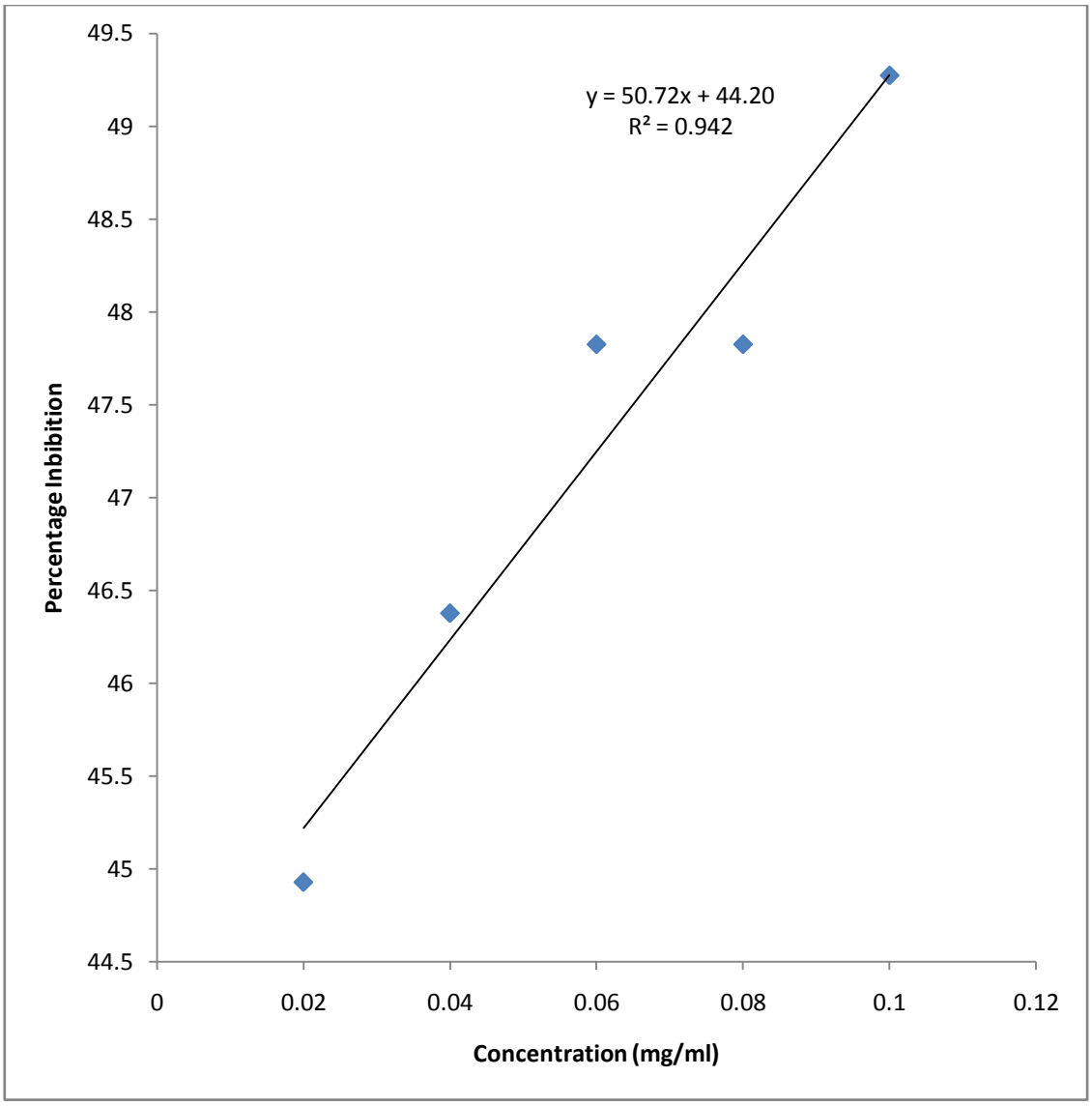
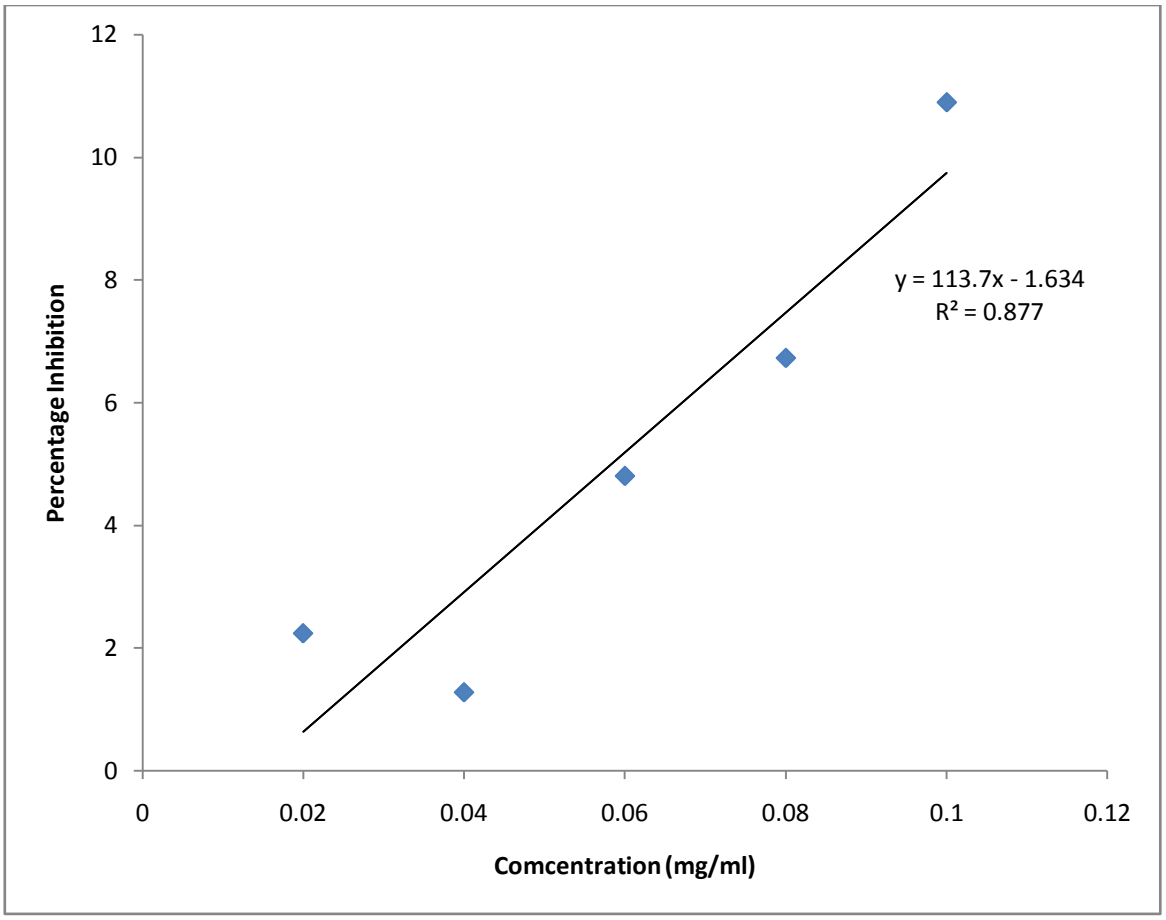
APPENDICES

