ADDIS ABABA UNIVERSITY

SCHOOL OF GRADUATE STUDIES

COLLEGE OF NATURAL SCINECES

CENTER FOR FOOD SCIENCE AND NUTRITION

Rat Hemoglobin Regeneration Efficiency of Teff Contaminated with Vertisol and Cambisol Soil Varieties

By:
Demeke Teklu

Advisor: Kaleab Baye (PhD)

A Thesis Submitted to the School of Graduate Studies of Addis Ababa University in Partial Fulfillment of the Requirements for the Degree of Master of Science in Food Science and Nutrition

June, 2016

Addis Ababa/Ethiopia

DECLARATION

I, the undersigned, declare that this thesis is my original work and that all sources of materials used for the thesis have been dully acknowledged.

Signature:____________________________ Date:_________________________

The thesis has been approved for submission by:

Name of Supervisor	Signature	Date
Kaleab Baye(PhD) (Advisor)	________________	_____________

Acknowledgment

First and for most the author would like to express his heartfelt thanks to Almighty God, for His wonderful and precious peace up on me throughout my entire life and for giving me wisdom and knowledge.

The author's special thank goes to Miss Roman Dale for her unreserved love, patience and kind support throughout the thesis work especially during sample collection.

The author is grateful to Dytes Inc., USA for their donation of 40kg of iron deficient AIN-93G rat diet and 100g of ferrous sulfate, Ethiopian public health institute department of traditional medicine and Addis Ababa University department of Biology for providing experimental rats.

The author's special gratitude goes to those kind peoples: Felekech Mulugeta, Abrham Gebretsadik, Tibebu Teklu, Amare Teklu, Teklu Senbetu and Eyerusalem Mantegaftot for their continuous prayer and kind support in one way or in other way.

Finally, the author would like to thank all the stuff members of Center for Food Science and Nutrition for their kind and friendly support in all direction of the thesis.

