THE HAEMOGLOBIN REGENERATION POTENTIAL OF FERMENTED AND UNFERMENTED *TELFAIRA OCCIDENTALISHOOK* AND *GNETUM AFRICANUM WELW* LEAVES IN IRON DEFICIENT ALBINO RATS

BY

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DECLARATION

The research work in this thesis entitled THE HAEMOGLOBIN REGENERATION POTENTIAL OF FERMENTED AND UNFERMENTED *TELFAIRA OCCIDENTALIS* AND *GNETUM AFRICANUM* LEAVES IN IRON DEFICIENT ALBINO RATShas been performed by me in the Department of Biochemistry, Ahmadu Bello University, Zaria under the supervision of Mr. O. A.Owolabi and Dr. A.B.Sallau.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.

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CERTIFICATION

This thesis entitled THE HAEMOGLOBIN REGENERATION POTENTIAL OF FERMENTED AND UNFERMENTED *TELFAIRA OCCIDENTALIS* AND *GNETUM AFRICANUM* LEAVES IN IRON DEFICIENT ALBINO RATSby IJEOMA OKOLO meets the regulations governing the award of the degree of Master of Science at Ahmadu Bello University, Zaria, and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

This work is dedicated to God Almighty and to all those who despite the numerous challenges facing them press on to achieve their goals in life.

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ABSTRACT

Haemoglobin regeneration potential of *Telfairaoccidentalis*and *Gnetumafricanum*leaves and their fermented derivatives was investigated in young albino rats of both sex in which nutritional anaemia was induced by feeding iron deficient diet and deionized water. Vitamin C content of *G. africanum*was significantly ($p<0.05$) higher than that of *T. occidentalis* but it decreased significantly from 28±0.02 to 14±0.01mg/100g in *T. occidentalis* while*G. africanum* had no significant ($p>0.05$) change after fermentation. The antinutrientcontent of phytate and oxalate was significantly ($p<0.05$) higher in *G. africanum* and fermentation significantly ($p<0.05$) decreased these antinutrients in both vegetables. The Fe, Zn, Ca, Cu and Mg content was significantly higher in *T. occidentalis* than *G. africanum* and fermentation significantly ($p<0.05$) increased the level of Zn, Ca, and Cu of both vegetable leaves while iron (Fe) significantly ($p<0.05$) increased and Mg significantly ($P<0.05$) decreased in *T. occidentalis*, Fe significantly ($p<0.05$) decreased with Mg showing no significant ($p>0.05$) difference in *G. africanum*. Speciation pattern analysis shows that most Fe in both vegetables were in the residual form and the *in-vitro* bioavailable Fe in both leaves were 57%, 65%, 43% and 31% for unfermented and fermented *T. occidentalis*and *G. africanum*leaves respectively. Supplementation study (3 weeks) shows that iron deficiency significantly ($p<0.05$) decreased relative weight gain, haemoglobin-Fe, ferritin, haemoglobin (Hb), and mean corpuscular haemoglobin concentration (MCHC), when compared to the Fe sufficient control rats. Rats fed with unfermented and fermented *T. occidentalis* significantly ($p<0.05$) increased serum ferritin but there was a non- significant ($p>0.05$) increase in the ferritin stores of rats fed with unfermented and fermented *G. africanun* leaves. The Hb, red blood cell, packed cell volume, white blood cell, mean corpuscular volume and MCHC levels in the iron deficient group were not significantly ($p>0.05$) different from the

iron sufficient and leaves supplemented groups. However, Hb concentration of rats in the iron sufficient, fermented and unfermented *T. occidentalis* and $FeSO_4$ groups were significantly ($p<0.05$) higher than the iron deficient and fermented and unfermented *G. africanum*supplemented groups. Percentage change in Haemoglobin-Fe was lower for rats supplemented with the fermented forms of both leaves respectively. This shows that *T. occidentalis* has a better haemoglobin regeneration potential than *G. africanum*and fermentation did not significantly ($p>0.05$) enhance the haemoglobin regeneration potential of both leaves.

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LIST OF ABBREVIATIONS

%: Percentage

AGP: Alpha 1 glycoprotein

BMP: Bone Morphogenic Protein

Ca: Calcium

CBC: Complete Blood Count

Cd: Cadmium

CDC: Centers for Disease Control

CRP: C-Reactive Protein

Cu: Copper

DCYTB: Duodemum Cytochrome B

DMT: Divalent Metal Transporter

FAO: Food and Agriculture Organization

Fe: Iron

G. africanum: *Gnetumafricanum*

H: Hydrogen

Hb: Haemoglobin

HCP: Human Carrier Protein

hDMT: Human Divalent Metal Transporter

HIF: Hypoxia Inducible Factor

LIP: Labile Iron Pool

MCH: Mean Corpuscular Haemoglobin

MCHC: Mean Corpuscular Haemoglobin Concentration

MCV: Mean Corpuscular Volume

Mg: Magnesium

Mn: Manganese

MTF: Metal Transcription Factor

NRf2: Nucleoid Factor Erythroid 2

NTBI: Non Transferrin Bound Iron

PCV: Packed Cell Volume

RBCs: Red Blood Cells

sTfR: Soluble Transferrin Receptor

SVCT1: Sodium Vitamin C Transporter

T. occidentalis:Telfairaoccidentalis

Tf: Transferrin

TIBC: Total Iron Binding Capacity

UNFPA: United Nations Fund for Population Activities

UNICEF: United Nations Children Education Fund

WBCs: White Blood Cells

WHO: World Health Organisation

Zn: Zinc

CHAPTER ONE

1.0 INTRODUCTION

The deficiency and excess of essential micronutrients and trace of toxic metals may cause serious effects on human health (Khan *et al.,* 2008). Iron deficiency ranges from iron depletion, which yields little physiological damage, to iron deficiency anaemia, which can affect the function of numerous organ systems (Joshi and Mathur, 2009). It is characterized by the reduction or absence of iron stores, low serum concentration of iron, poor haemoglobin concentration, haematocrit reduction and increased platelet count. This type of anaemia affects people of all ages and is prevalent in developing and developed countries (Nirjala and Korah, 2013). Due to its effects on development and growth, resistance to infections and association with the mortality of infants younger than 2 years, it is considered as a major public health problem and the most common nutritional deficiency in the world (Joshi and Mathur, 2009). It is the most common form of anaemic conditions in growing children due to increase in iron demand (Enaam *et al.,* 2014). The deficiency is also found in young girls and women due to excessive monthly menstrual bleeding, and also in pregnant women due to increased iron requirement (Majolagbe *et al.,* 2013). Iron deficiency anaemia in adults occurs typically due to a gradual decline in the iron content of the body due to a loss of haemoglobin and a depletion of iron stores. In the absence of malabsorption there is a good response to iron therapy. Blood loss from the gastro-intestinal tract or malabsorption of iron are common causes in men and post-menopausal women (Goddard *et al.,* 2000). People with asymptomatic colonic or gastric carcinoma may present with iron deficiency anaemia and it is essential to exclude these conditions as the cause of anaemia in elderly men and women (Goddard *et al.,* 2000). Iron deficiency varies greatly with geographical and socio-economic factors. Infections, excessive

sweating and staple food stuffs like vegetables or cereals with high bran and phytate content are some of the factors that account for high anaemic incidence in developing countries as compared to developed nations with temperate climatic conditions (Bjorn-Rasmussen, 1974). Regular body activities such as urination, defecation, sweating and menstruation in women lead to continuous loss of epithelial cells and iron. The consequential daily iron loss of body in adult male is 1.0 mg, while that of female is 0.8 mg (Mertz, 1987).

In order to balance this loss, and prevent iron deficiency, vulnerable populations are encouraged to consume iron-rich foods and breast-feed or use iron-fortified formula for infants. A daily absorption of 2.0 mg of iron is considered necessary (Anderson *et al.*, 1981). When the body has sufficient iron to meet its needs (functional iron), the remainder is stored for later use in the bone marrow, liver, and spleen.

Vegetables are the edible parts of plant that are consumed wholly or in parts, raw or cooked as part of main dish or salad. It includes leaves, stems, roots, flowers, seed, fruits, bulbs, tubers and fungi (Uwaegbute, 1989; Uzo, 1989).They add variety to the diet (Mepha *et al.,* 2007; Sobukola *et al*., 2007), and are valuable sources of nutrients especially in rural areas where they contribute substantially to protein, minerals, vitamins, fibers and other nutrients which are usually in short supply in daily diets (Mohammed and Sharif, 2011). It is worthwhile to note that consumption of numerous types of edible plants as sources of food could be beneficial to nutritionally marginal population especially in developing countries where poverty and climate is causing havoc to the rural populace (Mohammed and Sharif, 2011).

Telfairia occidentalis is a member of the family Cucurbitaceae commonly known as the fluted gourd, fluted pumpkin or ugwu in Nigeria. It has simple, dark green leaves that are as wide as

18 cm and long as 35 cm. *Telfairia occidentalis* has been reported to contain nutrients such as proteins, carbohydrates, vitamins, minerals and fiber. It also contains oxalates, saponins, glycosides, flavonoids, alkaloids and resins (Iweala and Obidoa, 2009). In Nigeria, the consumption of the leaf of *Telfairia occidentalis* as a leafy vegetable in the dietary food or as an infusion in medicinal preparation is being encouraged due to various medicinal properties such as antianaemic (Olaniyan and Adeleke, 2005), antidiaebetic, hepatoprotective effect (Olorunfemi *et al.*, 2005), and as a purgative leafy vegetable (Oboh, 2006).

Gnetum africanum is a dioecious plant belonging to the family Gnetaceae . It is a popular non-woody, wild dioecious vegetable that grows on trees and is commonly known in southeastern Nigeria as "Okazi" by the Igbo, and "Afang" by the Efik/Ibiobio. It is used in the treatment of nausea and is considered to be an antidote to some form of poison it is also used in the treatment of an enlarged spleen, sore throats and as a cathartic (Burkill, 1985). The leaves of *Gnetum* species are used as a disinfectant for wounds and to treat heamorrhoids. The leaves also increase blood production in humans (Ndam *et al.*, 2000). Phytochemistry of the leaves of the plant revealed that, it is an important source of protein, essential amino acids and mineral elements (Baker, 1972).



a. *Telfaira occidentalis* leaf (Adapted from; Darlinton Omeh, www.wealthresult.com)



b. *Gnetum africanum* leaf (Adapted from; Nki Lily Lete, www.nigerianfoodtv.com)

PLATE 1.1 Photographs of *Telfaira occidentalis* and *Gnetum africanum* leaves

1.1 Statement of Research Problem

Deficiencies of micronutrients are still a major public health problem in many developing countries with infants and pregnant women usually at risk (Salawu and Salimon, 2014). Iron deficiency anaemia is the most common nutritional problem worldwide (Grosbois *et al.,* 2005). It is the only nutrient deficiency which is also significantly prevalent in industrialized countries (WHO, 2013). It has also been documented as the commonest cause of nutritional anaemia in infancy and childhood (Bertil, 2004). Poor iron status affects billions of people worldwide (Abo-Zeid *et al.,* 2014). About 1.2 billion people worldwide demonstrate varying levels of iron deficiency (Emodi, 2007). Prevalence rates vary among countries; it affects 2.4 million children in the USA, (Brotanek *et al.,* 2007) 5.4% children in Spain, (Munoz *et al.,* 1995) 14.0% in Estonia, (Vendt *et al.,* 2007) 30.8% under five Brazilian children (Vieira *et al.,* 2008) and 22.3% under-five Nigerian children (Maziya *et al.,* 2004). Iron deficiency anaemia affects the development, growth, work capacity, resistance to infection and is associated with mortality of infants younger than 2 years.

In developing countries every second pregnant woman and about 40% of preschool children are estimated to be anaemic, this contributes 20% of all maternal deaths (WHO, 2014). The estimate of the global burden of deaths that is attributable to anaemia in women of reproductive age ranges from 16 800 to 28 000 annually with a greater risk of anaemia-related death in younger women (WHO, UNICEF, UNFPA, 1990-2008). In Nigeria studies have shown that anaemia contributes to the increased maternal mortality recorded in the nation (Ariba *et al.,*2004; Ujah *et al.,*2004).This ranges from 14.6% in North-Central Nigeria to 20% in the western region of the country (Ariba *et al.,*2004 and Ujah *et al.,*2004). Anaemia is estimated to contribute to 591 000 perinatal deaths globally per year (Ezzati *et al.,* 2004). The highest prevalence is in preschool-

age children and the lowest prevalence is in men 12.7%. However, the population group with the greatest number of individuals affected is non-pregnant women 468.4 million (WHO, 2008).

Although there are various drugs for the treatment of iron deficiency, they are not affordable by many people especially in developing countries, and their intake can easily result to toxicity and other side effects. In addition, most rural dwellers in developing countries do not have adequate access to these drugs hence depend more on use of plants for prevention and treatment of anaemia. However, not all plants can cure iron deficiency anaemia, therefore there is a need to research on plants that may have a high bioavailable iron content whose consumption will lead to the normal biosynthesis of haemoglobin, hence can be eaten as a protective or curative food based strategy against iron deficiency.

1.2 Justification

Iron is an absolute requirement for most forms of life, including humans and most bacterial species. Plants and animals all use iron, and it can be found in a wide variety of food sources. Iron is an essential micronutrient necessary for the transportation of respiratory gases via haemoglobin in the red blood cells. Iron also intervenes in the constitution of enzymatic systems such as catalases, peroxidases and cytochromes that play an essential role in cellular respiratory machanisms, in mitochondrial respiratory channel (Ghosh *et al*., 1995). It is also essential for the regulation of cell growth, and depletion of iron limits oxygen delivery to cells, resulting in fatigue, poor work performance, and decreased immunity. Iron deficiency can lead to nutritional anaemia and cause other health complications.

Among plant foods, vegetables are the cheapest and locally available food rich in iron and can be used as a part of the food based strategy to prevent nutritional anaemia. *Telfaira occidentalis* and

Gnetum africanun leaves are commonly consumed vegetables in Nigeria and they are known to contain iron, but the amount that becomes bioavailable is not known and this knowledge will reveal if consumption of these leaves could protect or cure iron deficiency anaemia. Hence, this research aims at evaluating the haemoglobin regeneration potential of these commonly consumed vegetables.

1.3 Aim and Objectives of the Study

1.3.1 Aim;

To evaluate the haemoglobin regeneration potential of fermented and unfermented *T. occidentalis* and *G. africanun* leaf supplemented diet on iron deficiency anaemic young rats.

1.3.2 Specific objectives;

I. To analyze for vitamin C, phytate and oxalate contents of the vegetables
II. To determine the mineral compositions of the vegetables
III. To determine the speciation pattern and *in-vitro* bioavailable iron content of the vegetables
IV. To determine the level of iron indices and the haemoglobin regeneration potential of the vegetables in iron deficient anaemic rats.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 *Telfaira occidentalis* and *Gnetum africanum*

2.1.1 Origin and geographic distribution of the plants

Telfaira occidentalis occurs in the forest zone of West and Central Africa, most frequently in Benin, Nigeria and Cameroon. It is a popular vegetable all over Nigeria and almost absent in the rest of East Africa. It has been suggested that it originated in south-east Nigeria and was distributed by the Igbos, who have cultivated this crop since time immemorial. It is, however, equally possible that fluted pumpkin was originally wild throughout its current range, but that wild plants have been harvested to local extinction and are now replaced by cultivated forms (Kayode and Kayode, 2010).

Gnetum africanum occurs naturally in the humid forest zones from Nigeria to the Central African Republic and to Angola. It can be found in rainforest from sea-level up to 1200 m altitude, and thrives on an annual rainfall of about 3000 mm. It is usually found with other climbers on middle- and under-storey trees, frequently forming thickets, it also grows on clay or sandy soils (Akintola *et al.*, 2012). 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. It belongs to the family, Gnetaceae and Order, Gnetales. Gnetum has several names as there are several ethnic groups that consume it in Nigeria and in Cameroon. In Nigeria, the Ibibios and Efiks call it "Afang", in Ikom of Cross River State, it is called"Nkani ", in Igbo it is called "Okazi" and in Cameroon, it is known as "Okok". *G. africanum* requires a large quantity of biomass and sunlight for its photosynthetic process.(Schippers and Besong, 2004).

2.1.2 General description of the plants

Telfairia occidentalis is a creeping vegetable shrub that spreads low across the ground with large lobbed leaves and long twisting tendrils. It is a dioecious, perennial herb climbing by coiled tendrils that often branch. The plant grows to a height of more than 20m; root system ramifying in the top surface of the soil, the stem are angular, and glabrous, becoming fibrous when old. Its habitat is the wet part of Nigeria and Africa in general. It thrives best in soils rich in organic matter and during the early part of the rainy season. It belongs to the family cucurbitaceae (Ehiagbonare, 2008). Locally it is called 'ugwu' by Igbos in eastern part of Nigeria, Yoruba, '*Egusi iroko*' and Benin, '*uwmenkhen*' (Verla *et al*., 2014). It is called fluted gourd or fluted pumpkin in English (Saalu *et al.*, 2010). The leaves are arranged spirally, with 3–5 leaflets; stipules are absent; the petiole is (2–) 4–11 (–15) cm long; the leaflets with petiolules is 0.5–3.5 cm long, the one in the central is the largest, with up to 15(–19) cm × 10(–12) cm, the lateral ones are asymmetrical, and usually dentate in the upper two-thirds or sometimes scabrid underneath, 3-veined from near the base (Odiaka and Schippers, 2004).

Gnetum africanum is a dioecious liana that grows up to 10 m long but sometimes longer;the branches are somewhat thickened at the nodes, and are glabrous. The leaves are decussately opposite, sometimes in whorls of 3, stipules are absent but petiole are up to 1 cm long. *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). The two African species, which are very similar, have been classified in section *Gnetum*, subsection *Micrognemones*. *Gnetum africanum* has leaves which are relatively thin and pale green (Schippers and Besong, 2004)

2.1.3 Nutritional composition

The proximate composition (%) of fluted pumpkin leaf extract was given as: crude protein (21.31), crude fibre (6.41), ether extract (5.50), ash (10.92), nitrogen free extracts (55.56), metabolisable energy (3121.00 kcal/kg), gross energy (4420.00 kcal/kg). Minerals in mg/100g; Ca (0.67), P(0.40), K(0.15), N (3.41), Mg (0.43), Na (0.02), Zn (7.50) , Fe (18.5) Mn (1.18., Antinutrients in mg/100g; Phytate (510.51), Tannins (0.184), Oxalate (0.0034) Total amino acid in *T. occidentalis* was 455.3 mg/g with total essential amino acid of 256.1 mg/g or 56.3 %, showing the plant proteins to be high in essential amino acids. Aqueous extract of the leaf contain 5.07 mg/100ml and 40mg/100ml of Vitamins E and C, respectively (Idris, 2011).

The proportions of the various minerals vary with the age of the plant. Anti-nutrient composition of the young stems and leaves of the plant was found to be higher than that found in the older leaves and stems (Akoroda, 1990).

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*.

2.1.4 Uses

The tender shoots, succulent leaves and immature seeds are cooked and consumed as a vegetable. The leaves are used alone or together with other vegetables such as okra (*Abelmoschus caillei* Stevels and *Abelmoschus esculentus*), dika nut (*Irvingia gabonensis*), or egusi seeds (*Citrullus lanatus*). They can also be mixed with *Gnetum africanum* and *Pterocarpus soyauxii* (Odiaka and Schippers, 2004).

Fresh leaves of *Gnetum africanum* and the very similar *Gnetum buchholzianum*, both called *Gnetum africanum* are widely used as a vegetable. They are usually cooked with meat or fish and occasionally consumed as a salad. Leaves are shredded into thin strips and are often eaten as part of a mixture in, for example, a groundnut-based sauce. To soften this rather tough vegetable, people often mix it with waterleaf (*Talinum triangulare*). Shredded leaves can be dried and preserved for later use (Schippers and Besong, 2004).

2.1.5 Health benefits

The sliced young leaves mixed with coconut water and salt could be stored in a bottle and used for the treatment of convulsion (Gbile, 1986). Also ,the leaf extract alone is useful in the management of hyperchelsterolaemia, liver problems, and impaired immune system

(Eseyin *et al.,* 2005; Adaramoye, 2007) but the oil from the seed could result in hyperlipidermias and hyperglycaemia if consumed excessively. The use of *T. occidentalis* in reproductive and fertility health is gaining ground. Nwangwa *et al.,* (2007) showed that it has potential to regenerate testicular damage and increase spermatogenesis. *Telfairia* is high in anti- oxidant and free radical scavenger properties and that may contribute to why many use the leave extract in oxidative damage condition such as cancers, liver and liver diseases. In Nigeria the fresh leaves are ground and the juice used as tonic by women that have just given birth; its high iron content assists in the replenishment of lost blood; being used for treatment of , chronic fatigue and diabetes (Aderibigbe *et al.,*1999; Dina *et al.,*2006; Alada, 2000). The blood schizontocidal activity of the root of *Telfairia* is comparable to that of chloroquine (Okokon *et al.,* 2007). The extract also shows inhibitory effect on growth of some bacteria (Odoemena and Onyeneke 1988; Oluwole *et al.,* 2003; Oboh *et al.,* 2006). *Telfairia* roots are very poisonous because of their high saponin content and are used to kill rats and mice as rodentcide and ordeal poison (Gills, 1992).

In Nigeria, *Gnetum africanum* is used for treatment of piles and high blood pressure and also as medicine against enlarged spleen, sore throat and as a purgative. In the Central African Republic the leaves are eaten to treat nausea and as an antidote to arrow poison made from *Periploca nigrescens*. In Cameroon the leaves are chewed to mitigate the effects of drunkenness and they are taken as an enema against constipation and to ease childbirth. They are also used to treat boils and fungal infections on the fingers (whitlows) (Schippers and Besong, 2004).

2.2 Iron

2.2.1 Description

Iron (Fe) is the second most abundant metal on earth and is a necessity for all life. It plays the key role in numerous enzymatic reactions due to its ease in shifting between the two common oxidation states, ferrous (Fe^{2+}) and ferric (Fe^{3+}) iron. In humans, iron carries off oxygen by means of haemoglobin in erythrocytes and myoglobin in myocytes, hence, it is indispensable for providing oxygen to the tissues. Severe iron deficiency leads to a number of symptoms such as tiredness, palpitations, and other signs associated with impaired oxygen transport. Also, iron overload is linked to several diseases involving functional changes of haemoglobin, such as thalassemia and sickle cell disease. Iron in excess is detrimental because of its propensity to cause oxidative damage. Except for the sloughing of epithelial cells, which removes iron confined in the absorptive cells, there is no other way to eliminate iron. It is therefore of major importance to tightly regulate iron absorption (Scheers, 2013).