Appendix 1



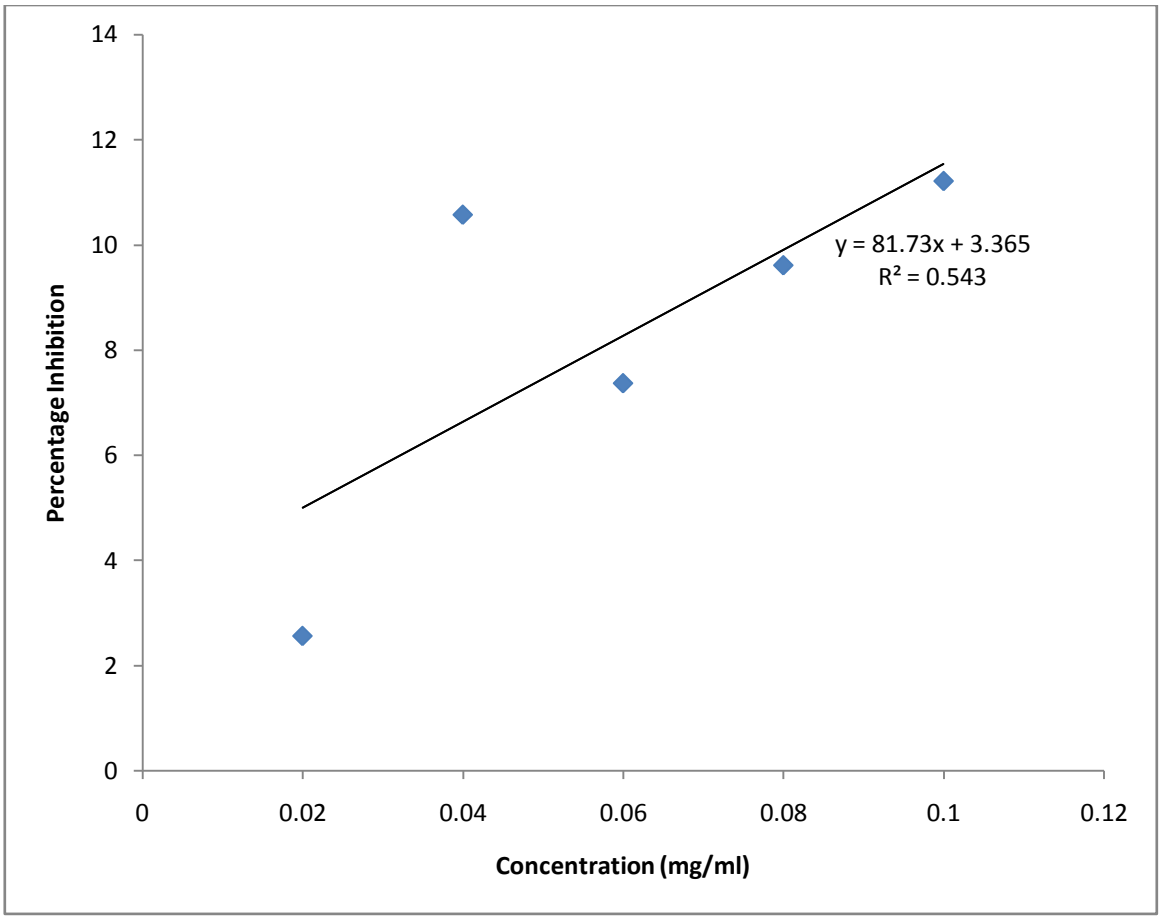
Plot of Percentage Inhibition of Free Radicals and Concentrations of Vitamin C

Appendix 2