Table of contents

Contents **Page**

Declaration i

Acknowledgment iii

Table of contents iv

List of abbreviations vii

List of tables ix

Abstract x

CHAPTER ONE 1

1. INTRODUCTION 2

   1.1.Background 2

   1.2. Statement of the problem 4

   1.3. Significance of the study 5

   1.4. Objective 6

      1.4.1. General objective 6

      1.4.2. Specific objectives 6

CHAPTER TWO 7

2. LITRATURE REVIEW 8

2.1. Iron 8

2.1.1. History 8

2.1.2. Function 9

2.1.3. Human iron requirement 11

2.1.4. Toxicity 14

2.1.5. Bioavailability and human iron regulation 15

2.1.5.1. Systemic iron regulation - Dietary iron uptake 15

2.1.5.2. Storage 17

2.1.5.3. Recycling and loss 17

2.1.6. Iron deficiency 18

2.1.6.1 Nutritional deficiency 19

2.1.6.2 Increased demand 19

2.1.6.3 Increased loss 19

2.1.6.4 Impaired absorption 19

2.1.7 Groups at risk of iron deficiency 20

2.1.8 Methods of measuring human iron status 21

2.1.9 Bioaccessibility and bioavailability assessment of iron 23

2.1.9.1 *Invitro* iron bioaccessibility assessment methods 23

I. Dialysis or solubility technique 23

II. Caco 2 cell model 24

III. Absorption prediction algorithm 24

IV. Phytic acid/Mineral molar ratio 25

2.1.9.2 *Invivo* iron bioavailability assessment methods 25

2.2. Soil 26

2.3. Teff (Eragrostisis tef(Zucc) Trotter 27

CHAPTER THREE 32

3. MATERIALS AND METHOD 33

3.1. Study area, sample collection and preparation 33

3.2. Moisture content analysis 34

3.3. Proximate composition analysis 34

3.3.1.Crude protein analysis 34

3.3.2.Crude fat analysis 37

3.3.3.Crude fiber analysis 38

3.3.4.Crude ash analysis 39

3.4.Iron content analysis 39

3.5.Rat hemoglobin repletion method 40

3.5.1.Study animals collection and screening 41

3.5.2.Study animals handling and ethical consideration 41

3.5.3.Rat hemoglobin depletion- repletion method 42

3.5.3.1.Experimental diets preparation and composition 42

3.5.3.2.Depletion phase (induction of iron deficiency-anemia) 44

3.5.3.3.Repletion phase 45

3.5.3.4.Measurement of iron bioavailability 46

I. Iron consumption 46

II. Hemoglobin iron pool 46

III. Hemoglobin regeneration efficiency 47

IV. Relative biological value 47

3.5.4. Statistical analysis 47

CHAPTER FOUR 49

4. RESULTS AND DISCUSSIONS 50

4.1. Proximate composition & total iron content of teff flour 50

4.2. Physicochemical property & iron content of vertisol & cambisol 52

4.3. Experimental rats baseline characteristics 53

4.4. Food & iron intake, body weight gain & feed efficiency ratio 53

4.5. Hemoglobin regeneration efficiency & relative biological value 55

4.6. Bioavailability of intrinsic iron originated from teff 61

CHAPTER FIVE 63

5. CONCLUSIONS AND RECOMMENDATIONS 64

REFERENCES 65

ANNEXS 85

Annex I: Annex I: Sample collection site, teff type and growing condition 85

Annex II: Ethical clearance letter from the Ethical Clearance Review Board of the College of natural sciences of Addis Ababa University 86

Lists of Abbreviations and Acronyms

AAS	Atomic Absorption Spectrophotometer
AIN-93G	American Institute of Nutrition Formula for Growing Rats
ANOVA	Analysis of Variance
AOAC	Association of Official Analytical Chemists
ATP	Adenosine Triphosphate
Caco-2	Human Colon Carcinoma Cell Line
CEC	Cation Exchange Capacity
CRP	C-Reactive Protein
CSAE	Central Statistics Agency of Ethiopia
Dcytb	Duodenal Cytochrome B
DM	Dry Matter
DMT1	Divalent Metal Transporter 1
DNA	Deoxyribonuclic Acid
DRI	Dietary Reference Intake
EDHS	Ethiopian Demographic Health Survey
EHNRI	Ethiopian Health and Nutrition Research Institute

ENS	Ethiopian Nutrition Survey
FAAS	Flam Atomic Absorption Spectrometry
FAO	Food and Agricultural Organization of United Nations
Hb	Hemoglobin
HRE	Hemoglobin Regeneration Efficiency
IFPRI	International Food Policy Research Institute
N	Normality
OC	Organic carbon
PPIs	Proton Pump Inhibitors
RBCs	Red Blood Cells
RBV	Relative Biological Value
SPSS	Software Statistical Package for Social Sciences
TB	Tuberculosis
TfR	Serum Transferring Receptor
UL	Tolerable Upper Intake Level
USA	United States of American
WHO	World Health Organization of United Nations

List of tables

Tables **Page**

Table 2.1. Iron intake required for growth under the age of 18 years, median basal iron losses, menstrual losses in women, and total absolute iron requirements (FAO, 2006) 13

Table 3.1. Nutritional composition of experimental diets (g/Kg of diet) 43

Table 4.1. Proximate composition and total iron content of laboratory threshed teff flour in dry base 51

Table 4.2. Physicochemical property and iron content of vertisol and cambisol soil (Meseret, 2015) 52

Table 4.3. Baseline rats profile at the end of depletion phase 53

Table 4.4. Food and iron intake, body weight gain and feed efficiency ratio during repletion phase 54

Table 4.5. The final hemoglobin, hemoglobin gain and hemoglobin regeneration efficiency of rats 56

Table 4.6. Comparison of relative biological value of current study with other studies 62