2.2.2 Dietary iron and bioavailability

Iron exists in two main forms in food; haem iron and non-haem iron. Haem iron is highly abundant in meat (myoglobin) and liver (haemoglobin). It is coordinated to a porphyrin ring deeply hidden inside a globular protein, which makes the access to iron restricted to small molecules such as O_2 and CO. This arrangement also protects iron from forming insoluble precipitates in the intestine and thus promotes iron bioavailability. Non-haem iron is present in vegetables, cereals, and fruits, which constitute the major part of a healthy diet. In the human intestine, non-haem iron is mostly present in soluble or insoluble chelate complexes. Organic acids such as ascorbic acid or citric acid form chelates with iron and enhance iron absorption

(Salovaara *et al.*, 2002). The diet also contains inhibitors of iron absorption. Phytate is an antinutrient which is abundant in legumes, cereals, and nuts it has a high binding capacity for metals and also has the ability to form large insoluble aggregates (Sandberg *et al.*, 1999). Since non-haem iron is absorbed as single ions, there are several parameters that determine the extent of absorption. In addition to the interactions with organic compounds, non-haem iron transport is pH-dependent and occurs in competition with other divalent ions to be transported by metal transmembrane carriers. Also, intracellular effects and factors specific to the host determine iron absorption (Scheers, 2013).

2.2.3 Iron requirements

An average individual contains about 50 mg Fe/kg, which is approximately 3.5 g for a 70 kg-person (Munoz *et* al., 2009). About 65% of these 3.5 g exist as haemoglobin-iron in erythrocytes. The remaining iron is present in myoglobin of muscle fibers, enzymes in various tissues, and stored in macrophages, liver, and bone marrow. After cellular turnover, most of the body iron is recycled primarily by macrophages of the reticuloendothelial system. Cellular sloughing into the gastrointestinal tract, bleeding, and respiration cause losses of 1–2 mg Fe per day. The iron levels are restored by intestinal absorption to maintain the balance. A typical western world diet provides 10–18 mg of iron per day. About 10% is absorbed, which does not give much of a margin in times of increased iron requirements, as in pregnancy or fast-growing children (Scheers, 2013).

2.2.4 Iron transport in and across the human enterocyte

2.2.4.1. Iron influx

Haem-bound iron may be absorbed from the gastrointestinal lumen through the haem-carrier protein HCP1. HCP1 is primarily a H+-coupled folate transporter suggesting that absorption of haem-iron is affected by folate availability (Laftah *et al.*, 2009). The absorbed haem-bound iron must be released in the cytosol by means of haem oxygenase (Raffin *et al*., 1974) in order to join the same pathway as elemental iron. In addition, haem is likely to be absorbed via receptor-mediated endocytosis (Grasbeck *et al.*, 1979). Dietary heme can also be transported across the apical membrane by a yet unknown mechanism (Wang and Pantopoulos, 2011; Abbaspour *et al*, 2014).

Divalent Metal Transporter 1 (DMT1), the main transporter for divalent cations, transports elemental iron across the lumenal membrane of absorptive cells. Human DMT1 (hDMT1) simultaneously imports hydrogen (H^+) and ferrous iron (Fe^{2+}) (Tandy *et al.*, 2000). It is still debated whether hDMT1 is specific for Fe^{2+}or also transports other metal ions (Espinoza *et al.*, 2012). Although, there is convincing evidence that hDMT1 transports Fe^{2+}in preference to Zn (Tandy *et al.*, 2000, Yamaji *et al.*, 2001, and Illing *et al.*, 2012), however, $Cd^{2+}$is preferred to Fe^{2+} (Illing *et* al., 2012). hDMT1 is expressed in four different isoforms (DMT1A, DMT1A-IRE, DMT1B and DMT1B-IRE), which are differently regulated and distributed (Hubert and Hentze, 2002). DMT1A and DMT1A-IRE are mainly concerned with the apical influx at polarized enterocytes while the DMT1B isoforms are involved in cytosolic iron sequestration from endosomal import by transferrin receptors at the basal membrane. Studies in the mouse have indicated that DMT1 is expressed along the entire small intestine but is only increased in

the proximal part of the duodenum in response to iron starvation (Canonne-Hergaux *et al.*, 1999). This suggests that the proximal duodenum is the major site for iron absorption, since elemental iron is mostly transported into the enterocyte through DMT1, the transport efficiency is partly determined by the availability of iron in the ferrous form. The availability of $Fe^{2+}$is assured by the expression of a brush border-associated ferric reductase (Scheers, 2013).

2.2.4.2 Intracellular transport and efflux

In the cytosol, newly absorbed iron initially joins the labile iron pool (LIP) also referred to as the chelatable iron pool, which consists of metabolically active forms of iron mainly loosely associated with macromolecules. Depending on the cellular requirements, iron may be distributed from the LIP to the major intracellular iron storage protein, ferritin, where iron is stored inside the hollow core as ferric phosphate salts. The ferritin molecule consists of L and H subunits, in which the H subunits possess ferroxidase activity required for the acquisition of iron salts. It has been proposed that a recently identified cytosolic iron chaperone, PCBP1 (hRNP/α-CP1), is responsible for the incorporation of iron to ferritin (Shi *et al*., 2008). However, it is not yet fully clear how the iron delivery to different intracellular compartments occurs (Scheers, 2013). In times of systemic shortage, iron is transported directly to the basal membrane for transmembrane export to the vascular compartment by the ferrous iron exporter ferroportin (SLC40A1/FPN1/IREG1) (Mckie *et al*., 2000). Iron is transported across the membrane as $Fe^{2+}$and is then oxidized to $Fe^{3+}$by a membrane-bound ferroxidase, hephaestin. The ferric iron is then transferred to transferrin (Tf) for systemic transportation.

2.3.5 Systemic regulation of iron absorption

2.2.5.1 Control of iron release

Systemic iron homeostasis is rigorously controlled, this is because iron is essential for survival, in addition to iron being highly toxic if overloaded. Hepcidin, a cysteine rich cationic peptide produced in the liver, is the key regulator of systemic iron levels. It controls iron release from the enterocytes into the systemic circulation by binding to the iron exporter ferroportin, thereby subjecting ferroportin to ubiquitin-dependent degradation (Qiao *et al.,* 2012). The removal of ferroportin at the enterocyte basal lamina blocks iron efflux and as a consequence enterocyte levels of ferritin increase, which reflect the intestinal iron load. Hepcidin is feedback-regulated by iron itself and is decreased during iron deficiency and increased in iron overload. The effect is thought to be mediated by the iron specific ligand Bone Morphogenic Protein (BMP) pathway (Babitt *et al.,* 2006; Andriopoulos *et al.,* 2009). Circulating iron-saturated transferrin interacts with transferrin receptor 2 (Tfr2) at the surface of hepatocytes, which induces hepcidin production through interaction of BMP6 and SMAD signaling . When iron-saturated transferrin levels are low the required stabilization of Tfr2 by the associated membrane protein HFE is lost and thus transcription of hepcidin mRNA is repressed. Hypoxia also regulates hepcidin expression in hepatocytes via the SMAD/BMP pathway (Chaston *et al.,* 2011). The hypoxia-inducible factor 2 (HIF2) has been proposed as a mediator, however HIF2 was recently observed as not being involved in the regulation of hepcidin transcription (Volke *et al.,* 2009; Mastrogiannaki *et al*., 2012).

Systemic iron is also absorbed in hepatocytes as nontransferrin-bound iron (NTBI), which requires the usual route of reduction to ferrous iron by membrane-bound reductases and uptake

through metal transporters. DMT1 is generally expressed in all tissues and may therefore play a role in hepatocyte iron influx. The zinc transporter ZIP14 is abundantly expressed in liver cells and has been shown to be involved in NTBI uptake (Liuzzi *et al.,* 2006). Later studies in the *Xenopus laevis* oocyte expression system have revealed that ZIP14 imports Fe^{2+}in addition to Zn, Cd and Mn. Interestingly, the transport of $Fe^{2+}$was dependent on Ca^{2+}, but not on Zn^{2+}(Pinilla-Tenas *et al*., 2011). It has also been observed that ZIP14 protein expression is down-regulated by the abundance of the HFE protein in liver cells, inhibiting the uptake of iron (Gao *et al*., 2008).

2.2.5.2 Recycling of iron by macrophages

Macrophages play an important role in executing the regulatory events leading to changes in systemic iron levels. Senescent or damaged erythrocytes are removed from the circulation by phagocytosis. Haem-iron is transported from the phagocytic vesicles into the cytosol by means of a transmembrane permease, HRG1 (White *et al*., 2013). Elemental iron is released by means of DMT1 into the cytosol where it associates with the Labile Iron Pool (LIP) or is incorporated into ferritin. Macrophages also scavenge iron by receptor-mediated endocytosis of haptoglobin-haemoglobin complexes or hemopexin-haem complexes retrieved from ruptured erythrocytes. Iron is eventually exported through ferroportin, which is partly controlled by hepcidin. In addition, the porphyrin ring of haem regulates the transcription of ferroportin by activating Nuclear Factor Erythroid 2 (NRf2) control of the ferroportin promoter (Marro *et al.,* 2010). This further strengthens the crucial role of hepcidin/ferroportin in regulating systemic iron levels.

2.2.5.3 Role of ascorbate in iron absorption

Ascorbate is an important regulator of iron transport; it assists Duodenal Cytochrome B (DCYTB) in the intestinal lumen by reducing ferric iron to the bioavailable ferrous form. In addition, cellular ascorbate status influences enterocyte protein levels of DMT1 and DCYTB in the absence of iron (Scheers and Sandberg, 2008). HIF2α, which regulates DMT1, DCYTB, and ferroportin transcription, is degraded in response to ascorbate, iron and oxygen. Also, low ascorbate, iron and oxygen levels stabilize HIF2α, providing a possible mechanism for the effects of ascorbate on DMT1 and DCYTB expression. Ascorbate may be important for the ferrireductase activity of DCYTB, as it has been suggested to donate electrons to the cytoplasmic domain for transmembrane propagation, taking part in the lumenal reduction of iron (Latunde-Dada *et al.,* 2002). It has been suggested that DCYTB also reduces copper in addition to iron, further indicating the regulatory role of copper in iron transport (Wyman *et al.,* 2008). Ascorbate is absorbed in the intestinal lumen by means of the ascorbate/sodium transporter; Sodium Vitamin C Transporter (SVCT1). The SVCT1 expression is regulated by ascorbate (MacDonald *et al.*, 2002) and also by iron (Scheers and Sandberg, 2008). Increased cellular iron levels up-regulate ascorbate uptake. Increased ascorbate levels then down-regulate DCYTB and DMT1 in response to the iron load, closing the feedback loop.

2.2.5.4 Role of Zinc in iron transport

Iron down-regulates its own uptake by the DMT1 transporter while zinc up-regulates uptake of iron by increasing the protein levels of DMT1 in human Caco-2 cells (Yamaji *et al.,* 2001). The basolateral efflux of iron and the mRNA expression of ferroportin are also increased by zinc supplementation. Zinc promotes ferroportin transcription by stimulating the binding of

Metal Transcription Factor 1 (MTF-1) to the ferroportin promoter (Troadec *et al.,* 2010). Iron is imported to hepatocytes via ZIP14 (Liuzzi *et al.,* 2005), which is also expressed in intestinal cells. In the state of inflammation when iron absorption is inhibited and iron is removed from the circulation, circulating zinc is removed to prevent its stimulation of iron absorption and efflux by ferroportin. The ZIP14 transporter in hepatocytes accomplishes the systemic removal of zinc and it is up-regulated by the acute phase cytokine interleukin-6 (Liuzzi *et al.,* 2005), further stating the central role of interleukin-6 in promoting inflammation. The Zip4 transporter is expressed along the intestine and is crucial for zinc absorption and a normal intestinal epithelium (Geiser *et al*., 2012). However, a specific role of the Zip4 transporter in iron absorption has not been established (Scheers, 2013).

2.2.5.5 Role of Calcium in iron absorption

Calcium is generally believed to interfere with iron absorption. It has also been established that this effect requires high calcium intake and occurs at the lumenal level. The mechanism involves the iron transporter hDMT1, of which transport is inhibited by Ca^{2+}(Shawki, and Mackenzie, 2010). Ca^{2+}inhibits Fe^{2+}transport non-competitively with low affinity. It is not clear how Ca^{2+}interferes with iron transport, but voltage dependence or intracellular Ca^{2+}signaling seems to be ruled out (Shawki and Mackenzie, 2010). Iron uptake, as indirectly estimated by intracellular ferritin content in Caco-2 cells, is only decreased with high concentrations (1.25–2.5 mM) of Ca^{2+}after a long exposure time (16–24 h) (Thompson *et al.,* 2010). The membrane expression of hDMT1 decreases accordingly, suggesting that the inhibition of iron uptake may involve regulation of transporter abundance. It has also been reported that short time Ca^{2+}incubations (1.5 h) of human intestinal Caco-2 cells down-regulated ferroportin levels at the basal membrane and the associated iron efflux (Lonnerdal, 2010). Several studies in humans

have shown a correlation with calcium and decreased Iron absorption. However, long-term high calcium intake does not correlate with impaired iron status, as concluded by Lonnerdal (2010) in an extensive review on the subject.

2.2.5.6 The importance of Copper for iron release

Individuals exposed to a copper-deficient diet develop iron deficiency anaemia as well as iron overload in specialized tissues such as the liver and intestine. The iron and copper homeostasis are linked by the inability to export iron in the absence of Copper to the systemic circulation. However, copper deficiency does not affect ferroportin expression (Prohaska and Broderius, 2012). Ferroportin activity is tightly controlled by the ceruloplasmin-homologue hephaestin (Vulpe *et al.,* 1999). Hephaestin is an integral transmembrane ferroxidase, which co-migrates with ferroportin to the basal membrane, in response to increased intracellular iron levels, in which they form a complex (Han and Kim, 2007; Yeh *et al.,* 2008). Exported ferrous iron requires oxidation to ferric iron, which is accomplished by the Cu-dependent ferroxidase activity of hephaestin.

Ceruloplasmin is another Cu-dependent ferroxidase important for iron metabolism. This is evident in the rare disease aceruloplasminemia, in which the absence of serum ceruloplasmin results in decreased mobilization of iron from body stores giving symptoms related to anaemia. It was recently shown that serum ceruloplasmin levels increase during iron deficiency in mice (Ranganathan *et al.,* 2011). Also, increased Copper consumption by the iron deficient mice raised the serum ceruloplasmin activity suggesting a compensatory route for increasing systemic iron levels in times of deprivation (Scheers, 2013).

2.2.6 Clinical diagnosis for iron disorders

The following tests are performed in the laboratory to determine iron status:

2.2.6.1 Haemoglobin concenration

Haemoglobin is the protein molecule in red blood cells that carries oxygen from the lungs to the body's tissues and returns carbon dioxide from the tissues to the lungs. It is made up of four protein molecules (globulin chains) that are connected together. The normal adult haemoglobin molecule contains 2 alpha-globulin chains and 2 beta-globulin chains. In fetuses and infants, there are only a few beta chains and the haemoglobin molecule is made up of 2 alpha chains and 2 gamma chains. As the infant grows, the gamma chains are gradually replaced by beta chains. Each globulin chain contains an important central structure called the haem molecule. Embedded within the haem molecule is iron that transports the oxygen and carbon dioxide in the blood. The iron contained in haemoglobin is also responsible for the red color of blood. Haemoglobin also plays an important role in maintaining the shape of the red blood cells. Abnormal haemoglobin structure can, therefore, disrupt the shape of red blood cells and impede its function and its flow through blood vessels. Haemoglobin is usually measured as a part of the complete blood count (CBC) from a blood sample. A low haemoglobin is referred to as anaemia. There are many reasons for anaemia. Some of the more common causes are: loss of blood (traumatic injury, surgery, bleeding colon cancer or stomach ulcer), nutritional deficiency (iron, vitamin B12, folate), bone marrow problems (replacement of bone marrow by cancer, suppression by chemotherapy drugs, kidney failure), and abnormal haemoglobin (sickle cell). Higher than normal haemoglobin levels can be seen in people living at high altitudes and in people who smoke. Dehydration produces a falsely high haemoglobin which disappears when proper fluid

balance is restored. Some other infrequent causes are: advanced lung disease (for example, emphysema), certain tumors, a disorder of the bone marrow known as polycythemia rubra vera, and abuse of the drug erythropoietin (Kotze *et al.*, 2009).

2.2.6.2 Serum ferritin concentration

Ferritin is currently considered the most important indicator of iron status as even in the first stage of iron deficiency, its concentration decreases (Knovich *et al.*, 2009). A ferritin molecule consists of 24 subunits of L-ferritin or H-ferritin. H-ferritin has ferroxidase activity, which is necessary for oxidising iron before it can be loaded onto ferritin. Ferritin is essential to iron homeostasis and is involved in a wide range of physiologic and pathologic processes, making iron available for critical cellular processes while protecting lipids, DNA, and proteins from the potentially toxic effects of iron. Alterations in serum ferritin are seen commonly in clinical practice, often reflecting perturbations in iron homeostasis or metabolism. In clinical medicine, ferritin is predominantly utilized as a serum marker of total body iron stores (Kotze *et al.*, 2009). In cases of iron deficiency and iron overload, serum ferritin serves a critical role in both diagnosis and management (Koulanouzidis *et al.*, 2009). Ferritin is directly implicated in potentially devastating diseases including familial haemochromatosis, sideroblastics, neurodegenerative diseases, and hematophagocytic syndrome. Elevated serum and tissue ferritin are linked to coronary artery disease, malignancy, inflammatory conditions and poor outcomes following stem cell transplantation. Additionally, recent research describes novel functions of ferritin independent of iron storage (Knovich *et al.*, 2009).

2.2.6.3 Serum transferrin saturation and transferrin concentration

Total iron binding capacity (TIBC) is typically measured along with serum iron to evaluate people suspected of having either iron deficiency or iron overload. The iron concentration

divided by TIBC gives the transferring saturation (Tf saturation), which is a more useful indicator of iron status than iron or TIBC alone. In healthy people, about 20-50% of available sites in Tf are used to transport iron. In iron deficiency, iron is low, but TIBC is increased, and Tf saturation becomes very low. In iron overload states such as haemochromatosis, iron were high and TIBC were low or normal, causing the Tf saturation to increase. The test has been found to be lacking in sensitivity, as linearity becomes questionable once saturation levels above 80% are reached, and the test has not yet proven to be useful in iron estimation for patients with transfusional iron. Although it may not be a reliable test for iron overload, elevated serum Tf saturation is a sensitive and specific disease marker for hereditary haemochromatosis. Fasting serum Tf saturation above 50% in women and above 60% in men, has a sensitivity of 0.92, a specificity of 0.93, and a positive predictive value of 86% for the diagnosis of hereditary haemochromatosis.

It is customary to test for Tf (instead of TIBC) when evaluating a patient's nutritional status or liver function. Because Tf is made in the liver, levels were low in patients with liver disease. Tf levels also drop when there is not enough protein in the diet, so this test can be used to monitor nutrition status.

Although serum ferritin measurement is the investigation of choice in iron deficiency, many laboratories continue to offer iron and TIBC/ Tf measurements. In iron deficiency, serum Tf concentrations are elevated while iron concentrations are decreased, resulting in a decreased Tf saturation (Kotze *et al.,* 2009). However serum iron concentrations follow a diurnal variation and may be increased after meat ingestion (Kotze *et al.,* 2009).

In some cases, iron and TIBC/ Tf are used instead of ferritin due to their lower cost while in other cases, ferritin is requested in addition to iron and TIBC/ Tf because of concerns regarding ferritin interpretation in inflammatory conditions. Tf can be measured either directly or indirectly by summing the results of serum iron and unsaturated iron binding capacity (UIBC) measurements to give total iron binding capacity (TIBC) (Kotze *et al.,* 2009).

2.2.6.4 Soluble transferrin receptor (stfr)

The soluble or serum transferrin receptor test (sTfR) is based on the fact that erythroblasts in the bone marrow will increase the presentation of membrane Tf receptor in the setting of iron deficiency, some of which come off and were detectable in the circulation. The sTfR correlates with this membrane expression of the Tf receptor and also tends to be elevated in the presence of increased erythroid activity. When a patient is noted to have an elevated sTfR, the clinician must determine whether it is due to iron deficiency or because the patient has increased erythroblastic activity which is increasing the membrane and ultimately the serum expression of the TfR. It is a reasonable index of erythropoietic activity (Chiang *et al*., 2002). Since sTfR is not affected by inflammation (Beerenhout *et al.,* 2002), it is a more reliable test than serum ferritin. However ambiguous results can occasionally be obtained and the use of sTfR-log ferritin index is recommended (Matsha *et al*., 2002). A value greater than 2 suggests iron deficiency, while a value less than 1.0 is consistent with of chronic disease (Knovich *et al.*, 2009).

2.2.6.5 Serum iron concentration

Serum iron determinations is widely believed to be unreliable as a result of circadian variation, clinicians often think that determinations of Hb, ferritin or direct Tf would provide better estimations of a patient's iron status (Kotze *et al.,* 2009). Hawkins (2007) advocates the abolishment of serum iron determinations and considers direct Tf measurements cost effective.

However, serum iron concentration is a useful measure for investigation of diseases that may involve inadequate delivery of iron to cells due to inhibition of iron uptake. The iron that is bound to Hb is not available to tissues such as the brain for incorporation into enzymes and proteins involved in energy production, e.g. mitochondrial iron-sulphur proteins and aconitases (Kotze *et al.,* 2009). The amount of iron reaching the cells can best be estimated by determining serum iron concentrations in combination with the other tests.

2.2.7 The role of iron in health and disease

Worldwide, more than 2 billion people suffer from iron deficiency (Shaha, 2004; Deokar *et al.,* 2013). The anaemia of chronic disease is frequently seen in patients with autoimmune disorders, malignancies and infectious conditions, due to iron retention in cells of the reticuloendothelial system (Kotze *et al.,* 2009). While iron deficiency is most common in developing countries, the most frequent inherited disorder in people of Northern-Western European origin is hereditary haemochromatosis (HH), a preventable iron overload disorder caused by mutations in the HFE gene. Iron loading in the presence of a genetic predisposition for HH depends on co-inheritance of other iron-related gene mutations and environmental factors. For example, alcohol consumption increases iron uptake significantly, while black tea has strong inhibitory effects due to the tannins it contains. Secondary iron overload may furthermore develop due to repetitive blood transfusion in patients treated for genetic haemoglobinopathies, cancer-related or myelodysplastic syndromes (Kotze *et al.,* 2009).

The balance of iron in the body depends on a complex interaction between genetic and environmental factors. Individuals with variant forms of genes involved in iron metabolism may respond differently to the intake of the same amounts of iron in the diet.

Iron deficiency, iron overload and the of inflammation are the commonest disorders of iron metabolism (Lynch, 2007).

2.2.7.1 Nutritional iron deficiency

This results from a diet that contains insufficient bioavailable iron to meet individual requirements. In developing countries, traditional foods usually contain large quantities of iron absorption inhibitors, particularly phytates and polyphenols. Conditions that cause blood loss, particularly hookworm infections, have an important contributory role leading to a high prevalence of iron deficiency in many developing countries (Kotze *et al.*, 2009).