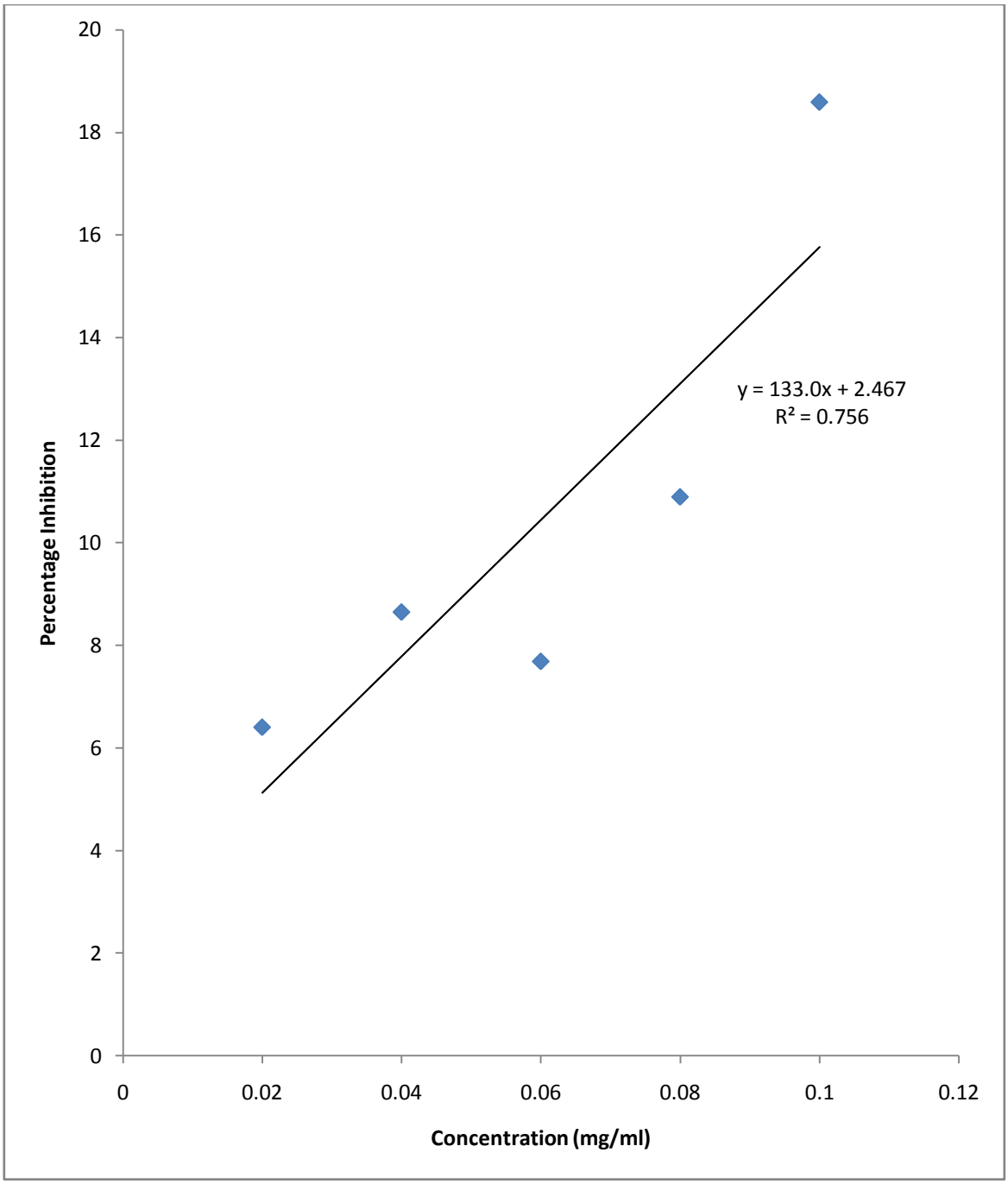
Plot of Percentage Inhibition of Free Radicals versus Concentrations of Aqueous Extract of *Gnetum africanum* Leaves