Abstract

It is an orthodox idea that teff has highest content of iron among the majority of cereal-based diet, which provides almost the entire staple food consumed in Ethiopia. However, now a day it is proven that it is due to soil contamination. Thus, these leads to two distinctive ideas on the contaminant iron bioavailability. The current study was, then, initiated with the objective of evaluating effect of different soil contamination on rat of hemoglobin regeneration efficiency. The *invivo* iron bioavailability of the uncontaminated teff, vertisol contaminated teff, and cambisol contaminated teff were analyzed and compared against the standard ferrous sulfate group. There was no significant deference in total food and iron intake, weight gain, feed efficiency ratio, initial and final hemoglobin, hemoglobin gain, hemoglobin regeneration efficiency between and among treatment and control groups ($P<0.05$). However, there was a significant difference in final hemoglobin, hemoglobin gain, and hemoglobin regeneration efficiency between teff and control group. The hemoglobin regeneration efficiency of control group was about 12% higher than teff group. The relative biological value of teff, vertisol and cambisol groups were 79.47%, 86.91% and 86.24% ($P<0.05$) respectively. The 6.77% and 7.44% of relative biological value and 3.97% and 4.31% of hemoglobin regeneration efficiency enhancements were observed due to 35mg/kg of extrinsic iron for vertisol and cambisol groups respectively. In summary, the current study demonstrates that intrinsic iron originated from teff has a good relative biological value, contamination of teff with both varieties of soil considerably improves the iron bioavailability; thus, contaminant iron has a good nutritional interest, and soil (vertisol and cambisol) contamination do not interfere with the intrinsic iron bioavailability.

Key words: *Bioavailability Cambisol, Hemoglobin Regeneration Efficiency, Invivo, Relative Biological Value, Vertisol*

Chapter One

Introduction

1. Introduction

1.1 Background

The high prevalence of iron deficiencies which accounts more than two billion people worldwide has several adverse effects on the population especially in developing countries, particularly on women and children (Gibson, 2006; Zimmermann and Hurrell, 2007). As a result, anemia leads to impaired psychomotor development in infants, impaired educational performance of schoolchildren, reduced working capacity in adults, and increased prenatal morbidity in pregnancy (Hall *et al*., 2000). Iron deficiency anemia yet remains one of the largest public health problems in both developing and developed countries (Ramakrishnan *et al.,* 2002; WHO, 2007). Iron deficiencies are common in populations whose diet is mostly composed of plant-based foods which is attributed to the low mineral content of plant-based foods, lower bioavailability of non-haem iron sources, and as well as to their high chelating agent contents (Lonnerdal, 2000; Hurrell and Egli, 2010) while in Ethiopia research indicates that all the six principal staple foods (Teff, wheat, sorghum, maize, barely and enset) which supplying more than 70 % of calories are plant source foods (Birhane *et al*., 2011).

Earlier study suggested that anemia was rare in Ethiopia and ascribed this with prevalence of teff consumption (Gebremedhin *et al.,* 1976). However, recent studies revealed that iron deficiency anemia is a mild to moderate public health problem and become a concern (Haidar and Pobocik, 2009; Haidar 2010; Umeta *et al*., 2008). Though a research by Baye *et al*., (2012) in northern Ethiopia suggested that children's iron intake was more than sufficient, the prevalence of anemia among under five children was

44% (EDHS, 2011). This may suggest the poorest bioavailability of iron intake from the food source.

Teff is an ancient tropical cereal that has its center of origin and diversity in Northern Ethiopian highlands from where it is believed to have been domesticated (Demissie 2001; Ketema, 1997). Teff is underutilized cereal crop worldwide, whereas in Ethiopia, it is a major food grain mainly used to make *injera* a traditional fermented Ethiopian pancake. In other countries like Australia, South Africa and United States, it is principally used as a forage crop for animal feed. In many regions of Ethiopia, teff is consumed by people in many traditional forms. Teff-based foods are commonly consumed by infants and adults and significantly contribute to the nutrient supply of the diet of millions of people. Teff represents 22.6% of the cereals grown in Ethiopia (CSAE, 2012).

According to Saturni *et al*, (2010) and Umeta *et al.*, (2005) teff which is one of the major staple food crop of Ethiopia, is believed to be an excellent source of iron for most of the population. However, in controversy of the above authors some authors argue that teff's highest content of iron is due to soil contamination as a result of threshing the crop on the ground (Abebe *et al*., 2007; Ambaw , 2013; Bokhari *et al*., 2012; Harvey *et al*., 2000). A significant proportion of the iron in the teff diets of Ethiopia is extrinsic to food, usually referred to as contaminant iron (Baye *et al*., 2013; Umeta *et al*., 2005).