2.2.7.2 Anaemia of inflammation

This type of anaemia is the second most prevalent after anaemia caused by iron deficiency and develops in patients with acute or chronic systemic illness or inflammation (Zarychanski and Houston, 2008; Abbaspour *et al*., 2014). This is the result of increased hepcidin expression induced by inflammatory cytokines which is generally considered to be a host response that evolved to make iron less available to pathogens. This condition is characterized by decreased release from iron stores, low plasma iron and transferrin concentrations, restriction of the available iron supply for red blood cell production and mild or moderate (Kotze *et al.*, 2009).

2.2.7.3 Primary iron overload

This is far less prevalent than iron deficiency. Primary systemic iron overload (haemochromatosis) is almost always the result of an inherited abnormality of the regulation of iron transport that affects hepcidin or its receptor ferroportin. The expression of hepcidin is induced independently by the accumulation of storage iron and by inflammation (Kotze *et al.*, 2009). Hepatic iron overload can also develop as a part of more complex genetic diseases,

including deficiencies of transferrin and ceruloplasmin and loss-of-functionmutations in DMT1 (Pietrangelo *et al.,* 2011). In these disorders, iron-restrictive anaemia develops because of diminished iron release from macrophages (ceruloplasmin deficiency), iron delivery to erythrocytes (transferrin deficiency), or iron utilization by erythrocyte precursors (DMT1 loss of function). In contrast, simple hereditary hemochromatosis in humans has only modest effects on erythropoiesis, limited to a slight increase in mean corpuscular volume (McLean *et al.*, 2007).

2.2.8 Iron deficiency

Iron deficiency, and anaemia resulting from iron deficiency, is considered to be one of the top ten contributors to the global burden of disease. The worldwide prevalence of anaemia in preschool aged children is approximately 47%, in pregnant women 41%, and in non-pregnant women 30% (McLean *et al.*, 2007). Globally, more than 800 million women and children suffer from anaemia, mostly in Africa, Asia and Latin America. It affects 22.3% under-five Nigerian children (Maziya-Dickson *et al.*, 2004; Ekwochi *et al.,* 2014)

Anaemia in pregnancy poses a great danger to both mother and child. Two-third of all pregnant and half of all non-pregnant women in Africa have anaemia (WHO/NHD, 2000). Anaemia in pregnancy arises from a variety of factors which include the physiological hemo-dilution of pregnancy, increased demand of the fetus on maternal stores of iron and folic acid, poor nutritional diet, infections and infestation such as malaria and hookworm and some adverse cultural practices(WHO/NHD, 2000; WHO/NHD, 2001). Iron deficiency anaemia accounts for 70-85% of cases of anaemia in pregnancy all over the world and more especially in Africa (Hassan *et al.,* 2014). Anaemia in pregnancy is associated with maternal problems such as hemorrhage and increased maternal morbidity and mortality (WHO/NHD, 2001; Merchant *et al.*, 2002). It is also associated with adverse fetal outcomes such as intrauterine growth retardation,

pre-term delivery, low birth weight and fetal death (Pallister, 1994; Merchant *et al.*, 2002). In Nigeria, Isah, *et al* (1985) found a prevalence of anaemia of 46% in non-pregnant and 52% of pregnant women respectively in Zaria and Iron deficiency was found in 54% and 25% of non-pregnant and pregnant women respectively. Gwarzo, *et al* (1994) found a prevalence of iron deficiency of 17% among pregnant women in Kano.

The fact that iron is the most abundant metal in the world, but also involves the most common dietary deficiency worldwide, may relate to a protective cellular mechanism that evolved in malaria endemic areas whereby iron used in the life cycle of parasites is effectively restricted (Biesalski and Erhard, 2007). Iron is essential for many metabolic processes including DNA, RNA and protein synthesis, the formation and maintenance of myelin, and is a co-factor of many haem and non-haem enzymes. Clinical consequences of iron deficiency include central nervous system dysfunction including cognitive impairment, decreased physical capacity, impaired work performance due to chronic fatigue, pregnancy complications, reduced immunity, gastrointestinal disturbances (e.g. glossitis, stomatitis, gastritis), and impaired temperature regulation in a cold environment.

Iron deficiency generally occurs in three sequential stages: depleted iron stores, iron deficiency erythropoesis and iron deficiency. All three stages can be analyzed biochemically with the measurement of Hb, ferritin and sTfR (Kotze *et al.,* 2009). Although there are some clinical indicators and the evaluation of iron intake might be helpful, the diagnosis relies mainly on these parameters.

The measurement of Hb is essential for the diagnosis of nutritional and is one of the most common, easiest and least expensive methods. Unfortunately, Hb measurement is not very

sensitive and specific for iron deficiency. Thus, to determine whether iron deficiency is responsible for anaemia, it is usually necessary to include other indicators.

Iron deficiency may result from inadequate intake, increased iron requirements due to genetic risk factors, increased blood loss or decreased absorption of iron, or a combination of these factors. Nutritional anaemia is also characterized by other nutritional deficiencies. Both copper and zinc are essential nutrients and deficiencies of both result in anaemia. Resistance to infections depends on a healthy immune function and copper and zinc are both necessary for normal function of the immune system. Copper deficit should be included in the differential diagnosis of anaemia unresponsive to iron supplementation (Kotze *et al.*, 2009).

Iron deficiency is linked to increased homocysteine levels, which is a marker of vitamin B deficiencies and inflammation. Low levels of vitamin B_{12} conversely lead to low iron uptake, which is associated with gastritis and atrophy of glands producing intrinsic factor in the stomach. (Kotze *et al.*, 2009).

2.2.8.1 Consequences of iron deficiency

Iron deficiency can exist with or without anaemia. Some functional changes may occur in the absence of anaemia, but the most functional deficits appear to occur with the development of anaemia (Wood and Ronnenberg, 2005). Even mild and moderate forms of iron deficiency anaemia can be associated with functional impairments affecting cognitive development (Beard and Connor 2003; Abbaspour *et al.*, 2014), immunity mechanisms (Failla, 2003) and work capacity (Viteri and Torun, 1974; Abbaspour *et al.*, 2014).

Iron deficiency during pregnancy is associated with a variety of adverse outcomes for both mother and infant, including increased risk of sepsis, maternal mortality, perinatal mortality, and

low birth weight (CDC, 2010). Iron deficiency and anaemia also reduce learning ability and are associated with increased rates of morbidity (CDC, 2010).

2.2.8.2 Prevention of iron deficiency

The four principle strategies for correcting micronutrient deficiencies in populations can be used for correcting iron deficiency, either alone or in combination. These are education combined with dietary modification, to improve iron intake and bioavailability; iron supplementation (provision of iron, usually in higher doses, without food), iron fortification of foods and the new approach of biofortification. However, there are some difficulties in the application of some of these strategies when considering iron (Abbaspour *et al*., 2014).

Food diversification: Dietary modifications involve increased intake of iron rich foods, especially flesh foods, increased consumption of fruits and vegetables rich in ascorbic acid to enhance nonhaem iron absorption, and reduced intake of tea and coffee, which inhibit nonhaem iron absorption (Hurrell, 2002 ; FAO/WHO, 2004). Another strategy is to reduce antinutrient contents in order to make the iron supplied from food sources more available. Iron bioavailability may be increased by techniques such as germination and fermentation, which promote enzymatic hydrolysis of phytic acid in whole grain cereals and legumes by enhancing the activity of endogenous or exogenous phytase enzymes (Cook, 2005). Even the use of nonenzymatic methods, such as thermal processing, soaking, and milling, for reducing phytic acid content in plant-based staples has been successful in improving the bioavailability of iron (and zinc) (Liang *et al*., 2008; Schlemmer *et al*., 2009).

Supplementation: For oral iron supplementation, ferrous iron salts (ferrous sulfate and ferrous gluconate) are preferred because of their low cost and high bioavailability (Larocque *et al*., 2005). Although iron absorption is higher when iron supplements are given on an empty stomach, nausea, and epigastric pain might develop due to the higher iron doses administered (usually 60 mg Fe/day). If such side-effects arise, lower doses between meals should be attempted or iron should be provided with meals, although food reduces absorption of medicinal iron by about two-thirds (Cavalli-Sforza *et al*., 2005). Iron supplementation during pregnancy is advisable in developing countries, where women often enter pregnancy with low iron stores. Although the benefits of iron supplementation have generally been considered to outweigh the putative risks, there is some evidence to suggest that supplementation at levels recommended for otherwise healthy children carries the risk of increased severity of infectious disease in the presence of malaria (Oppenheimer 2001; Sazawal *et al*., 2006).

Fortification: Fortification of foods with iron is more difficult than fortification with nutrients, such as zinc in flour, iodine in salt, and vitamin A in cooking oil (Larocque *et al.,* 2005). The most bioavailable iron compounds are soluble in water or diluted acid but often react with other food components to cause off-flavors, color changes or fat oxidation (Hurrell, 2002).Thus, less soluble forms of iron, although less well absorbed, are often chosen for fortification to avoid unwanted sensory changes (Larocque *et al*., 2005). Fortification is usually made with much lower iron doses than supplementation. It is closer to the physiological environment and might be the safest intervention in malarious areas (WHO, 2007).There is no concern over the safety of iron supplementation or iron fortification in non malarial endemic areas (Hurrell, 2007).

Iron compounds recommended for food fortification by the World Health Organization (WHO and FAO, 2006) include ferrous sulfate, ferrous fumarate, ferric pyrophosphate, and electrolytic

iron powder. Wheat flour is the most common iron fortified food and it is usually fortified with elemental iron powders which are not recommended by WHO (WHO and FAO, 2006; WHO, 2009). Hurrell and Egl, (2010) reported that of the 78 national wheat flour programs only eight would be expected to improve iron status. These programs used recommended iron compounds at the recommended levels. The other countries used non recommended compounds or lower levels of iron relative to flour intake. Commercial infant foods, such as formulas and cereals, are also commonly fortified with iron (Abbaspour *et al.,* 2014).

Biofortification: Iron contents vary from 25 to 56 mg/kg in the different varieties of wheat and 7-23 mg/kg in rice grains. However, most of this iron is removed during the milling process. Iron absorption from cereals and legumes, many of which have high native iron content, is generally low because of their high contents of phytate and sometimes polyphenols (Hurrell *et al*., 1999). Biofortification strategies include plant breeding and genetic engineering. Iron levels in common beans and millet have been successfully increased by plant breeding but other staple is more difficult or not possible (rice) due to insufficient natural genetic variation. Lucca *et al.,* (2002), increased the iron content in rice endosperm to improve its absorption in the human intestine by means of genetic engineering. They introduced a ferritin gene from *Phaseolus vulgaris* into rice grains, increasing their iron content up to twofold. To increase iron bioavailability, they introduced a thermotolerant phytase from *Aspergillus fumigates* into the rice endosperm. They indicated that this rice, with higher iron content and rich in phytase has a great potential to substantially improve iron nutrition in those populations where iron deficiency is so widely spread (Lucca *et al.,* 2002). Unfortunately the phytase did not resist cooking. The importance of various minerals such as zinc and iron needs more attention at individual and public health levels (Roohani *et al.,* 2013).

2.3 Fermentation

2.3.1 Definition

The term fermentation is derived from the Latin verb "fervere", to boil, which describes the appearance of the action of yeast on extracts of fruit or malted grain during the production of alcoholic beverages (Difo *et al.,* 2014). To the microbiologist, the term"fermentation" describes a form of energy-yielding microbial metabolism in which an organic substrate, usually a carbohydrate, is incompletely oxidized, and an organic carbohydrate acts as the electron acceptor (Adams, 1990). However to a biochemist, the term fermentation can be technically defined as the chemical transformation of organic compounds with the aid of enzymes particularly those made by microorganisms. It involves the conversion of large molecules to small molecules or molecular oxidation/ reduction mechanisms mediated by selected microorganisms (Yadav *et al.,* 2011). Thus, fermentation in food processing is defined as the conversion of carbohydrates to alcohol and carbon dioxide or organic acids using yeast and/or bacteria, under anaerobic conditions (William and Dennis, 2011).

2.3.2 Fermented foods

Fermented foods are those foods which have been subjected to the action of micro-organisms or enzymes so that desirable biochemical changes cause significant modification to the food. (Campbell-Platt, 1987). They are the source of alcoholic foods/beverages, vinegar, pickled vegetables, sausages, cheeses, yogurts, vegetable protein amino acid/peptide sauces and pastes with meat-like flavours, and leavened and sour-dough breads (Ojokoh *et al.,* 2013).

2.3.3 Benefits of fermentation

Several researches have shown that fermentation enhances the nutrient content of foods through the biosynthesis of vitamins, essential amino acids, micronutrient bioavailability and aids in degrading antinutritional factors (Oboh and Akindahunsi, 2003; Oboh, 2006). Many fermented foods, such as cheese, sauerkraut and fermented sausages, are preserved products, in that their shelf life is considerably extended over that of the raw materials from which they are made. In addition to being made shelf -stable, fermented foods have aroma and flavour characteristics that result directly or indirectly from the fermenting organisms (Steinkraus, 2002).

Fermentation of legumes enhances their nutritive value and antioxidant properties; reduces some anti-nutritional endogenous compounds such as phytic acid, and exerts beneficial effects on protein digestibility and biological value of legumes (Oboh *et al.*, 2012; Oyewole and Isah, 2012).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals

All chemicals and reagents used were of analytical grade.

3.1.2 Collection of plant materials

The leaves (*Telfaira occidentalis* and *Gnetum africanum*) were harvested from their natural habitat and were authenticated at the Herbarium, Department of Biological Sciences, Ahmadu Bello University, Zaria and a voucher number deposited, 23089 and 1259 for *Telfaira occidentalis* and *Gnetum africanum* leaves respectively.

3.1.3 Experimental animals

Forty (40) young Albino rats of both sexes were obtained and kept in well aerated plastic cages in the animal house, Department of Pharmacology and Therapeutics, Faculty of Pharmaceutical Sciences, Ahmadu Bello University, Zaria. The animals were acclimatized to the animal house environment for a period of one week before the commencement of the study. They were fed with growers mash and water *ad libitum* (during the acclimatization).

3.2 Methods

3.2.1 Preparation of the vegetable leaves

The harvested vegetables were washed and air dried at room temperature and then grinded using the grinding mill. The grinded leaves were used to form a supplemental diet for the experimental animals.

3.1.3 Fermentation of the vegetables

Open fermentation was done, the vegetables were soaked in deionized water for 72 hours at room temperature for the inherent fermenting microorganisms and environmental fermenting microorganisms to act. The fermented vegetables were then filtered out and oven dried, then grinded and stored for use in feed supplementation formulation.

3.1.4 Preparation of diet (iron deficient and iron sufficient diet)

Iron deficient and sufficient diets were prepared according to the method of Yi Ning *et al* (2002). (Modified)

Ingredient (g/kg diet)	**Iron sufficient**	**Iron deficient**
Cornstarch	625.0	625.0
Casein	200.0	200.0
Fiber	50.0	50.0
Groundnut oil	70.0	70.0

Mineral mix	35.0	35.0
Vitamin mix	10.0	10.0
Ferrous sulphate (g/kg)	10.0	0.00

3.2.2 Induction of anaemia

Iron deficiency anaemia was induced by feeding the animals exclusively on the formulated iron deficient diet for 8 weeks. Haemoglobin (Hb) concentration was used as index for anaemia and rats with Hb concentration of < 11g/dl (Velmurugan *et al.,* 2010) were used for the study.

3.2.3 Animal grouping

The experimental rats were divided into 8 groups, with 5 animals per group and fed on supplemented diet for 3 weeks.

Group 1: Normal rats (non anaemic) were fed with deionized water and iron sufficient diet *ad libitum*

Group 2: Anaemic rats continued on the iron deficient diet and deionized water *ad libitum*

Group 3: Anaemic rats were given iron sufficient diet and deionized water *ad libitum*

Group 4: Anaemic rats were given iron deficient diet supplemented with 10% *Telfaira occidentalis* and deionized water *ad libitum*

Group 5:*Anaemic rats were given* iron deficient diet *supplemented with 10% fermented Telfairia occidentalis* and deionized water *ad libitum*

Group 6: Anaemic rats were given iron deficient diet supplemented with 10% *Gnetum africanum* and deionized water *ad libitum*

Group 7: Anaemic rats were given iron deficient diet supplemented with 10% fermented *Gnetum africanum* and deionized water *ad libitum*

Group 8: Anaemic rats were given iron deficient diet and treated orally with ferrous sulphate tablets (2.79 mg/kg body weight) and deionized water *ad libitum*

3.2.4 Quantitative determination of mineral content

Flame Atomic Absorption Spectroscopy (FAAS) was used to determine the quantity of minerals (Fe, Ca, Mg, Cu and Zn) in the powdered leave samples.

3.2.4.1 Wet digestion of the leaves (Majolagbe et al., 2013)

Powdered plant sample (0.2g) was weighed into the digestion tube and 5ml of HNO_3-$HClO_4$ (2:1 by volume) mixture was added and mixed. The tube was placed into the digestion block inside a fume hood, the temperature was set at 150^0C and digestion was done for 1hr 30min. The temperature was then increased to 230^0C and digested for another 30 min (white fuming stage). The temperature was later reduced to 150^0C, 1ml of HCl was added and heated for another 30min. The digestor was switched off and 30ml of distilled water was added, more water was added to get to 50ml mark on the tube. The concentration of Fe, Mg, Zn, Cu and Ca was read using the atomic absorption spectrophotometer (AA 500 M0DEL).

3.2.5 Determination of the speciation pattern of iron (Zakir and Shikazono, 2011)

The speciation pattern of iron in the vegetables was determined using a modified combination of Tessier *et al* (1979) and Hall *et al* (1996) scheme.

3.2.5.1 Exchangable phase

Twenty ml of 1M solution of $MgCl_2$ pH 7.0, was added to 1g of the dry sample, the mixture was stirred continuously at room temperature for 1 hr, centrifuged at 3000rpm for 30min, filtered and the liquid extract was analyzed for iron using the FAAS

3.2.5.2 Carbonate phase (Exchangable phase)

Eight ml of 1M solution of NaOAc pH 5.0 (adjusted with HOAc) was added to the residue from step 1, the mixture was stirred continuously at room temperature for 6hr and then centrifuged at 3000rpm for 30min, filtered and the liquid extract was analyzed for iron using FAAS

3.2.5.3 Amorphous Fe oxyhydroxide phase (reducible)

Twenty ml of 0.25M solution of $NH_2OH.HCl$ was added to the residue from step 2, the mixture was heated at 60^0C for 2hr and vortexed then centrifuged at 3000rpm for 30min, filtered and the liquid extract was analyzed for iron using the FAAS.

3.2.5.4 Crystalline Fe oxide phase (reducible)

Thirty ml of 1M solution of $NH_2.HCl$ was added to the residue from step 3 and was heated for 3hr at 90^0C then vortexed 9 times, centrifuged at 3000rpm for 30 min, filtered

and the liquid extract was analyzed for iron using FAAS

3.2.5.5 Sulphides and organic phase (oxidizable)

Potassium trioxochlorate (v) (750mg) and hydrochloric acid (5ml) of 12M solution were added to the residue from step 4 and vortex. Hydrochloric acid (10ml) of 12M solution was added to the mixture then vortexed and left for 30min. Fifteen mls of deionised water was added and vortexed again. Finally, ten mls 4M solution of HNO_3 was added and vortexed. The mixture was heated for 20min at 90^0C, centrifuged at 3000rpm for 30min and the liquid extract was analyzed for iron using FAAS

3.2.5.6 Silicates and residual phase

Two ml of 16M solution of HNO_3 was added to the residue from step 5 and heated to dryness at 200^0C then cooled. Two mls of 12M solution of HCl was added and heated. Ten mls of aqua regia solution was added and the mixture heated to dryness at 120^0C then hydrochloric acid (1ml), trioxonitrate(v) acid (3ml) and 3ml of deionised water were added and warmed. The mixture was then centrifuged at 3000rpm for 30min, then the liquid extract was analyzed for iron using FAAS

3.2.5.7 In vitro bioavailable iron

This was obtained by summing the exchangeable, oxidizable and reducible iron content of the vegetables.

3.2.6 Quantitative determination of vitamin C

Vitamin C was determined using the UV Spectroscopy as described by Kunti *et al.*, 2012)

The plant sample (0.05g) was weighed and transferred into a test tube. Twenty five mls of methanol was added and the mixture was allowed to stand for 10min to extract properly. The sample was filtered and 1ml of the filterate was transferred into a cuvette and 3ml of the extracting solvent (methanol) was added, Vitamin C concentration was then determined using the UV spectrophotometer.

3.2.7 Quantitative determination of some antinutrients

3.2.7.1 Phytic acid determination

This was determined using the procedure described by Lucas and Maskakas (1975).

Each sample (2g) was weighed into 250ml conical flask, 100ml of 2% concentrated HCl was used to soak each sample for 3hrs. The mixture was filtered and 50ml of each filterate was placed in 250ml beaker and 107ml of distilled water was added to each solution, ten mls of 0.003M thiocyanate solution was then added as indicator and then titrated with standard iron chloride solution which contains 0.00195g iron per ml.

% Phytic acid= $Y \times 1.19 \times 100$

Where Y = Titre value × 0.00195

3.2.7.2 Oxalic acid determination

This was determined using the titration method described by Day and Underwood (1986).

The sample (1g) was weighed into 100ml conical flask where 75ml of 3M H_2SO4 was added and stirred for 1hr. It was then filtered using Whatman No 1 filter paper. From the filterate, 25ml was taken and titrated while hot (80-90^{0}C) against 0.1M $KMnO_4$ solution

until a faint pink colour persisted for at least 30 sec. The overall redox reaction is:

$MnO_4 + C_2O_4^{2} + 8H^{+} \quad Mn^{2+} + 4H_2O + 2CO_2$

Oxalate (mg/100g) = T × Vme × D.F × 100/Me × Ms (g)

Where T is the titre value of $KMnO_4$ (ml)

Vme is the volume mass equivalent ($1CM^3$ of 0.05M $KMnO_4$ solution is equivalent to 0.00225 anhydrous oxalic acid)

D.F is the dilution factor (Vt/A =75/25= 3)

Where Vt is the total volume of titrate(filterate 75ml) and

A is the aliquot used (25ml)

Me is the molar equivalent of $KMnO_4$ redox reaction

Ms is the mass of sample used

3.2.8 Determination of some haematological parameters

3.2.8.1 Packed cell volume

This was determined weekly by the microhematocrit method as described by Dacie and Lewis (2001).

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 pack 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 Estimation of haemoglobin concentration

This was determined bi-weekly by the Cyanomethaemoglobin method (Jain, 1986)

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 hematin. The acid hematin solution is then compared with a color standard.

Procedure: Sample solutions and standard solutions were prepared as follows.

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

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

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

The concentration of haemoglobin was measured 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 cyanmethaemoglobin, whose optical density was measured

photometrically.