Appendix 3



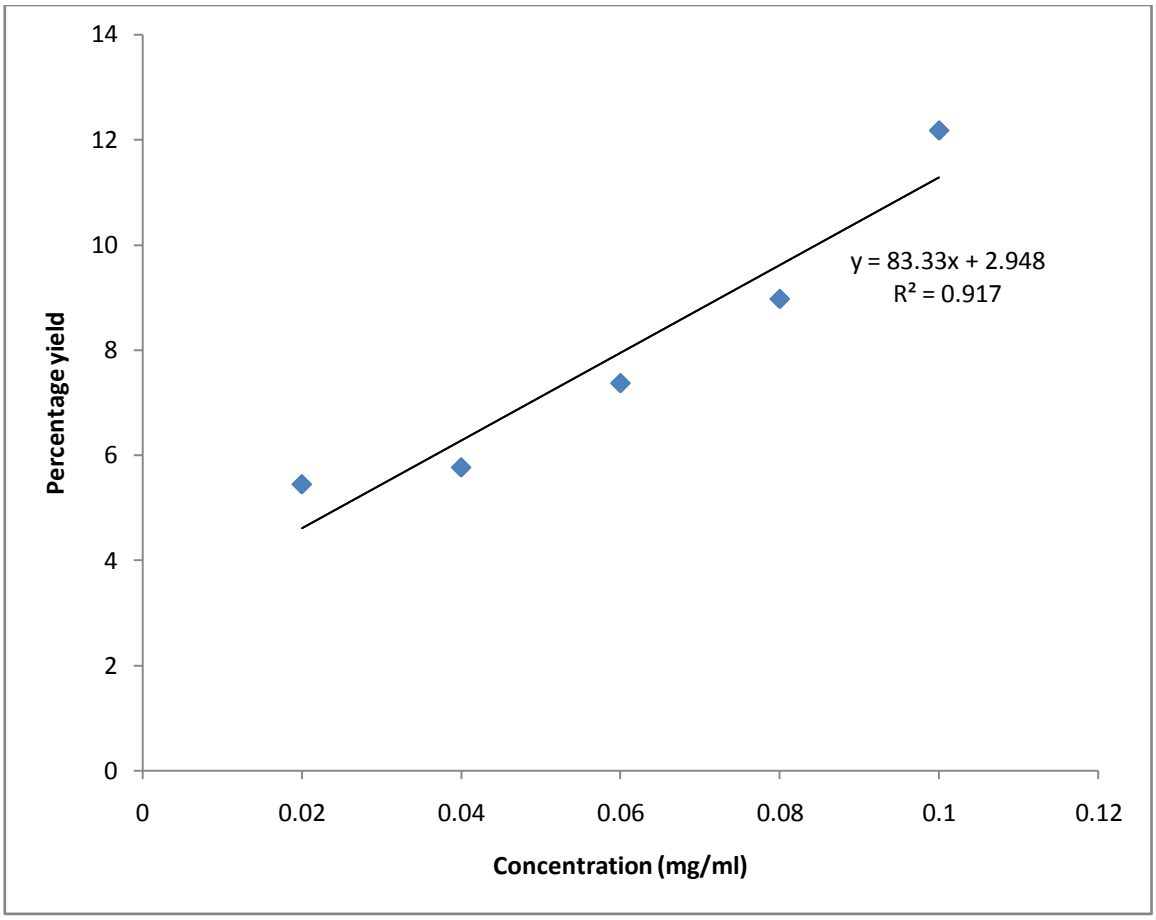
Plot of Percentage Inhibition of Free Radicals and Concentrations of Methanol Extract of *Gnetum africanum* Leaves