1.2 Statement of the problem

Compared to more common cereals like wheat, rice and maize, little is known about the nutritional composition and potential health benefits of teff. This, along with technological limitation in processing teff, has for long restricted its more wide spread consumption from its center of origin, Ethiopia. Thus, this limited the information on the nutritional benefit of teff available for the general public and lacks the global interest in teff. In turn the lack of the global interest in teff made prolonged thinking by Ethiopians that their grain is of interior nutritional quality (IFPRI, 2016).

Many research findings have been repeatedly reported that the Ethiopian traditional threshing of teff using cattle hooves on the ground contaminates teff with soil iron (Abebe *et al*., 2007; Ambaw , 2013; Baye *et al*., 2013; Bokhari *et al*., 2012; Harvey *et al*., 2000; Umeta *et al*., 2005). Nevertheless, the impact of this contaminant iron is left controversial yet and needs to be clarified with more evidential investigations. Otherwise the existing divisive ideas may lead to two extinct conclusions in turn which leads to harmful outcomes: for instance if it is concluded that contaminant iron is not bioavailable while it is, the supplement of iron will be suggested. Thus, this may lead to limited growth of children, exposure to infection, and oxidative stress due to excessive iron intake (Iannotti *et al*., 2006; Majudar *et al*., 2003; Sazawal *et al*., 2006), and if it is concluded that contaminant iron is bioavailable while it is not, this may lead to overestimation of the satisfaction of iron requirement while iron deficiencies remain the problem. Therefore, it is imperative to consider more tangible investigation to clear up the dilemma.

Beside the limited researches that are conducted on the bioavailability of contaminant iron in the diet of Ethiopians, the *invitro* methods were used for analysis which might be less appropriate to analyze bioavailability of contaminant iron in the diet (Baye *et al*., 2013). Therefore, this research activity was designed to study bioavailability of contaminant iron by employing the golden standard method *invivo*/rat hemoglobin repletion efficiency test method.

1.3 Significance of the study

It is an orthodox idea that teff has highest content of iron among the majority of diet consumed in Ethiopia, since other cereal based foods in Ethiopia which provides almost the entire staple food in the country blamed to have lower iron content. This research activity was one which aims to study bioavailability of iron by employing *invivo*/rat hemoglobin repletion efficiency test method. The research output will contribute much through the following points:

- help to promote teff utilization and marketing including export for its nutritional merits, since teff has been reported for its health claims one of which is its iron content, however, its iron utilizeability has not been confirmed and this research will answer this question;
- helps local consumers to attain dietary recommendations for iron and at the same time can reduce the risk of side effect and toxicity associated with taking high dose of iron supplements as iron utilizeability from teff and soil contamination will be known;

- since the government taking an effort to fortify iron to different flours as an approach for a strategy to control micronutrient deficiency (hidden hunger), this research will provide data on how much percent is needed to be fortified or even not;
- provide much needed information on teff and contaminant iron bioavailability to the academic and research world, thereby helps policy makers nationally and even internationally;
- provide information which helps to modify the agricultural practices if it is needed: i.e.- if the soil contamination interfere with the intrinsic iron absorption, then the traditional threshing process should be modified to which that minimizes the contamination

1.4 Objective

1.4.1 General objective

To evaluate the efficiency of rat hemoglobin regeneration of anemic rat fed with teff contaminated with vertisol and cambisol soil varieties.

1.4.2 Specific objectives

- ✓ To estimate the bioavailability of iron from cambisol and vertisol soil types,
- ✓ To predict the hemoglobin regeneration efficiency of teff’s intrinsic iron,
- ✓ To interrelate bioavailability of contaminant iron to vertisol and cambisol soil types, and
- ✓ To evaluate whether soil contamination block absorption of intrinsic iron

Chapter Two

Literature Review

2. Literature review

2.1 Iron

Iron is a metal in the first transition series. It is by mass the most common element on earth, forming much of earth's outer and inner core. It is the fourth most common element in the earth's crust and the second most abundant metal on earth. Its abundance in rocky planets like earth is due to its abundant production by fusion in high-mass stars, where the production of nickel-56 (which decays to the most common isotope of iron) is the last nuclear fusion reaction that is exothermic.