The concentration of haemoglobin 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 Estimation of Red Blood Cell (RBC) and White Blood Cell (WBC) count

These were determined after the animals were sacrificed using the improved Neubauer counting chamber (Dacie and Lewis, 2001).

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 diluting fluid was drawn to 11 and 101 marks respectively. The fluid and the blood were mixed gently and transferred into the counting chamber. It was allowed to settle for 2 mins 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 was done using the x 40 objective.

Calculation: Number of cell counted divided by volume of one square multiplying number of squares counted and multiply by dilution factor.

3.2.8.4 Estimation of RBC indices

These were calculated by the method of Dacie and Lewis, 2001.

Mean corpuscular Volume (MCV) in fl was calculated as:

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

Mean corpuscular haemoglobin (MCH) in pg was calculated as:

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

Mean corpuscular haemoglobin concentration (MCHC) in g/dl was calculated as:

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

3.2.9 Determination of *in-vivo* iron indices

3.2.9.1 Determination of serum ferritin

Serum feritin was determined using the Abcam's Ferritin (FTL) Rat ELISA kit according to the manufacturer's protocol.

Principle: The ferritin present in samples reacts with the anti- ferritin antibodies which have been adsorbed to the surface of polystyrene microtiter wells. After removal of unbound proteins by washing, anti-ferritin antibodies conjugated with horseradish peroxidase (HRP), are added. These enzyme labeled antibodies form complexes with the previously bound ferritin. Following another washing step, the amount of enzyme bound in complex is measured by the addition of a chromogenic substrate, 3,3',5,5'-tetramethylbenzidine (TMB). The quantity of bound enzyme varies proportionately with the concentration of ferritin in the sample tested; thus, the absorbance, at 450nm, is a measure of the concentration of ferritin in the test sample. The quantity of ferritin in the test sample can be interpolated from the standard curve constructed from the standards, and corrected for sample dilution.

Assay Procedure: Each standard including zero control, (100µl) was pipetted into pre designated wells, the microtiter plate was then incubated at room temperature for 60 minutes (the plate was kept covered and level during the incubation) and then the contents of the well was aspirated using diluted wash buffer. 1x Enzyme-Antibody (100µl) Conjugate was pippeted into each well and incubated at room temperature for ten (10±2) minutes, the plate was kept covered and level in the dark during incubation, the wells were then washed by aspirating the contents of the wells using diluted wash buffer. TMB Substrate solution (100µl) was then pipetted into each well, this was incubated in the dark for precisely ten (10) minutes after which 100 µl of stop solution was added to each well. The absorbance of each well content was read at 450 nm using the microplate reader.

Calculation: Control blank reading was subtracted from all the standard readings. The standard readings were plotted against their concentrations and the best smooth curve through these points was drawn to construct the standard curve. Protein concentrations for the samples were then extrapolated from the standard curve plotted.

3.2.9.2 Estimation of haemoglobin- iron (Hb-Fe) concentration

This was calculated by adopting the method of Park, 1983; Buchowski, 1989

Hb Fe (mg) = BW X 0.067 X Grams Hb per mL X 3.35 mg Fe

Note: the calculation assumes 6.7% body weight (BW) is blood and the

iron content of haemoglobin is 3.35 mg/g

3.3 Statistical Analysis

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

CHAPTER FOUR

4.0 RESULTS

4.1 Vitamin C Content of Fermented and Unfermented *Telfaira occidentalis* and *Gnetum africanum* Leaves

Result of the Vitamin C content in unfermented and fermented *Telfaira occidentalis,* and *Gnetum africanum* is presented in Figure 4.1. *Gnetum africanum* leaves had significantly ($p<0.05$) higer vitamin C concentrations than *Telfaira occidentalis* leaves. Fermented *Telfaira occidentalis* had the least concentration. Fermentation had no significant ($p>0.05$) change in *Gnetum africanum* leaves but led to a significant ($p<0.05$) decrease in *Telfaira occidentalis* leaves.

4.2 Antinutrient Content of Fermented and Unfermented *Telfaira occidentalis* and *Gnetum africanum* Leaves

Antinutrients content of unfermented and fermented *Telfaira occidentalis,* and *Gnetum africanum* leaves is presented in Figures 4.2 and 4.3. *Gnetum africanum* leaves had significantly ($p \square 0.05$) higher levels of phytate and oxalate. There was a significant ($p \square 0.05$ reduction in the levels of phytate and oxalate for both vegetables after fermentation.

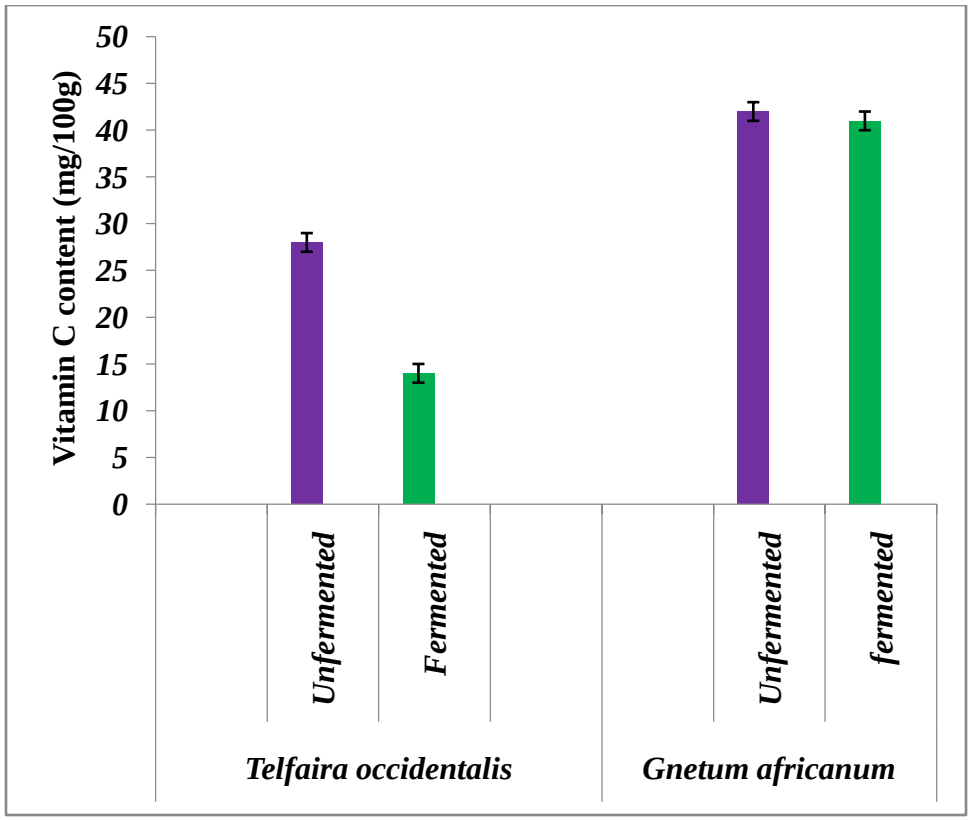


Fig.4.1: Vitamin C Content of Fermented and Unfermented *Telfaira occidentalis* and *Gnetum africanum* Leaves

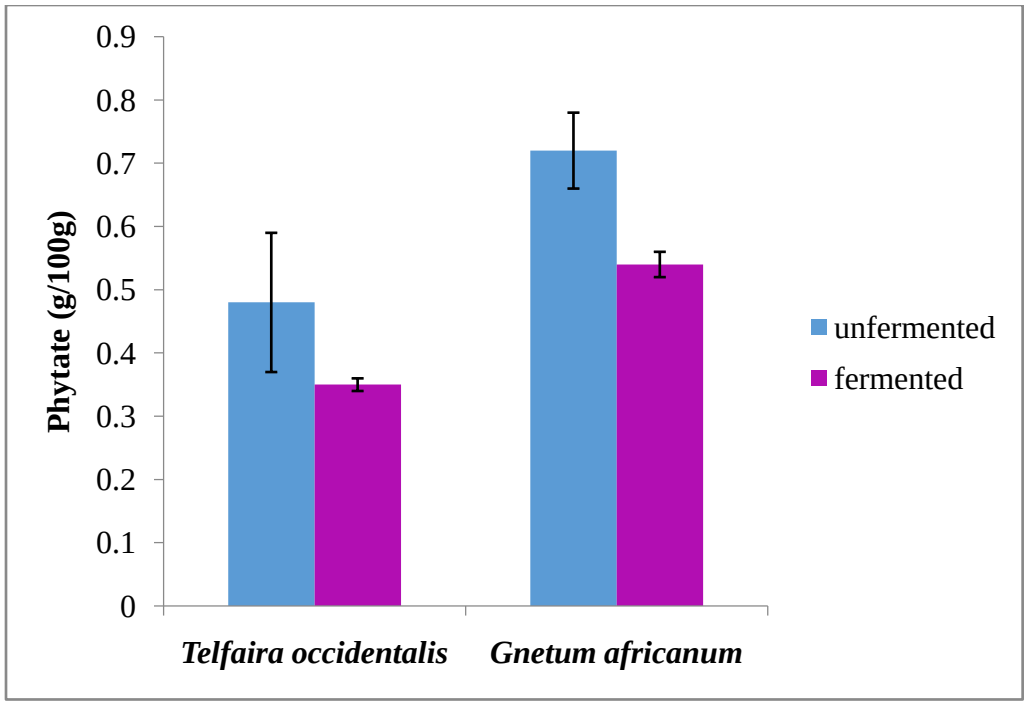


Fig.4.2. Phytate Content of Fermented and Unfermented *Telfaira occidentalis* and *Gnetum africanum* Leaves

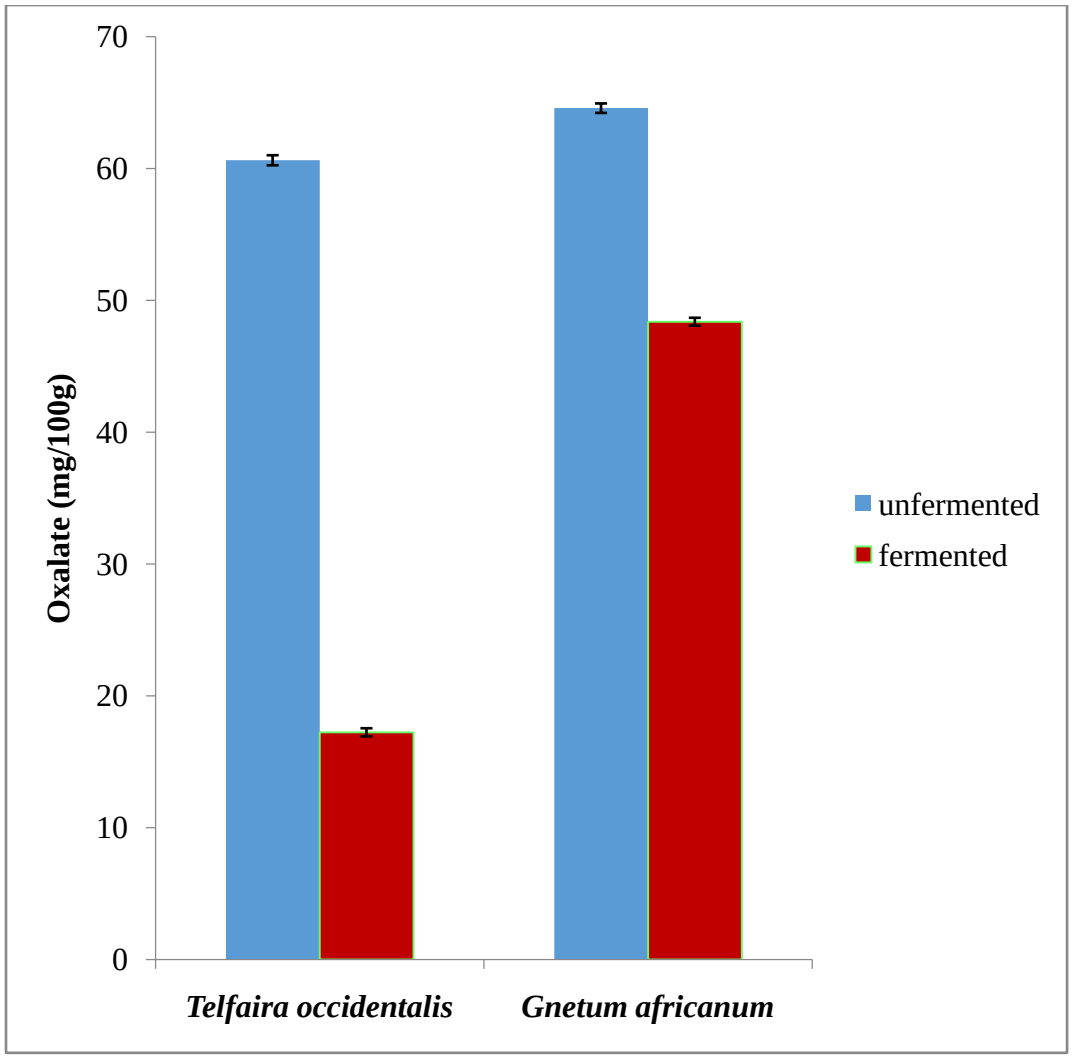


Fig.4.3: Oxalate Content of Fermented and Unfermented *Telfaira occidentalis* and *Gnetum africanum* Leaves

4.3 Mineral Content of Fermented and Unfermented *Telfaira occidentalis* and *Gnetum africanum* Leaves

The mineral content in unfermented and fermented *Telfaira occidentalis,* and *Gnetum africanum* leaves is shown in Table 4.1. *Telfaira occidentalis* had significantly ($p<0.05$) higher content of Fe, Ca, Cu, Zn and Mg than *Gnetum africanum* leaves. Fermentation significantly ($p<0.05$) increased the level of Zn, Ca, and Cu of both vegetable leaves. Iron (Fe) significantly ($p<0.05$) increased while Mg significantly ($p<0.05$) decreased in *Telfaira occidentalis*. In *Gnetum africanum*, the level of Fe significantly ($p<0.05$) decreased while Mg showed no significant ($p>0.05$) increase.

4.4 Speciation Pattern of Iron in Fermented and Unfermented *Telfaira occidentalis* and *Gnetum africanum* Leaves

Speciation pattern of iron in fermented and unfermented *Telfaira occidentalis,* and *Gnetum africanum* leaves is presented in Table 4.2. The result shows that the predominant iron species in the vegetables were in residual forms. Fermentation led to an increase in the total sum of iron in all the fractions. *Gnetum africanum* had the highest concentration of residual iron forms. Also, *Telfaira occidentalis* had a higher concentration of bioavailable iron than *Gnetum africanum,* however bioavailability was highest in the fermented vegetables.

Table 4.1: Mineral Content of Fermented and Unfermented *Telfaira Occidentalis* and *Gnetum Africanum* Leaves

SAMPLE	MINERAL ELEMENT (mg/100g)				
	Fe	**Mg**	**Zn**	**Ca**	**Cu**
Telfaira occidentalis	77.63 ± 0.05^{c}	54.88 ± 0.60^{b}	7.28 ± 0.61^{c}	1029.77 ± 0.03^{b}	1.12 ± 0.02^{c}
***Telfaira occidentalis,* Fermented**	157.07 ± 0.14^{d}	44.43 ± 0.51^{a}	17.12 ± 0.06^{d}	1596.42 ± 0.25^{d}	1.21 ± 0.06^{d}
Gnetum africanum	21.33 ± 1.53^{b}	45.20 ± 0.63^{a}	1.40 ± 0.06^{a}	884.13 ± 0.25^{a}	0.30 ± 0.01^{a}
***Gnetum africanum*, Fermented**	20.49 ± 0.13^{a}	45.43 ± 0.51^{a}	3.58 ± 0.01^{b}	1316.42 ± 0.21^{c}	0.91 ± 0.08^{b}

Values are mean ± S.D of three determinations. Values with different superscript down the column differ significantly($P<0.05$)

Table 4.2 Speciation Pattern of Iron in Fermented and Unfermented *Telfaira occidentalis* and *Gnetum africanum* Leaves

SAMPLE	IRON FORMS (mg/100g)						
	EXC	**RED**	**OXI**	**RES**	**SOF**	**BAL**	**%BAL**
***Telfaira occidentalis,* unfermented**	5.90	4.03	20.30	22.66	52.89	30.23	57
***Telfaira occidentalis,* Fermented**	4.03	7.76	33.36	24.74	69.89	45.15	65
***Gnetum africanum,* unfermented**	2.16	5.14	13.58	27.85	48.73	20.88	43
***Gnetum africanum,* Fermented**	3.28	3.65	15.45	49.31	71.69	22.38	31

EXC: Exchangable, **RED:** Reducible, **OXI:** Oxidizable, **RES:** Residual, **S.O.F:** Sum of Fractions, **BAL:** Bioavailable

4.5 Relative Weight Change of Iron Deficient Rats Supplemented With Fermented and Unfermented *Telfaira occidentalis* and *Gnetum africanum* Leaves

The relative weight change of iron deficient rats during supplementation with unfermented and fermented *Telfaira occidentalis* and *Gnetum africanum* leaves is shown in Figure 4.4. At day 7, there was a significant ($p<0.05$) decrease in the weight change of rats in the iron deficient control group when compared to rats in iron sufficient control group, weight change of anaemic rats on iron sufficient diet, and the vegetable supplemented groups were significantly ($p<0.05$) lower when compared to rats in iron sufficient control group, but not significant ($p>0.05$) when compared to rats in iron deficient control group, this trend also occurred at day 14 only that the weight change of rats on unfermented *G.africanum* and those treated with $FeSO_4$ did not differ significantly ($p>0.05$) from that of the iron sufficient control group.

At day 21, the weight change of rats in the treatment groups did not differ significantly ($p>0.05$) from the iron sufficient control group except for rats in groups supplemented with fermented *T.occidentalis* and fermented *G.africanum* respectively which did not differ significantly ($p>0.05$) from that of the iron deficient control group.

4.6 Haemoglobin Iron Concentration of Iron Deficient Rats Supplemented With Fermented and Unfermented *Telfaira occidentalis* and *Gnetum africanum* Leaves

The effect of fermented and unfermented *Telfaira occidentalis* and *Gnetum africanum* leaves supplementation on the haemoglobin- iron of iron deficient rats is presented in Table 4.3. There was a significant ($p<0.05$) decrease in the initial haemoglobin iron concentration of rats in all the treated groups except those supplemented with *Telfaira occidentalis* leaf when compared to rats in the iron sufficient control group. Percentage change in haemoglobin iron was significantly ($p<0.05$) higher in anaemic rats supplemented with iron sufficient diet, unfermented *T.occidentalis* and those treated with $FeSO_4$ when compared to that of rats in the iron deficient control group.

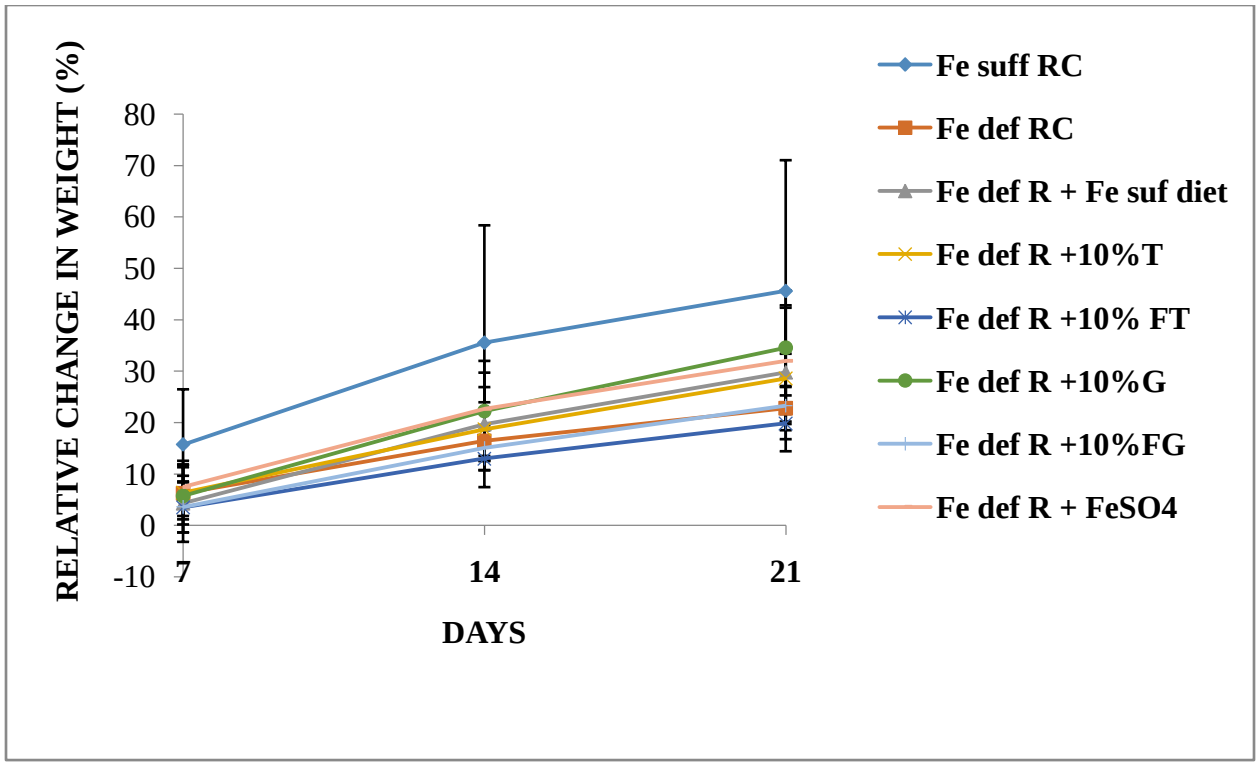


Figure 4.4: Effect of Fermented and Unfermented *Telfaira occidentalis* and *Gnetum africanum* Leaves Supplementation on Relative Change in Weight of Iron Deficient Rat

Fe suf RC: Iron sufficient Rat control, **Fe def. RC**: Iron deficient Rat control, **Fe def. R+ Fe suf. Diet**: Iron deficient Rat on iron sufficient diet, **Fe def. R+10%T:** Iron deficient Rat on iron deficient diet supplemented with 10% *Telfaira occidentalis*, **Fe def. R+10% FT:**Iron deficient Rat on on iron deficient diet supplemented with 10% fermented *Telfaira occidentalis*, **Fe def. R+10%G:** Iron deficient Rat on iron deficient diet supplemented with 10% *Gnetum africanum*, **Fe def. R+10%FG:** Iron deficient Rat on iron deficient diet supplemented with 10% fermented *Gnetum africanum* **, Fe def. R+$FeSO_4$:** Iron deficient Rat on iron deficient diet treated orally with 2.8mg/kg Ferrous Sulphate

Table 4.3: Effect of Fermented and Unfermented *Telfaira occidentalis* and *Gnetum africanum* Leaves Supplementation on Haemoglobin-Iron of Iron Deficient Rats

GROUPS n=5	INITIAL HB-FE (Day 0)	FINAL HB-FE (Day 21)	CHANGE IN HB	% CHANGE IN HB- FE
Fe suf. RC	2.51±0.19^{c}	3.97±0.54^{abc}	1.46±0.69^{a}	60.05±33.42^{a}
Fe def RC	2.01±0.41^{ab}	3.36±0.87^{ab}	1.35±0.48^{a}	65.10±16.50^{a}
Fe def R + Fe suf diet	1.69±0.27^{a}	3.81±0.53^{ab}	2.12±0.38^{ab}	126.75±24.81^{d}
Fe def R +10%*T*	2.45±0.33^{c}	4.96±0.83^{c}	2.51±0.51^{b}	102.03±12.54^{bcd}
Fe def R +10% *FT*	2.23±0.25^{bc}	4.16±0.43^{abc}	1.93±0.32^{ab}	87.33±15.17^{abc}
Fe def R +10%G	1.66±0.25^{a}	3.19±0.92^{a}	1.53±0.77^{a}	90.63±38.06^{abc}
Fe def R +10%FG	1.94±0.08^{ab}	3.49±0.34^{ab}	1.55±0.37^{a}	80.01±20.56^{ab}
Fe def R + FeSO4	1.99±0.40 ^{b}	4.36±0.74^{bc}	2.37±0.39^{b}	121.20±16.13^{cd}

Values with different superscript down the column differ significantly ($p<0.05$)

Fe suf RC: Iron sufficient Rat control, **Fe def. RC**: Iron deficient Rat control, **Fe def. R+ Fe suf. Diet**: Iron deficient Rat on iron sufficient diet, **Fe def. R+10%T:** Iron deficient Rat on iron deficient diet supplemented with 10% *Telfaira occidentalis*, **Fe def. R+10% FT:**Iron deficient Rat on on iron deficient diet supplemented with 10% fermented *Telfaira occidentalis*, **Fe def. R+10%G:** Iron deficient Rat on iron deficient diet supplemented with 10% *Gnetum africanum*, **Fe def. R+10%FG:** Iron deficient Rat on iron deficient diet supplemented with 10% fermented *Gnetum africanum* **, Fe def. R+$FeSO_4$:** Iron deficient Rat on iron deficient diet treated orally with 2.8mg/kg Ferrous Sulphate

4.7 Haemoglobin Concentration of Iron Deficient Rats Supplemented With Fermented and Unfermented *Telfaira occidentalis* and *Gnetum africanum* Leaves

The haemoglobin (Hb) concentration of iron deficient rats during supplementation with unfermented and fermented *Telfaira occidentalis* and *Gnetum africanum* leaves is shown in Figure 4.5. At day 0, the haemoglobin concentration of rats in all the treated groups were significantly ($p<0.05$) lower than that of the iron sufficient control group but was not significantly ($p>0.05$) different from that of the iron deficient control group. Hb concentration increased at day 7 and 21, respectively. The increase in all the treatment groups at day7 was significantly ($p<0.05$) higher when compared to the iron deficient control group except for those on unfermented *G.africanum* leaf, same trend occurred at day 21 except for rats on unfermented and fermented *G.africanum* leaves respectively which did not differ significantly ($p>0.05$) from the iron deficient control group.