Appendix 4



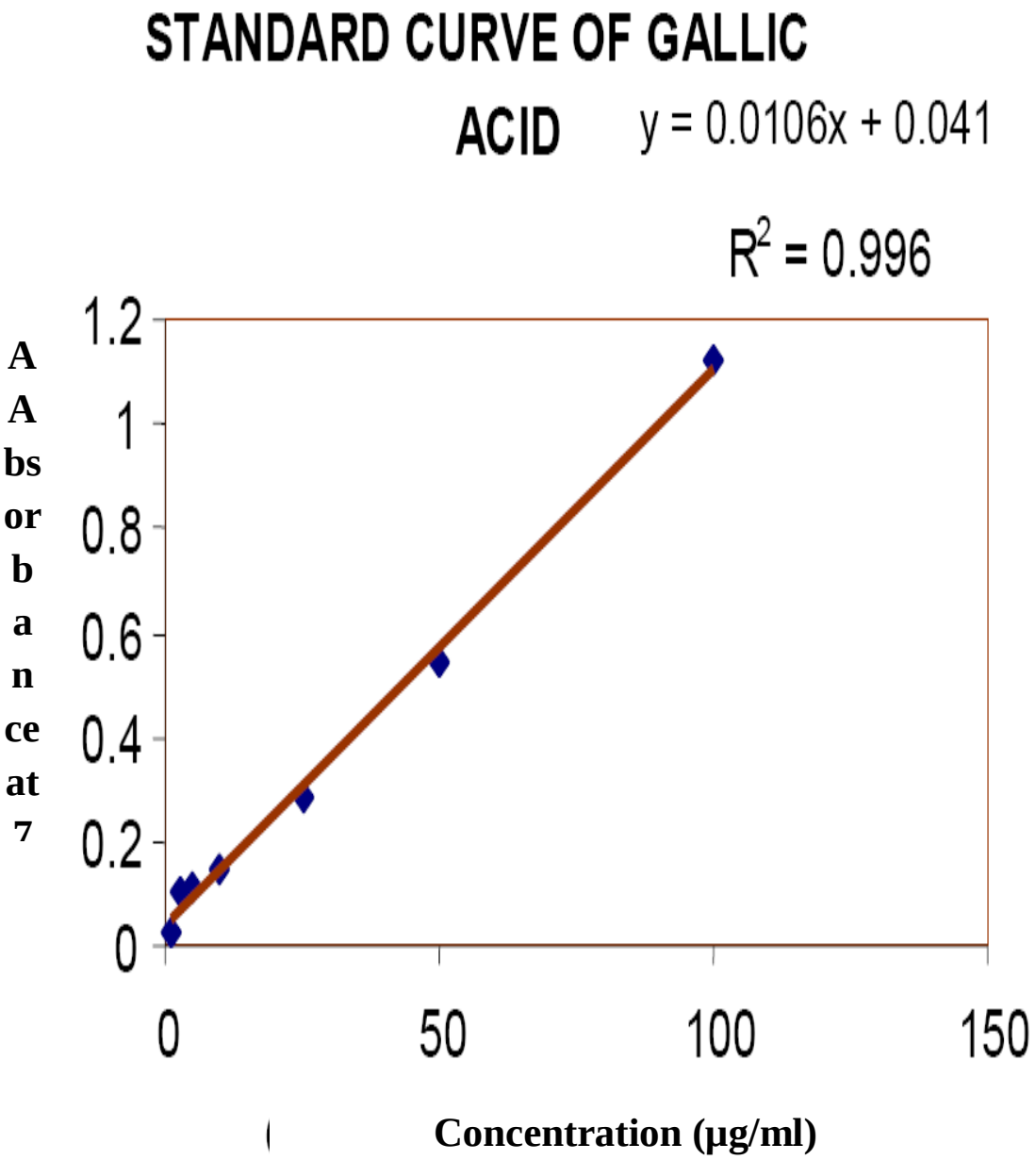
Plot of Percentage Inhibition of Free Radicals and Concentrations of Ethanol Extract of *Gnetum africanum* Leaves