2.1.1 History

In early 16th century, the relationship between dietary iron and blood disorder were recognized and history revealed that in 17th century iron was used to treat a disease called green disease or chlorosis (historical name of hypochromic anemia which is often caused by iron deficiency) (Guggenheim, 1995). During late 18th century Zinoffsky found that horse hemoglobin contains 0.335% iron which contributes to recognize the iron's physiological basis to blood (Suttle, 2010). Thought it was a debated work, in 1893 a study conducted by Stockman on anemic women showed that ferrous salts rapidly increase hemoglobin concentration (Bruner, 1996). Finally, in 19th century studies which had supported with a strong evidences have been shown that inorganic iron could function for blood hemoglobin synthesis (Suttle, 2010).

2.1.2 Function

Iron is an essential bioelement for most forms of life, from bacteria to mammals. Its importance lies in its ability to mediate electron transfer. In the ferrous state (Fe^{2+}), iron acts as an electron donor, while in the ferric state (Fe^{3+}) it acts as an acceptor. Thus, iron plays a vital role in the catalysis of enzymatic reactions that involve electron transfer (reduction and oxidation, redox) (Hentze *et al*., 2010; Muckenthaler *et al*., 2008; Wessling-Resnick, 2014).

Proteins can contain iron as part of different cofactors, such as iron-sulfur clusters (Fe-S) and heme groups, both of which are assembled in mitochondria. Human cells require iron in order to obtain energy as ATP from a multi-step process known as cellular respiration, more specifically from oxidative phosphorylation at the mitochondrial cristae. Iron is present in the iron-sulfur clusters and heme groups of the electron transport chain proteins that generate a proton gradient that allows ATP synthase to synthesize ATP (chemiosmosis).

Heme groups are part of hemoglobin, a protein found in red blood cells that serves to transport oxygen from the lungs to the tissues i.e. oxygen is transported from the lungs to the rest of the body bound to the heme group of hemoglobin in erythrocytes. Heme groups are also present in myoglobin to store and diffuse oxygen in muscle cells i.e. in muscles cells, iron binds myoglobin, which regulates oxygen release (Abbaspour *et al*., 2014; Arosio and Levi, 2002; Finney and O'Halloran , 2003; Ganz, 2005).

In response to a systemic bacterial infection, the immune system initiates a process known as iron withholding. If bacteria are to survive, then they must obtain iron from their environment. Disease causing bacteria do this in many ways, including releasing iron-binding molecules called siderophores and then reabsorbing them to recover iron, or scavenging iron from hemoglobin and transferrin. The harder they have to work to get iron, the greater a metabolic price they must pay. That means that iron-deprived bacteria reproduce more slowly. So our control of iron levels appears to be an important defense against most bacterial infections; there are some exceptions however. TB causing bacterium can reside within macrophages which are an iron rich environment and *Borrelia burgdorferi* utilizes manganese in place of iron. People with increased amounts of iron, like people with hemochromatosis, are more susceptible to some bacterial infection (Ganz, 2005). Although this mechanism is an elegant response to short-term bacterial infection, it can cause problems when inflammation goes on for longer. Since the liver produces hepcidin in response to inflammatory cytokines, hepcidin levels can increase as the result of non-bacterial sources of inflammation, like viral infection, cancer, auto-immune diseases or other chronic diseases. When this occurs, the sequestration of iron appears to be the major cause of the syndrome of anemia of chronic disease, in which not enough iron is available to produce enough hemoglobin-containing red blood cells (Andrews, 1999).

Iron is also known to be very essential for improved cognitive development as it is essential for all tissues that are found in brain from very early life it functions in the neural myclination processes. Therefore, during pregnancy its adequacy is associated with improved outcomes for the infant as well as the mother (Abbaspour *et al*., 2014; Lannotti *et al*., 2006; Rouault and Cooperman , 2006).