4.8 Mean Corposcular Haemoglobin Concentration of Iron Deficient Rats Supplemented With Fermented and Unfermented *Telfaira occidentalis* and *Gnetum africanum Leaves*

The mean corpuscular haemoglobin concentration (MCHC) of iron deficient rats during supplementation with unfermented and fermented *Telfaira occidentalis* and *Gnetum africanum* leaves is shown in Figure 4.6. At day 0 and 7, there was a significant ($p<0.05$) decrease in the MCHC of rats in iron deficient control when compared to rats in the iron sufficient control group while the MCHC of rats in the other groups shows no significant ($p>0.05$) change. The MCHC of rats in the treatment groups shows a significant ($p<0.05$) decrease when compared to rats in the iron sufficient control group. At day 21, there was no significant ($p \square 0.05$) difference in the MCHC of rats between all the groups.

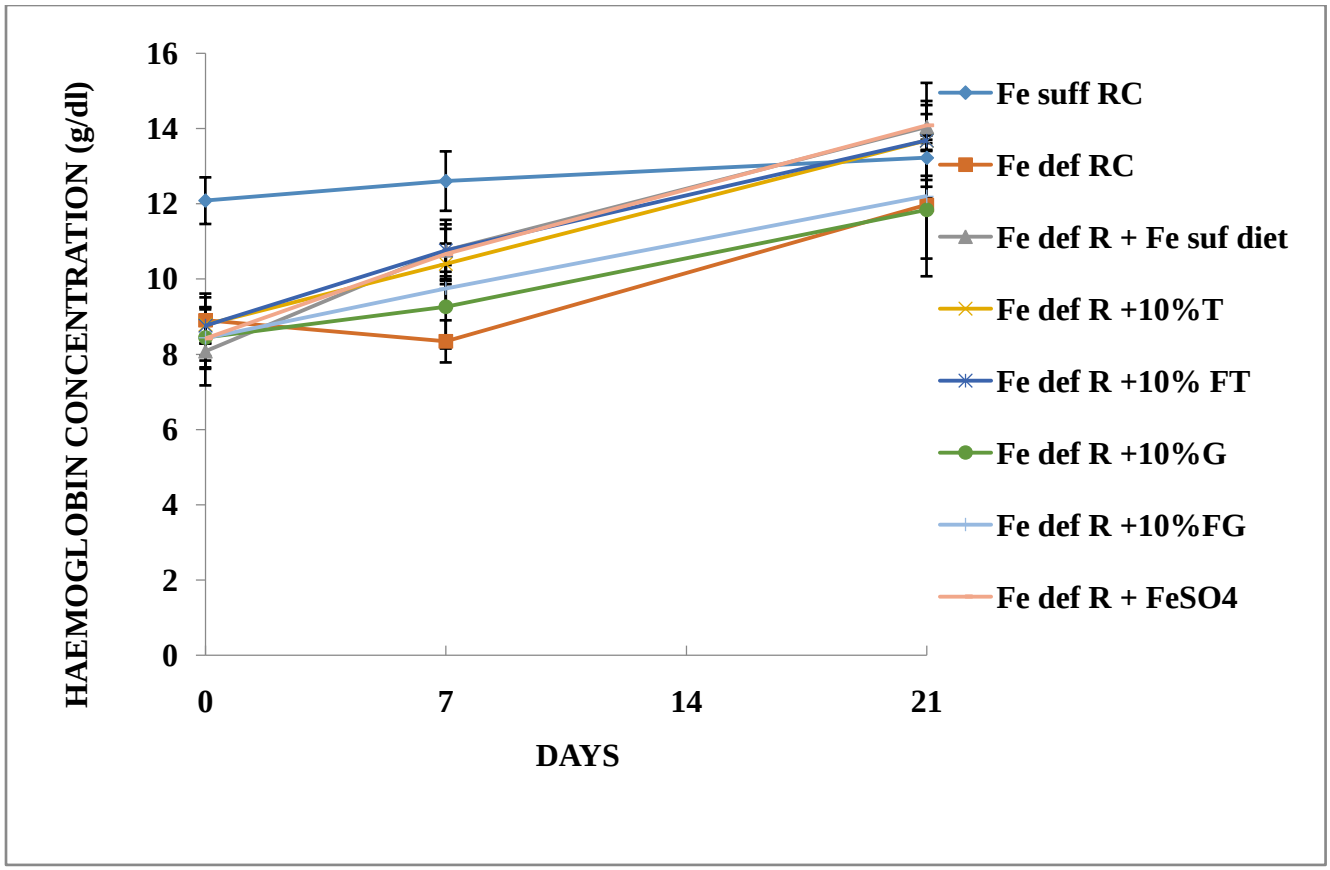


Figure 4.5: Effect of Fermented and Unfermented *Telfaira occidentalis* and *Gnetum africanum* Leaves Supplementation on Haemoglobin Concentration of Iron Deficient Rats

Fe suf RC: Iron sufficient Rat control, **Fe def. RC**: Iron deficient Rat control, **Fe def. R+ Fe suf. Diet**: Iron deficient Rat on iron sufficient diet, **Fe def. R+10%T:** Iron deficient Rat on iron deficient diet supplemented with 10% *Telfaira occidentalis*, **Fe def. R+10% FT:**Iron deficient Rat on on iron deficient diet supplemented with 10% fermented *Telfaira occidentalis*, **Fe def. R+10%G:** Iron deficient Rat on iron deficient diet supplemented with 10% *Gnetum africanum*, **Fe def. R+10%FG:** Iron deficient Rat on iron deficient diet supplemented with 10% fermented *Gnetum africanum* **, Fe def. R+$FeSO_4$:** Iron deficient Rat on iron deficient diet treated orally with 2.8mg/kg Ferrous Sulphate

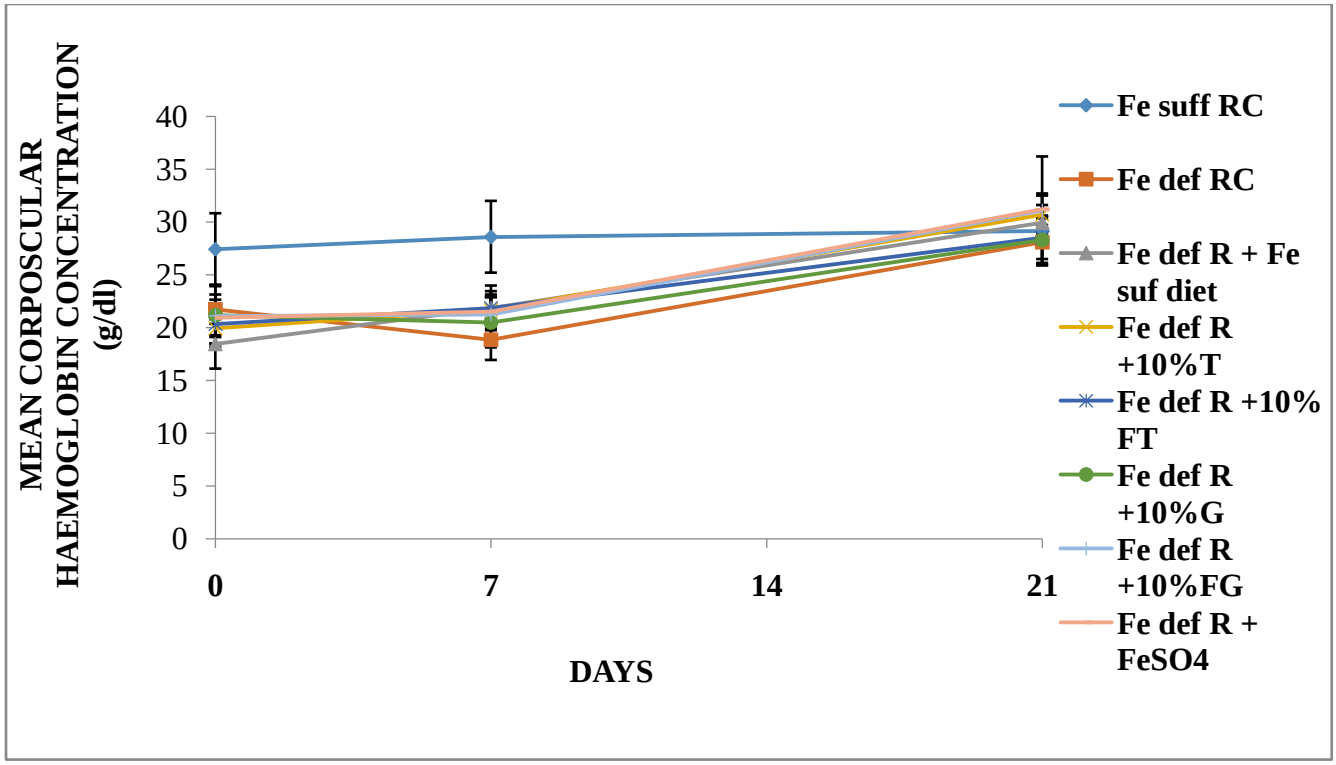


Figure 4.6: Effect of Fermented and Unfermented *Telfaira occidentalis* and *Gnetum africanum* Leaves Supplementation on Mean Corposcular Haemoglobin Concentration of Iron Deficient Rats

Fe suf RC: Iron sufficient Rat control, **Fe def. RC**: Iron deficient Rat control, **Fe def. R+ Fe suf. Diet**: Iron deficient Rat on iron sufficient diet, **Fe def. R+10%T:** Iron deficient Rat on iron deficient diet supplemented with 10% *Telfaira occidentalis*, **Fe def. R+10% FT:**Iron deficient Rat on on iron deficient diet supplemented with 10% fermented *Telfaira occidentalis*, **Fe def. R+10%G:** Iron deficient Rat on iron deficient diet supplemented with 10% *Gnetum africanum*, **Fe def. R+10%FG:** Iron deficient Rat on iron deficient diet supplemented with 10% fermented *Gnetum africanum*, **Fe def. R+$FeSO_4$:** Iron deficient Rat on iron deficient diet treated orally with 2.8mg/kg Ferrous Sulphate

4.9 Serum Ferritin Concentration of Iron Deficient Rats Supplemented With Fermented and Unfermented *Telfaira occidentalis* and *Gnetum africanum Leaves*

The serum concentration of ferritin in iron deficient rats supplemented with fermented and unfermented *Telfaira occidentalis* and *Gnetum africanum* leaves is presented in Figure 4.7. Serum ferritin shows significantly ($p<0.05$) higher concentration in rats in groups supplemented with iron sufficient diet and unfermented and fermented *T.*occidentalis leaves respectively when compared to the iron deficient control group. Ferritin concentration of rats in groups supplemented with unfermented and fermented *G.africanum* leaves respectively and those treated with $FeSO_4$ did not differ significantly ($p>0.05$) from that of the iron deficient control group.

4.10 Some Haematological Parameters of Iron Deficient Rats Supplemented With Fermented and Unfermented *Telfaira occidentalis* and *Gnetum africanum* Leaves

The haematological parameters of iron deficient rats supplemented with fermented and unfermented *Telfaira occidentalis* and *Gnetum africanum* leaves at the end of 21 days is presented in Table 4.4. Red blood cell count was significantly ($p<0.05$) higher in group treated with $FeSO_4$ when compared to iron deficient control group and unfermented *G.africanum* group. White blood cell count did not differ significantly ($p>0.05$) between all the groups. Packed cell volume was significantly ($p<0.05$) lower in fermented *G.africanum* group when compared to group on iron sufficient diet and unfermented *G.africanum* leaf respectively, haemoglobin concentration was significantly ($p<0.05$) higher in groups on iron sufficient diet, unfermented and fermented *Telfaira occidentalis* respectively and those treated with $FeSO_4$ when compared to iron deficient control group and rats on unfermented *G.africanum* leaf. MCV, MCH and MCHC did not differ significantly ($p>0.05$) between all the groups.

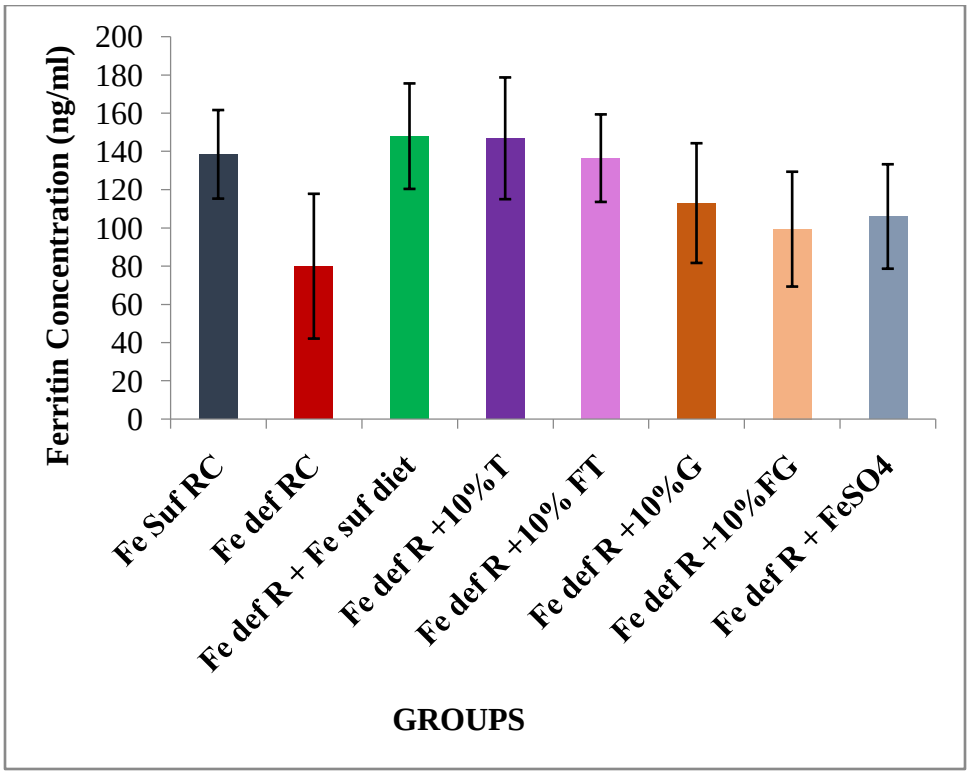


Figure 4.7: Effect of Fermented and Unfermented *Telfaira occidentalis* and *Gnetum africanum* Leaves Supplementation on Serum Ferritin Concentration of Iron Deficient Rats

Fe suf RC: Iron sufficient Rat control, **Fe def. RC**: Iron deficient Rat control, **Fe def. R+ Fe suf. Diet**: Iron deficient Rat on iron sufficient diet, **Fe def. R+10%T:** Iron deficient Rat on iron deficient diet supplemented with 10% *Telfaira occidentalis*, **Fe def. R+10% FT:**Iron deficient Rat on on iron deficient diet supplemented with 10% fermented *Telfaira occidentalis*, **Fe def. R+10%G:** Iron deficient Rat on iron deficient diet supplemented with 10% *Gnetum africanum*, **Fe def. R+10%FG:** Iron deficient Rat on iron deficient diet supplemented with 10% fermented *Gnetum africanum*, **Fe def. R+$FeSO_4$:** Iron deficient Rat on iron deficient diet treated orally with 2.8mg/kg Ferrous Sulphate

Table 4.4: Effect of Fermented and Unfermented *Telfaira occidentalis* and *Gnetum africanum* Leaves Supplementation on Some Haematological Parameters in Iron Deficient Rats

GROUP n=5	RBC count ($x10^6$µl)	WBC count ($x10^6$µl)	PCV %	Hb (g/dl)	MCV (fl)	MCH (pg)	MCHC (g/dl)
Fe suf RC	4.46 ± 0.19^{abc}	5.80 ± 2.01^{a}	45.40 ± 2.30^{ab}	13.22 ± 0.59^{abc}	101.18 ± 9.25^{a}	29.80 ± 2.25^{a}	29.15 ± 1.44^{a}
Fe def. RC	4.20 ± 0.37^{ab}	7.26 ± 1.81^{a}	42.71 ± 4.86^{ab}	11.97 ± 1.43^{a}	101.89 ± 11.26^{a}	28.47 ± 1.87^{a}	28.10 ± 2.23^{a}
Fe def. R + Fe suff. Diet	4.68 ± 0.18^{bc}	8.76 ± 1.50^{a}	47.00 ± 2.45^{b}	14.04 ± 0.34^{c}	100 ± 6.90^{a}	30.02 ± 1.02^{a}	29.94 ± 1.66^{a}
Fe def. R+10%T	4.43 ± 0.39^{abc}	7.23 ± 2.21^{a}	46.00 ± 2.65^{ab}	13.68 ± 1.53^{bc}	96.2 ± 4.95^{a}	29.53 ± 1.68^{a}	30.70 ± 1.79^{a}
Fe def. R+10% FT	4.62 ± 0.29^{abc}	8.04 ± 0.83^{a}	48.00 ± 0.71^{b}	13.68 ± 0.94^{bc}	104.26 ± 7.41^{a}	29.62 ± 0.45^{a}	28.51 ± 2.03^{a}
Fe def. R+10%G	4.08 ± 0.75^{a}	8.38 ± 1.10^{a}	42.00 ± 6.60^{ab}	11.84 ± 1.77^{a}	104.04 ± 13.5^{a}	29.22 ± 1.74^{a}	28.30 ± 2.19^{a}
Fe def. R+10%FG	4.32 ± 0.29^{abc}	6.85 ± 1.33^{a}	40.00 ± 6.16^{a}	12.20 ± 0.25^{ab}	92.54 ± 8.83^{a}	28.28 ± 1.69^{a}	31.09 ± 5.11^{a}
Fe def. R+$FeSO_4$	4.78 ± 0.14^{c}	9.60 ± 6.33^{a}	45.20 ± 2.59^{ab}	14.08 ± 0.65^{c}	94.54 ± 4.14^{a}	29.84 ± 0.48^{a}	31.20 ± 1.49^{a}

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

Fe suf RC: Iron sufficient Rat control, **Fe def. RC**: Iron deficient Rat control, **Fe def. R+ Fe suf. Diet**: Iron deficient Rat on iron sufficient diet, **Fe def. R+10%T:** Iron deficient Rat on iron deficient diet supplemented with 10% *Telfaira occidentalis*, **Fe def. R+10% FT:**Iron deficient Rat on on iron deficient diet supplemented with 10% fermented *Telfaira occidentalis*, **Fe def. R+10%G:** Iron deficient Rat on iron deficient diet supplemented with 10% *Gnetum africanum*, **Fe def. R+10%FG:** Iron deficient Rat on iron deficient diet supplemented with 10% fermented *Gnetum africanum*, **Fe def. R+$FeSO_4$:** Iron deficient Rat on iron deficient diet treated orally with 2.8mg/kg Ferrous Sulphate

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

CHAPTER FIVE

5.0 DISCUSSION

Fermentation is one of the oldest and most economical methods of food production and preservation (Oyewole and Isah, 2012). It involves the conversion of large molecules such as sugars and carbohydrate to less complex products such as carbondioxide and alcohol through the mediation of selected microorganisms (William and Dennis, 2011; Yadav *et al.,* 2011).

Fermentation improves the nutrient content of foods. Several experiments have demonstrated that fermentation of legumes enhances their nutritive value and antioxidant properties; reduces some anti-nutritional endogenous compounds such as phytic acid, and exerts beneficial effects on protein digestibility and biological value of legumes (Oboh *et al,* 2012; Oyewole and Isah, 2012).

Vitamins serve as essential components of the specific coenzymes participating in metabolism and other specialized activities (Qasim *et al.,* 2009). Among the vitamins, vitamin C which is also known as ascorbic acid is a water soluble antioxidant which is found in variable quantities in fruits and vegetables. Human and other primates have lost the ability to synthesize vitamin C as a result of a mutation in the gene coding for L-gulonolactone oxidase, an enzyme required for the biosynthesis of vitamin C via the glucuronic acid pathway (Woodall and Ames, 1997). Thus, vitamin C must be obtained through the diet. Vitamin C plays an important role as a component of enzymes involved in the synthesis of collagens and carnitine. It also reduces ferric iron in foods to aid absorption of micronutrients like iron (Scheers, 2013).