Appendix 5

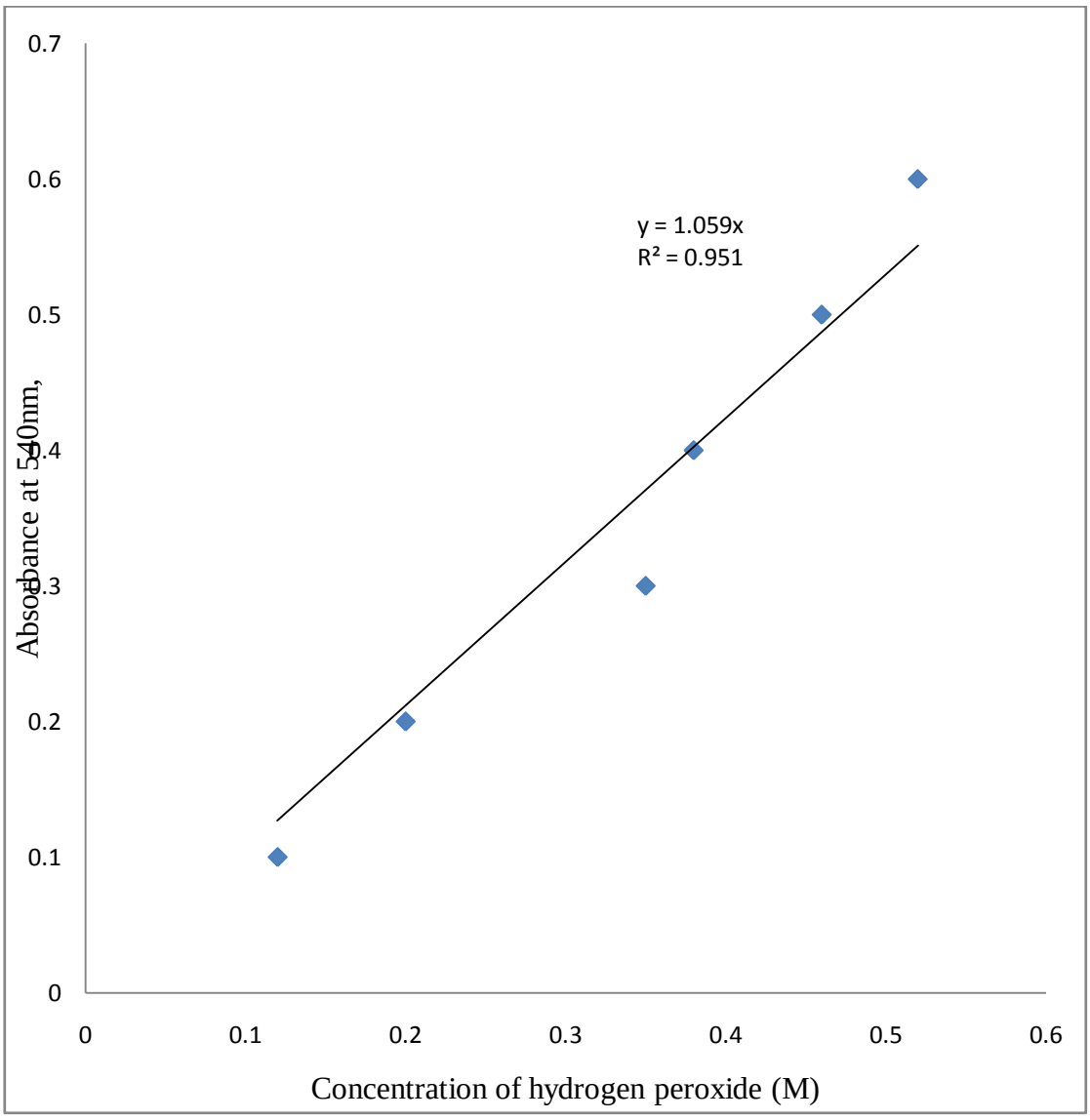


Plot of Percentage Inhibition of Free Radicals and Concentrations of Acetone Extract of *Gnetum africanum* Leaves

Appendix 6

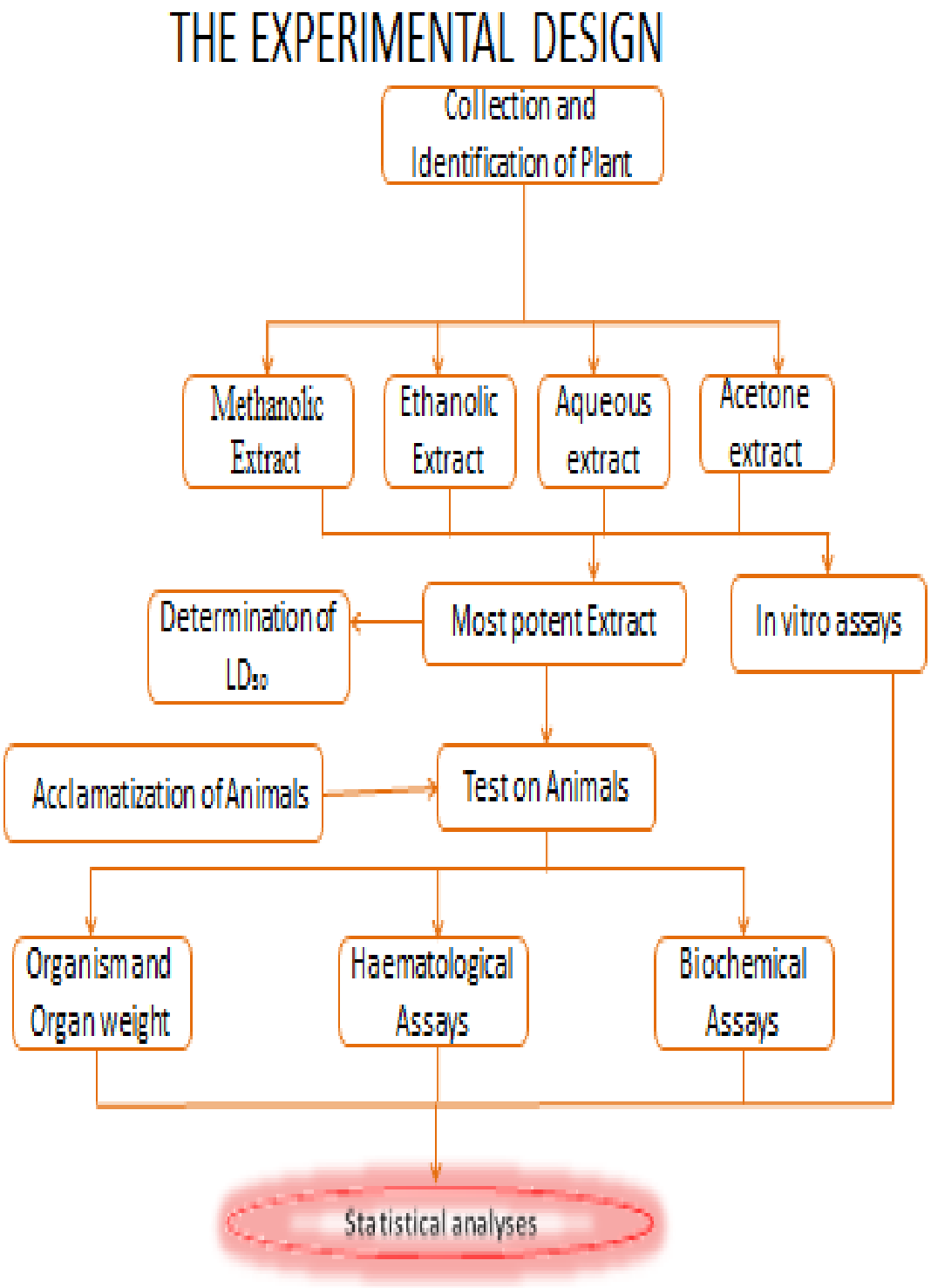

STANDARD CURVE OF GALLIC ACID
y = 0.0106x + 0.041
R2 = 0.996
A Absorbance at 7
1.2
1
0.8
0.6
0.4
0.2
0
0
50
100
150
Concentration (µg/ml)