Telfaira occidentalis and *Gnetum africanum* are commonly consumed vegetables in Nigeria. Vitamin C content was significantly higher in *Gnetum africanum* than *Telfaira occidentalis*, this

may be because of the nature of the environmental conditions where the plants were grown. *Gnetum africanum* grows in the wild therefore it is exposed to harsh conditions hence the plant is equipped to produce high vitamin C which serves as an antioxidant defence. Fermentation of these vegetables led to a significant ($p<0.05$) reduction in the vitamin C content of *Telfaira occidentalis* but no siginificant ($p>0.05$) decrease in *Gnetum africanum* leaf. The difference in vitamin C decrease might be attributed to the difference in the maturation of the individual vegetables when the samples were taken (Agbemafle *et al.,* 2012) hence *Gnetum africanum* leaf due to its tough texture probably require more fermentation time beyond the 48hrs used in this research to ferment properly as loss of vitamin C increases with increased fermentation time as Ifensan *et al* (2014) reported an 87.5% decrease in the Vitamin C content of *T. occidentalis* leaf fermented for 5days. Muchoki *et al* (2007) also reported an 85% decrease in Vitamin C after fermenting cowpea leaves for 21 days.

Antinutrients are chemicals which have been evolved by plants for their own defense, among other biological functions and reduce the maximum utilization of nutrients especially proteins, vitamins, and minerals, thus preventing optimal exploitation of the nutrients present in a food and decreasing the nutritive value (Habtamu and Negussie, 2014).

Phytate, which is also known as inositolhexakisphosphate, is a phosphorus containing compound that binds with minerals and inhibits mineral absorption.The presence of phytate in foods has been associated with reduced mineral absorption due to the structure of phyate which has high density of negatively charged phosphate groups which form very stable complexes with mineral ions such as Zn^{2+}, $Fe^{2+/3+}$, Ca^{2+}, Mg^{2+}, Mn^{2+}, and Cu^{2+}causing non-availability for intestinal absorption (Walter *et al.,* 2002). They are generally found in food high in fibre especially in wheat bran, whole grains and legumes (Lori *et al.,* 2001). Phytate is hydrolysed via intermediate

myo-inositolphosphates ($InsP_5$ to $InsP_1$) into myo-inositol and inorganic phosphate. Degradation of phytate can be achieved during food processing through the addition of exogenous phytases (Konietzny and Greiner, 2002), by micro-organisms producing phytase or by endogenous cereal phytases (Greiner and Larsson, 2000).

Oxalate is an anti-nutrient which under normal conditions is confined to separate compartments. However, when it is digested, it comes into contact with the nutrients in the gastrointestinal tract (Noonan and Savage, 1999) and binds with minerals, such as Ca^{2+}, Fe^{2+}, and Mg^{2+} rendering these minerals unavailable to the body.

The content of phytate and oxalate were significantly higher in *G.africanum* leaf than *T. occidentalis* leaf this is probably because phosphorus which is stored as phytate might be higher in *G.africanum* leaf and maybe there is a greater need for Ca regulation, protection against herbivory and plant survival in *G.africanum* leaf hence more oxalate was produced. (Franceschi and Nakata, 2005; Nakata, 2012)

There was a significant ($p<0.05$) reduction in the antinutrient content of both vegetables after fermentation. Phytic acid reduced by 27% in *T. occidentalis* and 25% in *G.africanum* . Oxalate reduced by 71% in *T. occidentalis* and 25% in *G.africanum*. Reduction was higher for *T. occidentalis* leaf than *G. africanum* leaves probably because of the nature of the,leaves, duration of fermentation and nature of the fermenting microorganisms. The loss in phytic acid during fermentation was possibly due to the action of fermenting microorganisms which released phytase to hydrolyze phytate into inositol and orthophosphate (Sandberg and Andlind, 2002). Similar reduction level of phytic acid after fermentation was observed by Giami (2004), and Ugwu and Oryanye (2006) for fluted pumpkin seed and breadfruit seeds, respectively. Significant reduction in phytate after fermentation of *Cassia obtusifolia* leaves were also

reported by Nuha *et al.,* (2010). Adeniran *et al.,* (2013) also reported a significant decrease in phytate content of *Phaseolus lunatus* after fermentation for 72 hours. Ekundayo *et al* (2013) reported a significant decrease in the phytate and oxalate content of bush mango (*Irvingia gabonensis*) seed after fermentation for 72 hours. The significant reduction in oxalate might be that they leached out of the vegetables due to the acidic medium created during fermentation. Reduction was far lower for *G.africanum* leaf probably because of the nature of the leaves and the degree to which the leaves fermented. Igbabul *et al* (2014) reported a significant decrease in oxalate content after fermenting cocoyam flour (*Colocasia esculenta*) for 24, 48 and 72 hours.

Minerals, classified as micronutrients are needed in small amounts. They cannot be synthesized by animals and must be provided from plants or mineral-rich water (Anjorin *et al.,* 2010) Deficiency in minerals, however, can have a major impact on health such as anaemia and osteoporosis that commonly occur in both developed and developing countries (Norhaizan and Nor Faizadatul, 2009). This study focused only on iron (Fe), zinc (Zn), copper (Cu), magnesium (Mg) and calcium (Ca).

Mineral contents were significantly ($p<0.05$) higher in *T. occidentalis* than *G.africanum* leaf. This is probably because of the the differences in botanical structure, as well as in the mineral composition of the soil in which the plants are cultivated. Other factors responsible for a variation in elemental content are preferential absorbability of the plant, use of fertilizers, irrigation water and climatological conditions (Rajurka *et al.,* 1997; Lokhande *et al.,* 2010).

The major minerals analyzed were calcium and magnesium, these are required in amounts greater than 100mg per day (Imelouane *et al.,* 2011; Ogunkunle *et al.,* 2014). Calcium is essential for healthy bones, teeth and blood (Charles, 1992; Hughes, 1972 and Lokhande *et al.,*

2010). The health of the muscles and nerves depends on calcium. It is required for the absorption of dietary vitamin B, for the synthesis of the neurotransmitter acetylcholine, for the activation of enzymes such as the pancreatic lipase. It helps to regulate the activity of skeletal muscle, heart and many other tissues. Deficiency of calcium causes rickets, osteomalacia and scurvy. The recommended dietary allowance of calcium for children is between 500 and 1000 mg and 800 mg for adults (Lokhande *et al.,* 2010). Hence, both vegetables and their fermented variants can be used to combat calcium deficiency.

Magnesium (Mg) is a major mineral which is required for energy production, oxidative phosphorylation, and glycolysis. It contributes to the structural development of bone and is required for the synthesis of DNA, RNA, and the antioxidant glutathione. It also plays a role in the active transport of calcium and potassium ions across cell membranes, a process that is important to nerve impulse conduction, muscle contraction, and normal heart rhythm (Rude, 2012). The recommended dietary allowance is 400-420mg in adult males and 310-320mg in adult females (Institute of Medicine, 1997). Both vegetables and there fermented derivatives are not rich sources of magnesium since their contents are far below the recommended values.

The minor minerals analysed were copper, zinc and iron these are required in amounts less than 100mg per day (Imelouane, 2011; Ogunkunle *et al.,* 2014). Copper has the main role in immune system, connective tissue and skeleton, blood formation, the blood vessels and nervous system. Zinc and copper are cofactors of the antioxidant enzyme, superoxide dismutase (Cu/Zn-SOD) which detoxifies the toxic superoxide radical (Cavdar *et al.*, 2002; Ross *et al.*, 2011). The daily dietary requirement for copper is 0.9mg in adults, 1mg and 1.2mg in pregnant and lactating women respectively (Institute of Medicine, 2001). *T.occidentalis* leaves contain higher copper contents which significantly ($p<0.05$) higher than the requirement for adults however the

unfermented form meets the requirement during pregnancy and the fermented form meets the requirement for lactating women. The copper content of *G.africanum* leaf is not a rich source of copper since its content is significantly ($p<0.05$) lower than the copper requirement for adults however, its fermented variant can be used as a rich source of copper.

Zinc is necessary for the growth and multiplication of cells (enzymes responsible for DNA and RNA synthesis), for skin integrity, bone metabolism and functioning of taste and eyesight (Thunus and Lejeune, 1994). Zinc deficiency is characterized by recurrent infections, lack of immunity and poor growth. Growth retardation, male hypogonadism, skin changes, poor appetite and mental lethargy are some of the manifestations of chronically zinc-deficient human subjects (Prasad, 1982). The requirement for zinc is 1 mg/day in infancy;10 mg/day between 1 and 10 years of age, and 15 mg/day in adults. Pregnant and lactating women require 20 to 25 mg, while normal adult require 15 mg of zinc every day (Lokhande, *et al.,* 2010). The unfermented variant of both plants could only meet the zinc requirement in infancy, fermentation significantly ($p<0.05$) increased the zinc content in both leaves, however, the increase in *G.africanum* did not meet the requirement for children and adults but that of *T. occidentalis* met the requirement, hence fermented *T. occidentalis* can be used to combat zinc deficiency.

Iron is an essential component of haemoglobin (Wessling-Resnick, 2014) and myoglobin (Angett, 2012). It is necessary for growth, development, normal functioning and synthesis of some hormones and connective tissues (Murray-Kolbe and Beard, 2010; Angett, 2012). Its deficiency ranges from iron depletion to iron deficiency anaemia which affects numerous organs and systems (Joshi and Mathur, 2009). Recommended intakes for children 4-8yrs is 10mg, 14-18yrs is 11mg (male), 15mg (female), for adults 19-50yrs is 8mg (males), 18mg (females), for those that are 51yrs and above is 8mg for both males and females (Institue of Medicine, 2001).

Therefore, the iron content of these vegetables and there fermented derivatives are good iron sources.

Fermentation increased the iron content of *T. occidentalis* significantly ($p<0.05$) whereas it led to significant ($p<0.05$) decrease in *G.africanum*, there was a significant ($p<0.05$) decrease in magnesium content of *T. occidentalis* and a non significant ($p>0.05$) increase for *G.africanum*. Zinc increased significantly ($p<0.05$) for both vegetables. The observed increase might be due to contribution by the fermenting microorganisms since the reduced antinutrients might have led to the release of some of the minerals bound to them, while the decrease might be due to leaching of the minerals into the fermentation medium probably due to the duration of fermentation. Similar changes in mineral content following fermentation were reported by Nuha *et al* (2010) where iron and zinc content increased significantly after *Cassia obtusifolia* leaf was fermented. However, Ifensan *et al* (2014) reported a significant decrease in the mineral contents of *T. occidentalis* leaves after fermentation for 5 days. Raihanatu *et al*, (2011) also reported a significant decrease in mineral contents of *Sorghum bicolor* grains after fermentation for 3 days.

Sequential extraction is an analytical process that chemically and selectively leaches metal out of soil, sediment and sludge samples. The purpose of sequential or "selective" extraction is to simulate the release of the selective metals into solution under various environmental conditions. The theory behind sequential extraction is that the most mobile metals are removed in the first fraction and continue in order of decreasing of mobility, showing the speciation pattern of the metal (Ahntrom and Parker, 1999). It is well known that metals are present in soil in different chemical forms, which influence their reactivity and hence their mobility and bioavailability in plants, hence humans (Uduma and Jumoh, 2013).

Bioavailable fraction is the fraction of the total amount of a chemical present in a specific environmental compartment that, within a given time span, is either available or can be made available for uptake by microorganisms or plants, from either the direct surrounding of the organism or the plant, or by ingestion of food (Peijnenburg and Jager, 2003).

Speciation pattern shows that residual iron is predominant in all the vegetables, this might indicate that most of the iron content of the leaves is bound in the plant tissues, this agrees with the findings of Majolagbe *et al* (2013). Percent *in-vitro* bioavailability was higher in *Telfaira occidentalis* than *Gnetum africanum* leaves. This may be because iron content and antinutient reduction was higher in *Telfaira occidentalis,* also the amount of residual iron which is usually unavailable was higher in *Gnetum africanum.*

The primary cause of anaemia in this study was feeding iron deficient diet for a long period (8 weeks). Anaemia is considered as one of the most common index of nutritional deficiency worldwide and is caused by iron deficiency store or iron deficiency erythropoiesis (Lin *et al.,* 2003; Soliman, *et al.,* 2010). Several authors have reported that iron deficiency anaemia is mainly caused by some food constituents that may contribute to inhibition of iron absorption, hence contribute to the high prevalence of iron deficiency, and iron deficiency anaemia (Lin *et al.,* 2003; Soliman *et al.,* 2010).

There was a significant decrease in the weekly relative weight gain in all the iron deficient rats when compared to the iron sufficient control, this might be due to an elevation in metabolic rate in iron-deficient anaemic animals, Beard *et al* (1995) concluded that iron-deficient rats are hypermetabolic and attributed this to a decreased ability to thermoregulate compared to control rats. This agrees with the reports of Nora, (2008) who investigated the protective effect of

soybean and thyme on iron deficiency anaemia in rats and found that iron deficient rats had reduced weight gain and stated that it may be due to lower plasma thyroid hormone levels (Beard *et al.,* 1998).

The measurement of Hb is essential for the diagnosis of nutritional anaemia and is one of the most common, easiest and least expensive methods (Kotze *et al.,* 2009). Haemoglobin is the protein molecule in red blood cells that carries oxygen from the lungs to the body's tissues and returns carbon dioxide from the tissues to the lungs. It is made up of four protein molecules (globulin chains) that are connected together. The normal adult haemoglobin molecule contains 2 alpha-globulin chains and 2 beta-globulin chains. In fetuses and infants, there are only a few beta chains and the haemoglobin molecule is made up of 2 alpha chains and 2 gamma chains. As the infant grows, the gamma chains are gradually replaced by beta chains.Each globulin chain contains an important central structure called the haeme molecule. Embedded within the haeme molecule is iron that transports the oxygen and carbon dioxide in the blood. The iron contained in haemoglobin is also responsible for the red color of blood. Haemoglobin also plays an important role in maintaining the shape of the red blood cells. Abnormal haemoglobin structure can, therefore, disrupt the shape of red blood cells and impede its function and its flow through blood vessels. Haemoglobin is usually measured as a part of the complete blood count (CBC) from a blood sample. A low haemoglobin is referred to as anaemia. There are many reasons for anaemia. Some of the more common causes are: loss of blood (traumatic injury, surgery, bleeding colon cancer or stomach ulcer), nutritional deficiency (iron, vitamin B12, folate), bone marrow problems (replacement of bone marrow by cancer, suppression by chemotherapy drugs, kidney failure), and abnormal haemoglobin (sickle cell anaemia). Higher than normal haemoglobin levels can be seen in people living at high altitudes and in people who

smoke. Dehydration produces a falsely high haemoglobin which disappears when proper fluid balance is restored. Some other infrequent causes are: advanced lung disease (for example, emphysema), certain tumors, a disorder of the bone marrow known as polycythemia rubra vera, and abuse of the drug erythropoietin (Kotze *et al.,* 2009).

The haemoglobin concentration of the animals on iron deficient diet met the cut-off value (<11g/dl) after eight weeks of feeding, this is in contrast with the work of Solimon *et al* (2010) and Nora, (2008), who reported that iron deficiency anaemia was induced by the third week of feeding. This might be because the diet used in this study was not completely deficient in iron, also, the amount of iron stores in the rats could also delay the induction. The haemoglobin concentrations for all the rats except those in the iron sufficient control group were significantly low, this agrees with Solimon *et al* (2010), Nora (2008), Joshi and Mathur (2009). Decreased haemoglobin might be due to the effect of a shortage of iron available to the erythroid precursors in the bone marrow for haemoglobin synthesis (Cook, 2005).

Haemoglobin iron is an index of the iron content of haemoglobin. The percentage change in haemoglobin iron was higher in rats treated with iron sufficient diet, unfermented *T.occidentalis* and $FeSO_4$ respectively, this is probably because absorbed iron was made more available to erythroid precursors for haemoglobin synthesis, this is further shown by their significant ($p<0.05$) higher Hb concentration.

Haematological paramaters are routinely checked to diagnose anaemia which is characterized by decreased haemoglobin concentration in circulating blood, less than 12g/dl and less than 13g/dl in females and males respectively (Okochi *et al*., 2004). Iron deficiency anaemia may be effectively diagnosed in most cases by full blood examination and serum ferritin level

(Pasricha *et al.*, 2010). The Mean corpuscular volume (MCV), represents the **average volume of a red cell** mean corpuscular haemoglobin (MCH), the mass of haemoglobin per average red cell irrespective of the cell size and mean corpuscular haemoglobin concentration (MCHC), describes the average concentration of haemoglobin per red cell, it takes into account red cell size. These indices are used to classify anaemia based on the blood cell morphology. Depending on the MCV, anaemia is classified as microcytic, normocytic or macrocytic. These may be further subdivided according to the average amount of RBC haemoglobin (MCHC) into hypochromic or normochromic (Hoffman and Benze, 2000; Abdelgader *et al.*, 2014). MCHC is a better index for anaemia classification than MCH because it shows the haemoglobin content in each red blood cell. Therefore, three groups of anaemia are distinguished, Microcytic hypochromic, normocytic normochromic and macrocytic anaemia. The MCHC of all rats decreased significantly ($p<0.05$) at the point of induction, this shows that a hypochromic anaemia was induced. This contrasts the reports of Joshi and Mathur, (2009), Solimon *et al.*, (2010) and Salawu and Salimon, (2014), who reported a non significant ($p>0.05$) change in MCHC.

By day 21 of supplementation, there was a non significant ($p>0.05$) change in RBC, WBC, Hb, MCV, MCH, MCHC and PCV of rats in the treatment groups when compared to the iron sufficient control group, this is propably due to the significant increase in haemoglobin-iron. Haemoglobin iron occupies a dominant position in all animals, and high proportion present as haemoglobin indicates that any condition influencing the level of this compound in the blood greatly affect the iron status of the body (Joshi and Mathur, 2009). However, rats in the iron deficient control group which continued on the iron deficient diet showed a non significant ($p>0.05$) increase in Hb concentration, this might be because iron deficiency usually increase

the rate of iron absorption hence there was an increase in the rate of absorbing the little iron contained in the diet this also combined with increased depletion of available iron stores (Bothwell *et al.,* 1979; Joshi and Mathur 2009). This agrees with the findings of Joshi and Mathur (2009) who reported an increase in the blood parameters after 10 days of depletion though the rats were kept on iron deficient diet.

Ferritin is currently considered the most important indicator of iron status as even in the first stage of iron deficiency, its concentration decreases (Knovich *et al.,* 2009). A ferritin molecule consists of 24 subunits of L-ferritin or H-ferritin. H-ferritin has ferroxidase activity, which is necessary for oxidising iron before it can be loaded onto ferritin for storage (Koulanouzidis *et al*., 2009).

The serum ferritin concentrations of rats in the iron deficient control group was significantly ($p<0.05$) low, this might be because the rats in the face of insufficient iron from diet began to deplete their iron stores in order to meet up with some of their iron needs this probably explains the non significant ($p>0.05$) increase in Hb. At the end of the 21 days supplementation, ferritin concentration remained significantly low for rats in the iron deficient control group, serum ferritin concentrations of rats in unferrmented and ferrmented *Gnetum africanum* group and rats in the $FeSO_4$ group had a non significant ($p>0.05$) increase when compared to the iron deficient control group. This may be because the iron content and absorption from *Gnetum africanum* leaves were low as revealed by the mineral and *in-vitro* iron bioavailability analysis, the unfermented leaves had higher antinutrients yet its iron bioavailability as shown by the ferritin concentration was higher than that of the fermented leaves, this might be because the unfermented *Gnetum africanum* leaf had a significantly ($p<0.05$) higher Vitamin C content, and Vitamin C enhances iron absorption. The fermented leaves had a lower absorption as shown by

the ferrritin concentration probably because fermentation reduced the total iron content and increased the calcium content which is also a strong inhibitor to iron absorption (Leif, 1998).

Ferritin concentration was significantly ($p<0.05$) higher in rats supplemented with unferrmented *Telfaira occidentalis* than ferrmented *Telfaira occidentalis* this might be because vitamin C which enhances iron absorption significantly decreased and calcium which inhibits iron absorption significantly increased in the fermented leaf, this probably accounts for the variation in both forms of the leaves. Ferritin concentrations was significantly ($p<0.05$) higher in rats found in the iron sufficient diet control and *T.occidentalis* groups probably because the diet had a higher iron content and the iron were better absorbed hence rats in this group had higher iron stores. The non significant ($p>0.05$) change in serum ferritin concentration in iron deficient rats on ferrous sulphate drug when compared to rats in the iron deficient control group and the rats in the iron sufficient diet control and *T.occidentalis* groups probably indicates that the iron from the drug was not efficiently absorbed this might be because the action of the first pass effect on drugs taken orally reduced the amount of the iron that finally got into circulation. Also, the drug is known to produce intestinal side effects such as constipation, nausea, and bloating in many users (Hansen, 1994; Soliman *et al.,* 2010). Some forms of ferrous sulfate are enteric-coated to delay tablet dissolving and prevent some side effects, but enteric-coated iron may not absorb as well as iron from standard supplements (Rickettes, 1993; Soliman *et al.,* 2010).

CHAPTER SIX

6.0 SUMMARY, CONCLUSION AND RECOMMENDATION

6.1 SUMMARY

Gnetum africanum leaf has significantly ($p<0.05$) higher vitamin C and antinutrient contents and fermentation significantly ($p<0.05$) decreased the antinutrient content of both leaves, but significantly ($p<0.05$) decreased the vitamin C content of only *Telfaira occidentalis* leaf.

T. occidentalis leaf contains significantly ($p<0.05$) higher mineral content than *G. africanum* and fermentation significantly ($p<0.05$) increased the level of the minerals in both leaves except magnesium in *T. occidentalis* and iron in *G. africanum* leaf.

Residual iron was higher in *G. africanum* leaf while *in-vitro* bioavailable iron was higher in *T. occidentalis* leaf. Fermentation increased the unavailable iron of both leaves but increased the *in-vitro* bioavailable iron in *T. occidentalis* leaf by 14% and decreased that of *G. africanum* leaf by 28%.

The experimental iron deficient diet induced a hypochromic anaemia in the rats, which caused a significant decrease in haemoglobin concentration. Supplementation of the rats diet with 10% *T. occidentalis* leaf had a significant ($p<0.05$) increase in haemoglobin, haemoglobin iron, and ferritin when compared to the rats in the iron deficient diet control group. The increase in these parameters were not significantly ($p>0.05$) different from the iron deficient diet control group when 10% *G. africanum* leaf was used. There was no significant ($p>0.05$) difference between the fermented and unfermented derivatives of both leaves.

6.2: CONCLUSION

Telfaira occidentalis leaf at 10% supplementation regenerated haemoglobin in nutritionally anaemic rats on iron deficient diet while 10% *G. africanum* leaf supplementation had no significant ($p>0.05$) haemoglobin regeneration potential. Fermentation of both leaves did not significantly ($p>0.05$) enhance their haemoglobin regeneration potentials.

6.3: RECOMMENDATION

i. The induction of nutritional anaemia in young rats is a classical study on its own. There is need to embark on how to formulate a diet that will induce the desired anaemic state in 4-6 weeks, shorter than was achieved in this study.

ii. The success of *Telfaira occidentalis* leaf at regenerating haemoglobin at 10% supplementation should be investigated further; specifically at a lower supplementation level of 5% and also cooked, as consumed.

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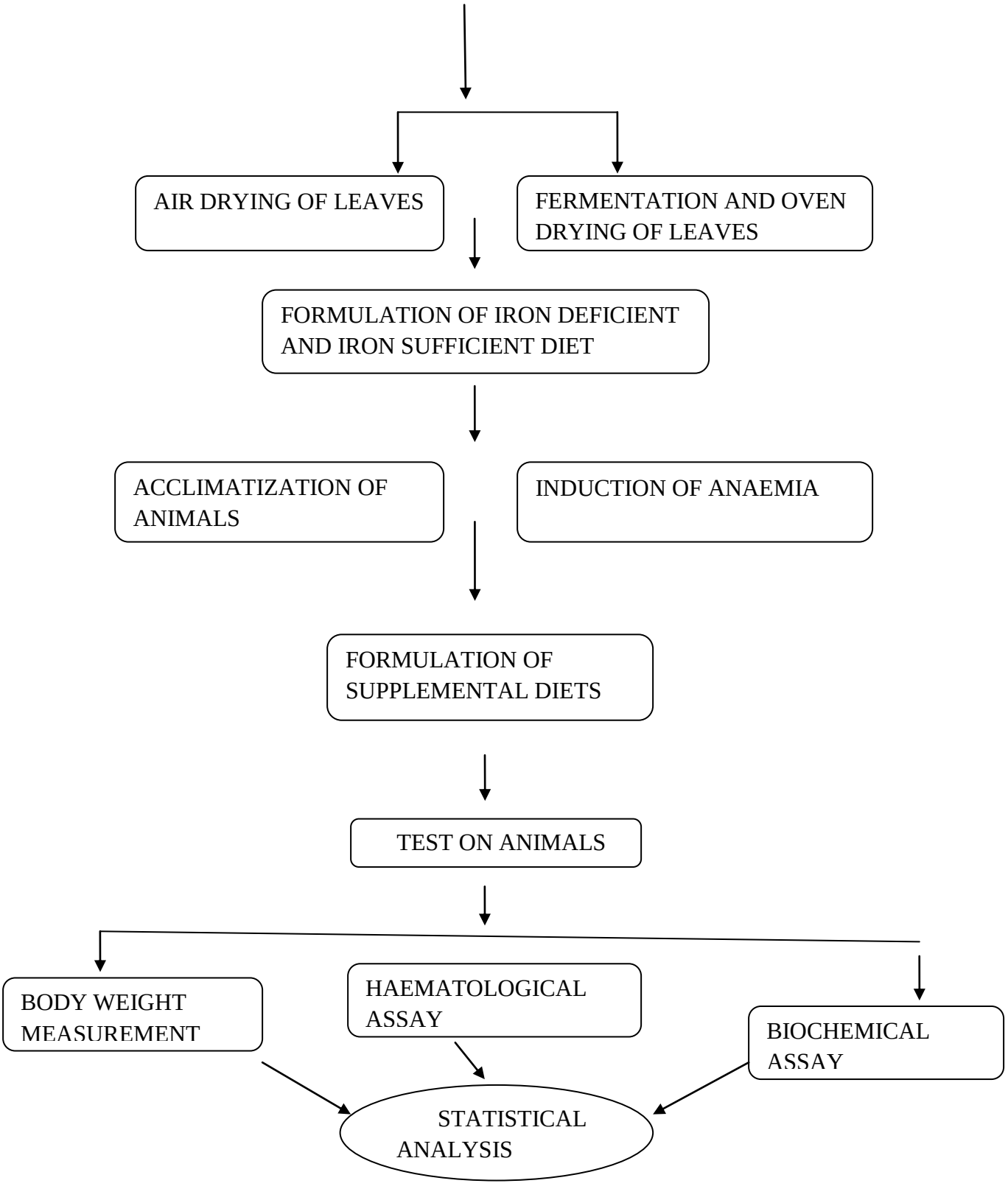
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APPENDICES:

Appendix 1

EXPERIMENTAL DESIGN

COLLECTION AND IDENTIFICATION OF LEAVES


AIR DRYING OF LEAVES
FERMENTATION AND OVEN DRYING OF LEAVES
FORMULATION OF IRON DEFICIENT AND IRON SUFFICIENT DIET
ACCLIMATIZATION OF ANIMALS
INDUCTION OF ANAEMIA
FORMULATION OF SUPPLEMENTAL DIETS
TEST ON ANIMALS
BODY WEIGHT MEASUREMENT
HAEMATOLOGICAL ASSAY
BIOCHEMICAL ASSAY
STATISTICAL ANALYSIS

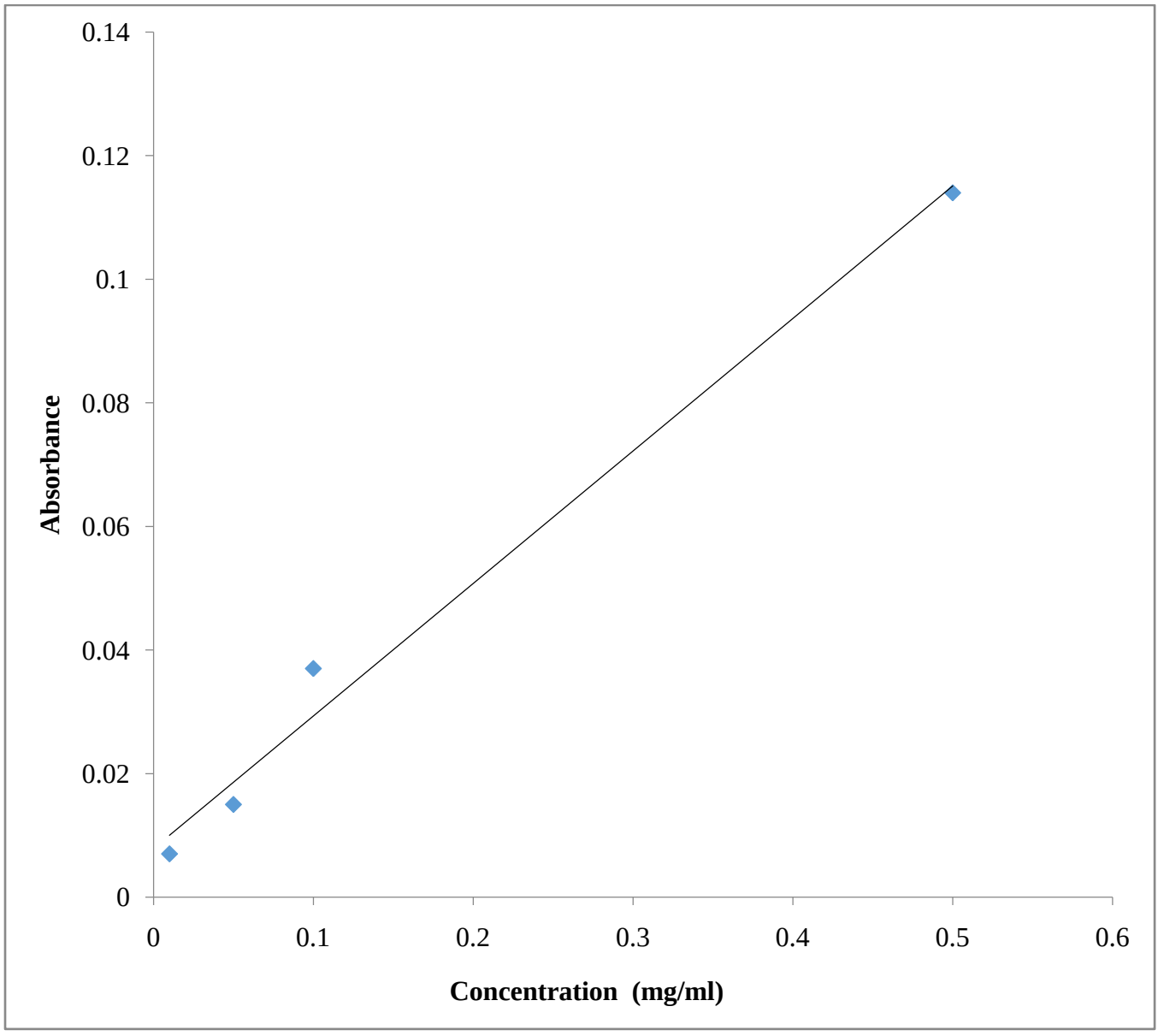
Appendix 2

Content of Vitamin and Mineral Mix (Vitalyte Extra ™)

Specification per 1000g

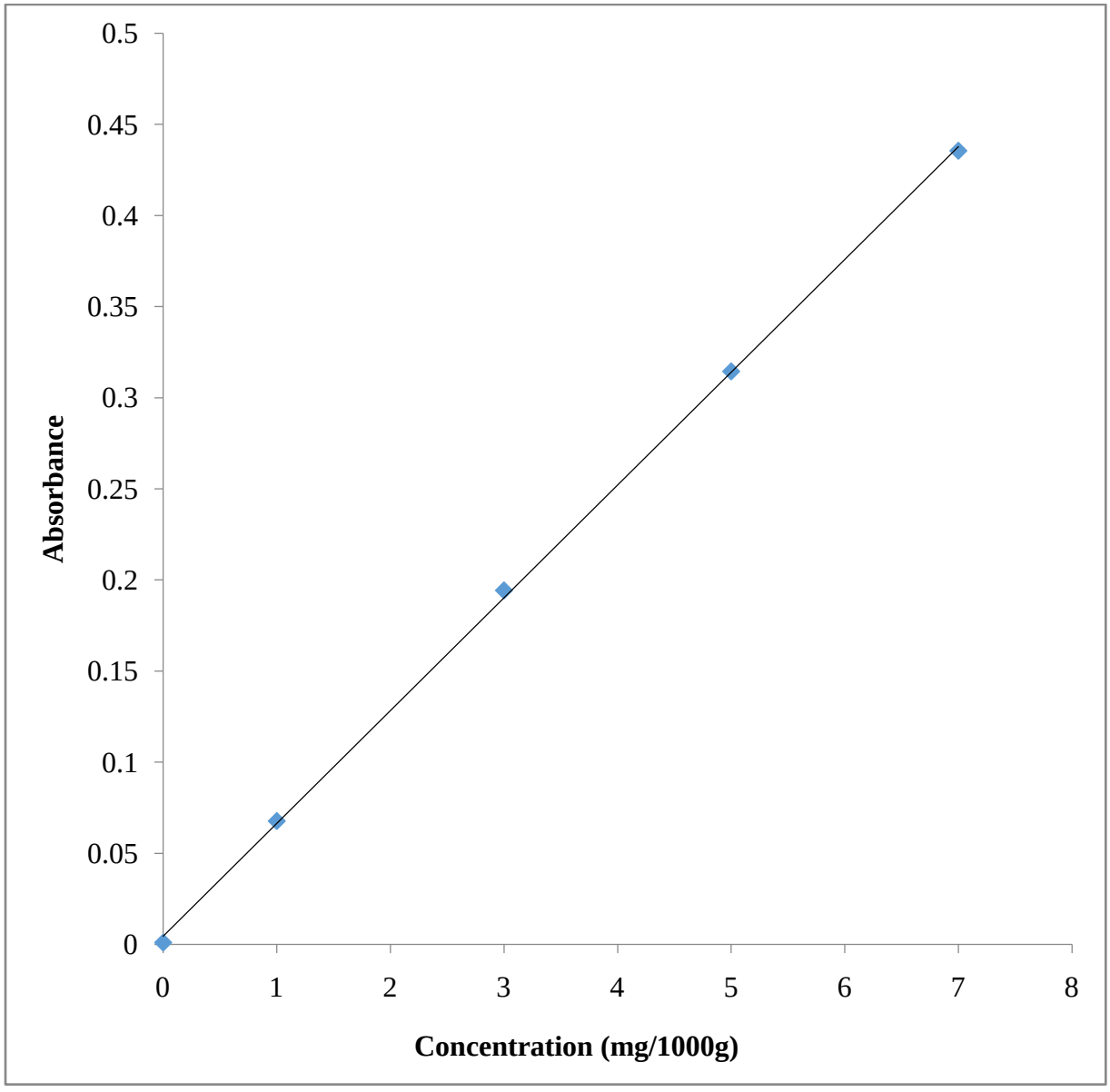
Vitamin A	15,000,000 IU
Vitamin D3	4, 400,000 IU
Vitamin E	2,500 IU
Vitamin K	4,350 mg
Vitamin B2	4,350 mg
Vitamin B6	2,350 mg
Vitamin B12	11,350 mg
Vitamin C	1000 mg
Nicotinamide	16,700 mg
Calcium Pantothenate	5,350 mg
Potassium Chloride	87,000 mg
Sodium Sulphate	212,000 mg
Sodium Chloride	50,000 mg
Magnesium Sulphate	12,000 mg
Copper Sulphate	12,000 mg
Zinc Sulphate	12,000 mg
Manganese Sulphate	12,000 mg
Lysine Hydrochloride	15,000 mg
Methionine	10,000 mg
Expicients Q.S	1,000 g