APPENDIX 7



Standard Curve for Hydrogen Peroxide

Appendix 8


THE EXPERIMENTAL DESIGN
Collection and Identification of Plant
Methanolic Extract
Ethanolic Extract
Aqueous extract
Acetone extract
Determination of LD_{50}
Most potent Extract
In vitro assays
Acclamatization of Animals
Test on Animals
Organism and Organ weight
Haematological Assays
Biochemical Assays
Statistical analyses

Appendix 9

Calculations and Conversions Used in Some Analyses

(A) Calculations and Conversions Required For Analysis of Total Polyphenol Content of Extracts.

(1) Folin-ciocaltau reagent was diluted 10 times to prepare a 15ml solution by mixing 1.5 ml of Folin-ciocaltau reagent with 13.5 ml of distilled water.

(2) 0.75g of Na_2CO_3 was dissolved in distilled water and volume made up to 10ml solution to produce 75g/l solution of Na_2CO_3.

(3) To determine the mass of sample to be dissolved in distilled water producing stock from which 6050µl of sample solution was pipetted and used, the following procedure was followed.

- The percentage yield of the extract was obtained. Let the percentage yield of the extract be y
- This implies out of 100g of sample, y gram of extract was obtained. Therefore out of 10g of sample, y/10g of extract is obtained.
- y/10 g of extract obtained from 10g of sample is dissolved in 100ml of distilled water

Therefore, Xg is dissolved in 6050µl (6.05ml)

X=[y/10] x 6.05/100

(B) Conversions and Calculations Required in the Preparation of Varying Concentration of Extracts for DPPH Radical Scavenging Activity of the Extracts

An electronic balance was used to weigh 100µg (0.1mg) of extract. The extract was dissolved in distilled water to produce 10ml stock solution of concentration 0.1mg/ml. Stock solution was further diluted in distilled water to obtain desired concentrations of extract as demonstrated in the table below. Each dilution process was done in triplicate.

Volume of extract solution (ml)	**0.2**	**0.4**	**0.6**	**0.8**	**1.0**
Volume of distilled water (ml)	0.8	0.6	0.4	0.2	0.0
Final concentration of solution (mg/ml)	0.02	0.04	0.06	0.08	0.1

(C) Conversions and Calculations Used for Dose of Phenylhydrazine and Extracts Administered to Animals Based on Body Weight

Let the dose of administration = Dmg/Kg body weight

Let the weight of the rats = T grams

If, Xmg is administered to 1000g of animal, then a mass (M mg) will be administered to Tg of rats

This implies M (in mg) = $(TxD) \div 1000$

(a) For Determination of Volume of Extract Which Corresponds to the Respective Dose

1g of extract was dissolved in 10ml solution.

This implies 10ml of extract solution contains 1000mg of extract. Therefore, M mg of extract will be contained in a volume (V_1ml) of extract solution.

$V_1 = (M \times 10) \div 1000$

$V_1 = M \div 100$

But $M = (TxD) \div 1000$

Therefore $V_1 = [(T \times D) \div 1000]/100$

$\mathbf{V_1 = (TXD) \div 100,000}$

For instance if the weight of the rat is 150g and the dose of extract to be administered is 200mg/Kg body weight, then the volume to be administered is calculated as

V_1 = 150 x200/100,000

V_1= 0.3 ml.

(B) For Determination of Volume of Phenlyhydrazine Corresponding to Dose (40mg/kg)

1g of phenylhydrazinium chloride was dissolved in 5ml solution.

This implies 5ml of extract solution contains 1000mg of extract. Therefore, M milligram of extract will be contained in a volume (V_2ml) of extract solution.

V_2 of phenylhydrazine required is half of volume of extract at the same dose (Dmg/Kg body weight) and weight of rats (T g).

Therefore the V_2 is $V_1/2$

$V_2 = (T \times D) \div 50{,}000$

For instance the weight of rat is 150g and the dose of phenylhydrazium chloride is 40mg/Kg, the volume (V_2) of phenylhydrazinium chloride will be

V_2 = 150X40/50,000

V_2 = 0.12ml of phenylhydrazinium chloride solution