Appendix 3



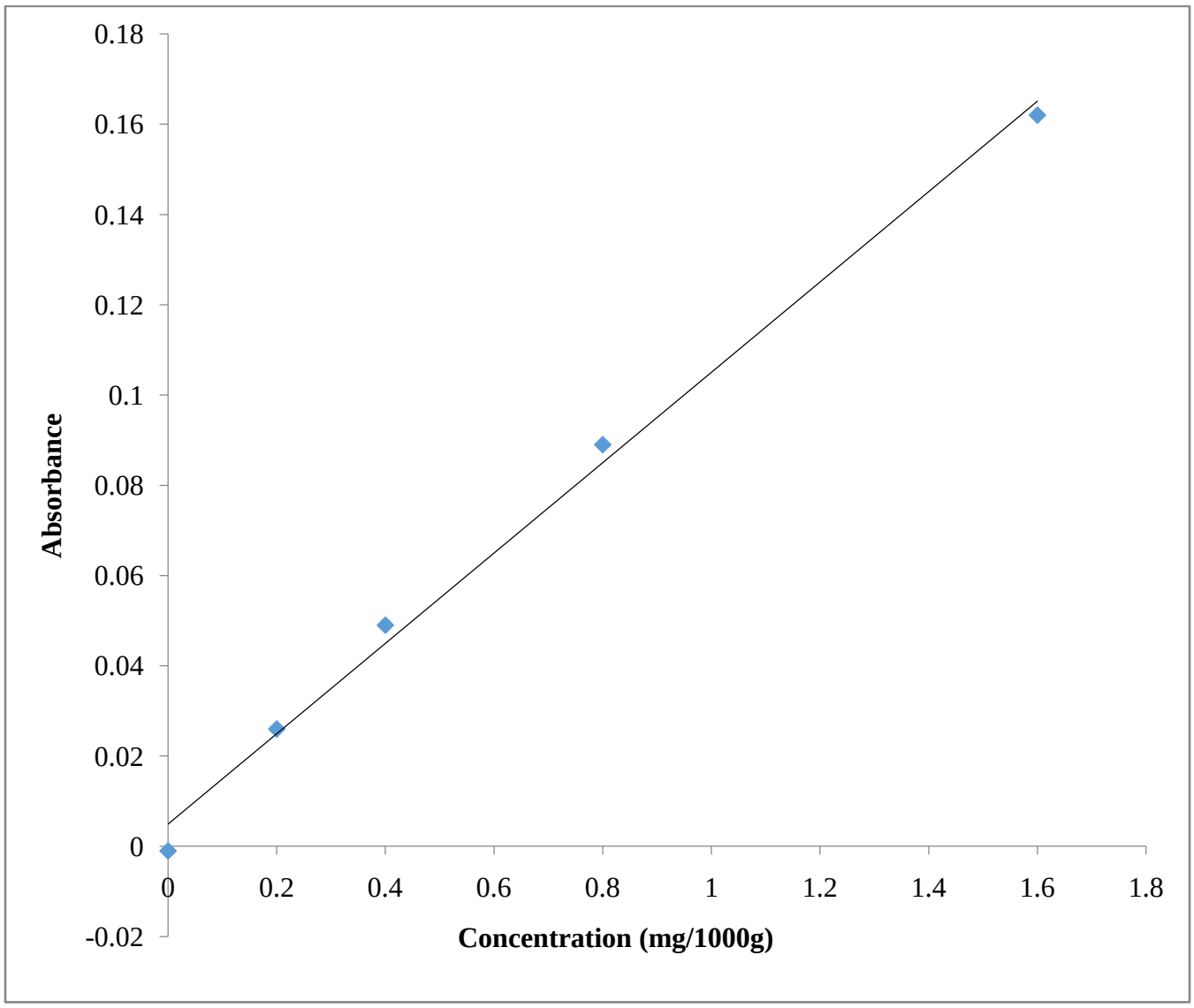
Standard Curve for Vitamin C

Appendix 4



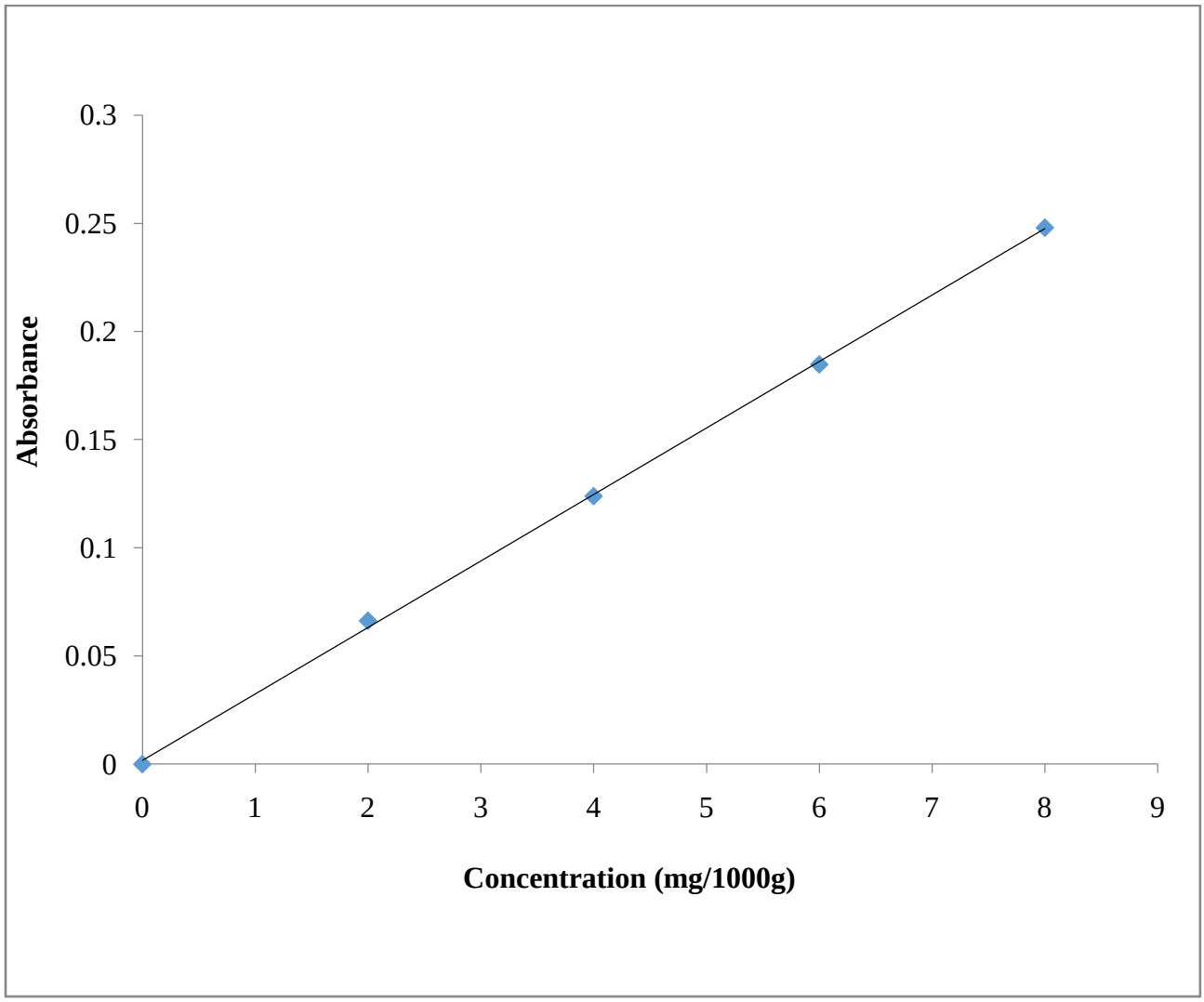
Standard Curve for Fe

Appendix 5



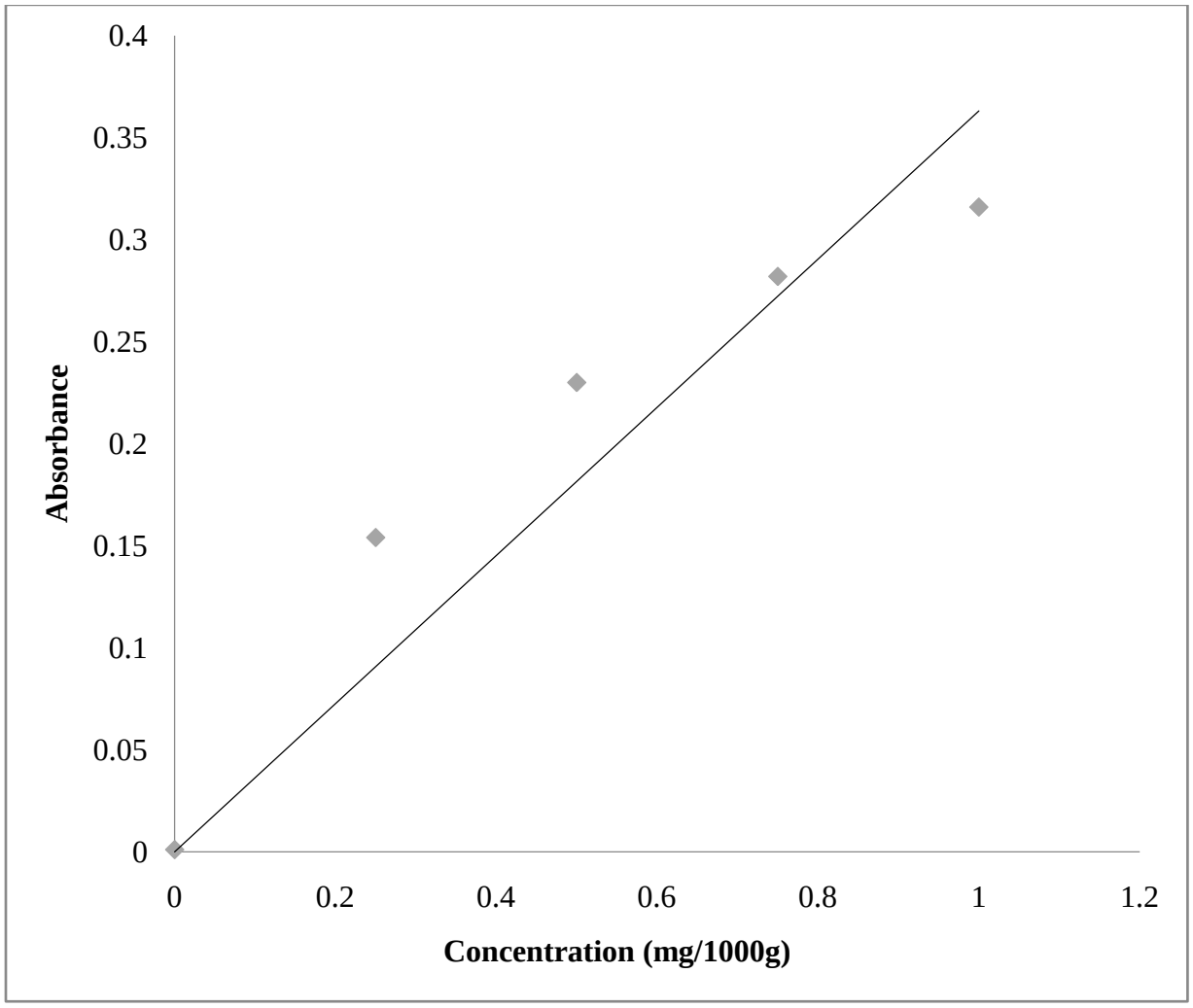
Standard Curve for Zn

Appendix 6



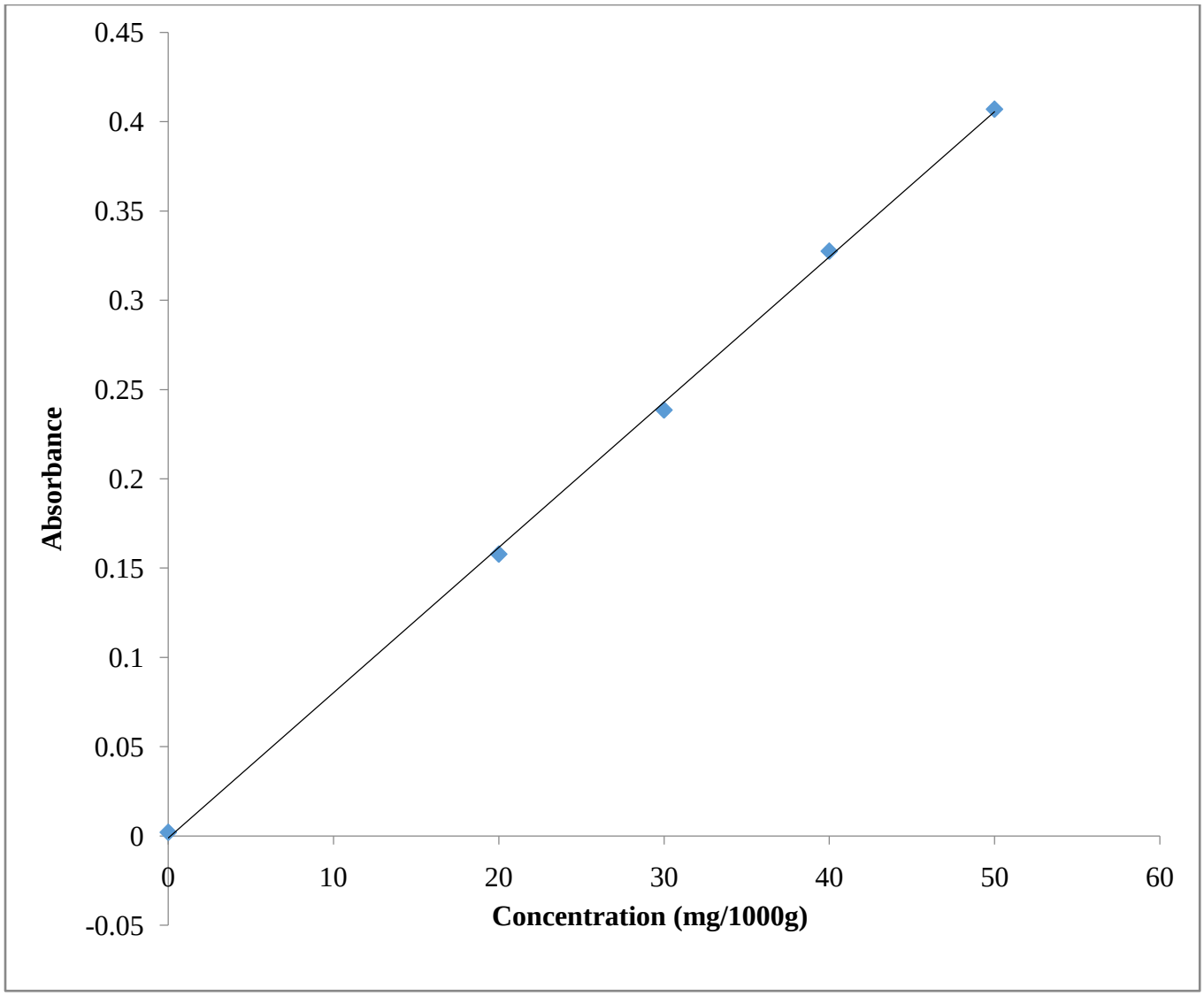
Standard Curve for Cu

Appendix 7



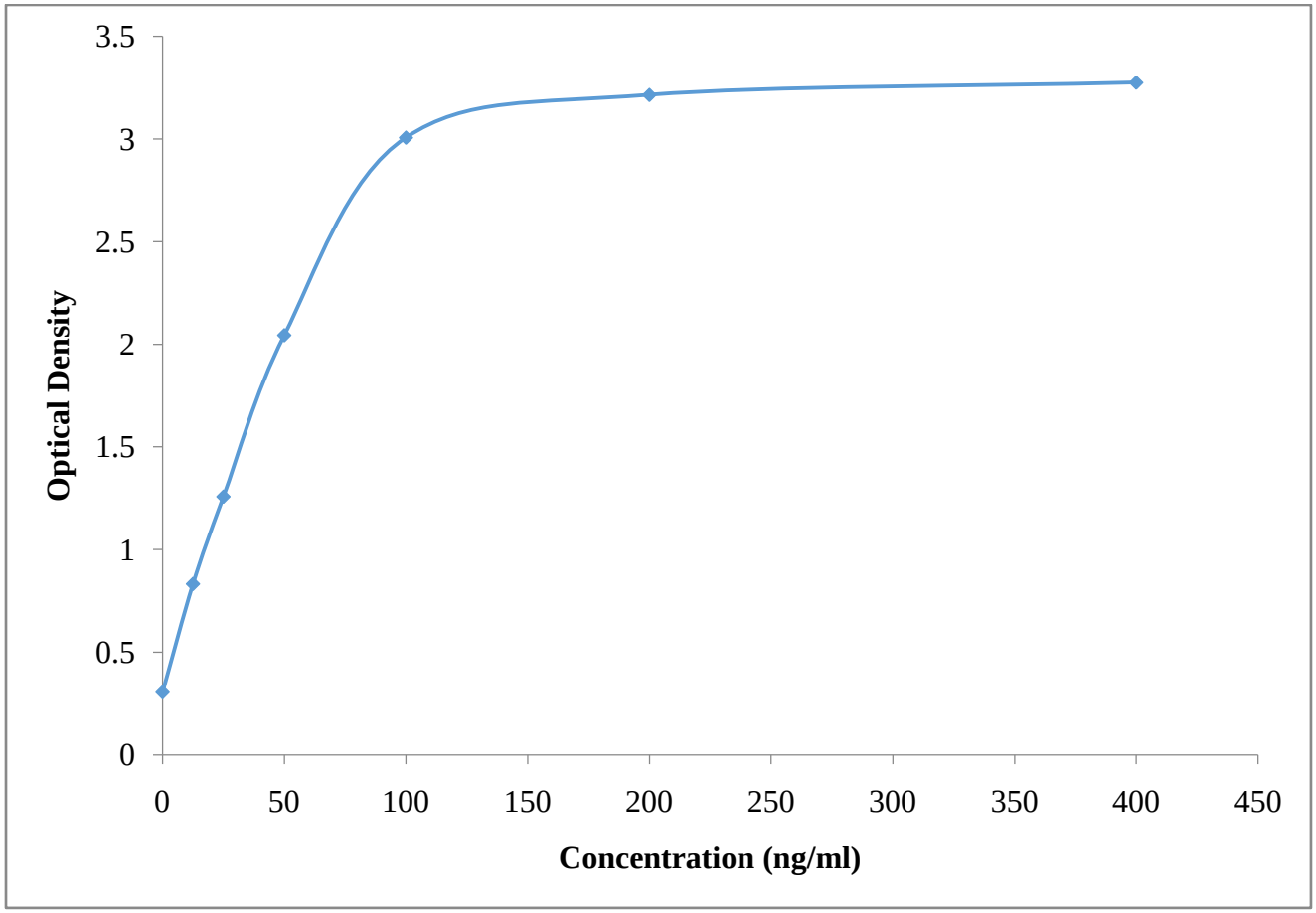
Standard Curve for Mg

Appendix 8



Standard Curve for Ca

Appendix 9



Standard Curve for Serum Ferritin

Appendix 10

Vitamin C content in Fermented and Unfermented *Telfaira occidentalis* and *Gnetum africanum* Leaves

VEGETABLE (mg/100g)	VITAMIN C
TELFAIRA OCCIDENTALIS	28 ± 0.02^{b}
***TELFAIRA OCCIDENTALIS,* Fermented**	14 ± 0.01^{a}
GNETUM AFRICANUM	42 ± 0.01^{c}
***GNETUM AFRICANUM,* Fermented**	41 ± 0.00^{c}

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

Appendix 11

Antinutrient Content of Fermented and Unfermented *Telfaira occidentalis* and *Gnetum africanum* Leaves

VEGETABLE	ANTINUTRIENT	
	PHYTATE(g/100g)	OXALATE(mg/100g)
TELFAIRA OCCIDENTALIS	0.48 ± 0.11^{b}	60.63 ± 0.38^{c}
***TELFAIRA OCCIDENTALIS* (fermented)**	0.35 ± 0.01^{a}	17.32 ± 0.31^{a}
GNETUM AFRICANUM	0.72 ± 0.06^{d}	64.58 ± 0.36^{d}
***GNETUM AFRICANUM* (fermented)**	0.54 ± 0.02^{c}	48.38 ± 0.30^{b}

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

Appendix 12

Effect of Fermented and Unfermented *Telfaira occidentalis* and *Gnetum africanum* Leaves Supplementation on Relative Change in Weight of Iron Deficient Rat

GROUPS **n=5**	**DAY 7**	**DAY 14**	**DAY 21**
Fe suf RC	$15.72\pm10.74_{a}^{b}$	$35.54\pm22.82_{ab}^{b}$	$45.61\pm25.42_{c}^{b}$
Fe def RC	$6.23\pm5.07_{a}^{a}$	$16.44\pm5.68_{a}^{a}$	$22.8\pm4.30_{b}^{a}$
Fe def R + Fe suf diet	$4.32\pm7.53_{a}^{a}$	$19.72\pm7.17_{b}^{a}$	$29.78\pm13.02_{c}^{ab}$
Fe def R +10%*T*	$6.36\pm6.19_{a}^{a}$	$18.72\pm5.22_{a}^{a}$	$28.60\pm4.78_{b}^{ab}$
Fe def R +10% *FT*	$3.48\pm4.86_{a}^{a}$	$12.97\pm5.54_{b}^{a}$	$19.84\pm5.43_{c}^{a}$
Fe def R +10%G	$5.73\pm3.91_{a}^{a}$	$22.25\pm9.74_{a}^{ab}$	$34.56\pm11.14_{b}^{ab}$
Fe def R +10%FG	$3.58\pm4.96_{a}^{a}$	$15.09\pm4.39_{b}^{a}$	$23.31\pm3.60_{c}^{a}$
Fe def R + FeSO4	$7.48\pm4.26_{a}^{ab}$	$22.67\pm7.03_{b}^{ab}$	$31.99\pm10.34_{c}^{ab}$

Values with different superscript down the column differ significantly ($p<0.05$)

Values with different subscript across the row differ significantly ($p<0.05$)

Fe suf RC: Iron sufficient Rat control, **Fe def. RC**: Iron deficient Rat control, **Fe def. R+ Fe suf. Diet**: Iron deficient Rat on iron sufficient diet, **Fe def. R+10%T:** Iron deficient Rat on iron

deficient diet supplemented with 10% *Telfaira occidentalis*, **Fe def. R+10% FT:**Iron deficient Rat on on iron deficient diet supplemented with 10% fermented *Telfaira occidentalis*, **Fe def. R+10%G:** Iron deficient Rat on iron deficient diet supplemented with 10% *Gnetum africanum*, **Fe def. R+10%FG:** Iron deficient Rat on iron deficient diet supplemented with 10% fermented *Gnetum africanum*, **Fe def. R+$FeSO_4$:** Iron deficient Rat on iron deficient diet treated orally with 2.8mg/kg Ferrous Sulphate

Appendix 13

Effect of Fermented and Unfermented *Telfaira occidentalis* and *Gnetum africanum* Leaves Supplementation on Haemoglobin Concentration of Iron Deficient Rats

GROUPS **n=5**	**DAY 0**	**DAY 7**	**DAY 21**
Fe suf RC	$12.08\pm0.62_{a}^{b}$	$12.60\pm0.79_{ab}^{d}$	$13.22\pm0.59_{c}^{abc}$
Fe def RC	$8.90\pm0.61_{a}^{a}$	$8.34\pm0.56_{b}^{a}$	$11.97\pm1.43_{c}^{a}$
Fe def R + Fe suf diet	$8.08\pm0.91_{a}^{a}$	$10.76\pm0.69_{b}^{c}$	$14.04\pm0.34_{c}^{c}$
Fe def R +10%*T*	$8.78\pm0.83_{a}^{a}$	$10.40\pm0.54_{b}^{c}$	$13.68\pm1.53_{c}^{bc}$

Fe def R +10% *FT*	$8.76\pm0.48_{a}^{a}$	$10.76\pm0.81_{b}^{c}$	$13.68\pm0.94_{c}^{bc}$
Fe def R +10%G	$8.44\pm0.61_{a}^{a}$	$9.26\pm1.11_{b}^{ab}$	$11.84\pm1.77_{c}^{a}$
Fe def R +10%FG	$8.43\pm0.82_{a}^{a}$	$9.75\pm0.44_{b}^{bc}$	$12.20\pm0.25_{c}^{ab}$
Fe def R + FeSO4	$8.42\pm0.77_{a}^{a}$	$10.66\pm0.67_{b}^{c}$	$14.08\pm0.65_{c}^{c}$

Values with different superscript down the column differ significantly ($p<0.05$)

Values with different subscript across the row differ significantly ($p<0.05$)

Fe suf RC: Iron sufficient Rat control, **Fe def. RC**: Iron deficient Rat control, **Fe def. R+ Fe suf. Diet**: Iron deficient Rat on iron sufficient diet, **Fe def. R+10%T:** Iron deficient Rat on iron deficient diet supplemented with 10% *Telfaira occidentalis*, **Fe def. R+10% FT:**Iron deficient Rat on on iron deficient diet supplemented with 10% fermented *Telfaira occidentalis,* **Fe def. R+10%G:** Iron deficient Rat on iron deficient diet supplemented with 10% *Gnetum africanum,* **Fe def. R+10%FG:** Iron deficient Rat on iron deficient diet supplemented with 10% fermented *Gnetum africanum,* **Fe def. R+$FeSO_4$:** Iron deficient Rat on iron deficient diet treated orally with 2.8mg/kg Ferrous Sulphate

Appendix 14

Effect of Fermented and Unfermented *Telfaira occidentalis* and *Gnetum africanum* Leaves Supplementation on Mean Corposcular Haemoglobin Concentration of Iron Deficient Rats

GROUPS n=5	DAY 0	DAY 7	DAY 21
Fe suf RC	$27.43\pm3.39_{a}^{b}$	$28.59\pm3.40_{a}^{b}$	$29.15\pm1.44_{a}^{a}$
Fe def RC	$21.73\pm1.39_{b}^{a}$	$18.83\pm1.91_{a}^{a}$	$28.10\pm2.23_{c}^{a}$
Fe def R + Fe suf diet	$18.44\pm2.34_{a}^{a}$	$21.85\pm2.12_{b}^{a}$	$29.94\pm1.66_{c}^{a}$
Fe def R +10%*T*	$19.93\pm1.44_{a}^{a}$	$21.68\pm1.26_{a}^{a}$	$30.70\pm1.79_{b}^{a}$
Fe def R +10% *FT*	$20.33\pm1.24_{a}^{a}$	$21.85\pm1.03_{a}^{a}$	$28.51\pm2.03_{b}^{a}$
Fe def R +10%G	$21.18\pm2.73_{a}^{a}$	$20.49\pm2.38_{a}^{a}$	$28.30\pm2.19_{b}^{a}$
Fe def R +10%FG	$21.10\pm2.93_{a}^{a}$	$21.25\pm2.19_{a}^{a}$	$31.09\pm5.11_{b}^{a}$
Fe def R + $FeSO_4$	$20.95\pm1.68_{a}^{a}$	$21.51\pm1.61_{a}^{a}$	$31.20\pm1.49_{b}^{a}$

Values with different superscript down the column differ significantly ($p<0.05$)

Values with different subscript across the row differ significantly ($p<0.05$)

Fe suf RC: Iron sufficient Rat control, **Fe def. RC**: Iron deficient Rat control, **Fe def. R+ Fe suf. Diet**: Iron deficient Rat on iron sufficient diet, **Fe def. R+10%T:** Iron deficient Rat on iron deficient diet supplemented with 10% *Telfaira occidentalis*, **Fe def. R+10% FT:**Iron deficient Rat on on iron deficient diet supplemented with 10% fermented *Telfaira occidentalis*, **Fe def. R+10%G:** Iron deficient Rat on iron deficient diet supplemented with 10% *Gnetum africanum*, **Fe def. R+10%FG:** Iron deficient Rat on iron deficient diet supplemented with 10% fermented

Gnetum africanum, **Fe def. R+$FeSO_4$:** Iron deficient Rat on iron deficient diet treated orally with 2.8mg/kg Ferrous Sulphate

Appendix 15

Effect of Fermented and Unfermented *Telfaira occidentalis* and *Gnetum africanum* Leaves Supplementation on Serum Ferritin Concentration of Iron Deficient Rats

Group n=5	Ferritin Concentration(ng/ml)
Fe suff. RC	138.50 ± 23.16^{bc}
Fe def. RC	80.00 ± 37.86^{a}
Fe def. R + Fe suff. Diet	148.00 ± 27.58^{c}
Fe def. R + 10%*T.O*	146.88 ± 31.84^{c}
Fe def. R+ 10% F. *T.O*	136.50 ± 22.89^{bc}
Fe def. R+ 10%*G.A*	113.00 ± 31.30^{abc}
Fe def.R +10% F.*G.A*	99.38 ± 30.03^{ab}
Fe def.R + $FeSO_4$	106.00 ± 27.31^{abc}

Values with different superscript down the column differ significantly ($p<0.05$)

Fe suf RC: Iron sufficient Rat control, **Fe def. RC**: Iron deficient Rat control, **Fe def. R+ Fe suf. Diet**: Iron deficient Rat on iron sufficient diet, **Fe def. R+10%T:** Iron deficient Rat on iron deficient diet supplemented with 10% *Telfaira occidentalis*, **Fe def. R+10% FT:**Iron deficient Rat on on iron deficient diet supplemented with 10% fermented *Telfaira occidentalis*, **Fe def. R+10%G:** Iron deficient Rat on iron deficient diet supplemented with 10% *Gnetum africanum*, **Fe def. R+10%FG:** Iron deficient Rat on iron deficient diet supplemented with 10% fermented *Gnetum africanum*, **Fe def. R+$FeSO_4$:** Iron deficient Rat on iron deficient diet treated orally with 2.8mg/kg Ferrous Sulphate

Appendix 16

Preparation of Reagents Used for Speciation Pattern Analysis

Magnesium chloride (1M) was prepared by weighing 20.33g of the salt in a 100ml volumetric flask , little deionized water was added to dissolve it afterwhich more water was added to make it up to the 100ml mark.

Sodium acetate (1M) was prepared by weighing 8.203g of the salt in a 100ml volumetric flask , little deionized water was added to dissolve it afterwhich more water was added to make it up to the 100ml mark.

Hydroxylamine hydrochloride (1M) was prepared by weighing 17.37g into a 250ml volumetric flask , little deionized water was added to dissolve it afterwhich more water was added to make it up to the 250ml mark.

Hydroxylamine hydrochloride (0.25M) was obtained by taking 14.39ml from the already prepared 1M Hydroxylamine hydrochloride

Hydrochloric acid (12M) was prepared by measuring 248.70ml of the acid and deionized water was added by the sides to make the volume up to the 250ml mark.

Aqua Regia Solution was prepared by measuring 1.2 litres of distilled water into a 2 litres volumetric flask 400ml concentration HCl and 133ml of 70% Nitric acid was added carefully, and was diluted to 2 litres.

Appendix 17

Preparation of standard drug stock solution

A stock solution of $FeSO_4$ with a concentration of 19.5 mg per ml was prepared by dissolving 3 tablets of the drug and made up to 10ml with deionized water. 2.86ml was taken from the stock solution and made up to 20ml with deionized water to prepare a concentration of 2.79 mg. The drug was administered to the rats at a dose of 2.79mg/kg

Appendix 18

Mineral content of the control and supplemented diets

DIETS	**MINERALS (mg/80g)**			
	Fe	**Zn**	**Cu**	**Ca**
Fe def. diet	13.92	3.06	10.20	22.02
Fe suf. diet	286.9	2.86	4.58	40.58
Fe def. diet + 10% *T. occidentalis*	18.74	4.17	9.27	100.76
Fe def. diet + 10% *T. occidentalis* fermented	25.09	5.15	9.28	146.09
Fe def. diet + 10% *G. africanum*	14.23	3.58	9.20	89.11
Fe def. diet + 10% *G. africanum* fermented	14.17	3.80	9.25	123.69

Fe def: Iron deficient, **Fe suf**: Iron sufficient