2.1.3 Human iron requirement

The human iron requirement varies according to age, sex, and body weight (Table 2.1). The newborn term infant has an iron content of about 250-300 mg (75 mg/kg body weight). During the first 2 months of life, hemoglobin concentration falls because of the improved oxygen situation in the newborn infant compared with the intrauterine foetus. This leads to a considerable redistribution of iron from catabolised erythrocytes to iron stores. This iron will cover the needs of the term infant during the first 4-6 months of life and is why iron requirements during this period can be provided by human milk, which contains very little iron. Because of the marked supply of iron to the foetus during the last trimester of pregnancy, the iron situation is much less favourable in the premature and low-birth-weight infant than in the term infant. An extra supply of iron is therefore needed in these infants even during the first 6 months of life (FAO/WHO 1988; FAO/WHO 2004; Zimmermann and Hurrell, 2007)

In the full-term infant, iron requirements will rise markedly after age 4-6 months and amount to about 0.7-0.9 mg/day during the remaining part of the first year. These requirements are therefore very high, especially in relation to body size and energy intake (European Communities, 1993).

In the first year of life, the full-term infant almost doubles its total iron stores and triple its body weight. The change in body iron during this period occurs mainly during the first 6-12 months of life. Between 1 and 6 years of age, the body iron content is again doubled. The requirements for absorbed iron in infants and children are very high in relation to their energy requirements. For example, in infants 6-12 months of age, about

1.5 mg of iron need to be absorbed per 4.184 MJ and about half of this amount is required up to age 4 years (Abbaspour *et al*., 2014; FAO/WHO 1988; FAO/WHO 2004; FAO, 2006).

In the weaning period, the iron requirements in relation to energy intake are the highest of the lifespan except for the last trimester of pregnancy, when iron requirements to a large extent have to be covered from the iron stores of the mother. The rapidly growing weaning infant has no iron stores and has to rely on dietary iron. It is possible to meet these high requirements if the diet has a consistently high content of meat and foods rich in ascorbic acid. In most developed countries today, infant cereal products are the staple foods for that period of life. Commercial products are regularly fortified with iron and ascorbic acid, and they are usually given together with fruit juices and solid foods containing meat, fish, and vegetables. The fortification of cereal products with iron and ascorbic acid is important in meeting the high dietary needs, especially considering the importance of an optimal iron nutrition during this phase of brain development (Dallman and Siimes, 1979; Mascotti *et al*., 1995).

Iron requirements are also very high in adolescents, particularly during the period of rapid growth (Rossander-Hulthén and Hallberg, 1996). There is a marked individual variation in growth rate and the requirements may be considerably higher than the calculated mean values given in table 2.1. Girls usually have their growth spurt before menarche, but growth is not finished at that time. Their total iron requirements are therefore considerable. In boys during puberty there is a marked increase in haemoglobin mass and concentration, further increasing iron requirements to a level above the average iron requirements in menstruating women (Hallberg and Rossander-Hulthénm, 1991; Hallberg *et al.*, 1997; FAO/WHO**,** 1988, FAO, 2006; Mascotti *et al.*, 1995).

Table 2.1. Iron intakes required for growth under the age of 18 years, median basal iron losses, menstrual losses in women, and total absolute iron requirements (FAO, 2006)

Group	Age (Years)	Body weight (Kg)	Required iron intake for growth (mg/day)	Basal iron loss (mg/day)	Menstrual loss (mg/day)		Total requirement (mg/day) †	
		Mean		Median	Median	95th percentile	Median	95th percentile
Children	0.5-1	9	0.55	0.17			0.72	0.93
	1-3	13.3	0.27	0.19			0.46	0.58
	4-6	19.2	0.23	0.27			0.5	0.63
	7-10	28.1	0.32	0.39			0.71	0.89
Males	11-14	45	0.55	0.62			1.17	1.46
	15-17	64.4	0.6	0.9			1.5	1.88
	18+	75		1.05			1.05	1.37
Females	11-14[b]	46.1	0.55	0.65			1.2	1.4
	11-14	46.1	0.55	0.65	0.48[C]	1.9[C]	1.68	3.2
	15-17	56.4	0.35	0.79	0.48[C]	1.9[C]	1.62	3.1
	18+	62		0.87	0.48[C]	1.9[C]	1.46	2.94
Post-menopausal		62		0.87			0.87	1.13
Lactating		62		1.15			1.15	1.5

† Total Absolute Requirements = Requirement for growth + basal losses + menstrual losses (females only). b Non-menstruating. c Effect of the normal variation in haemoglobin concentration not included in this figure.

2.1.4 Toxicity

Large amounts of ingested iron can cause excessive levels of iron in the blood. High blood levels of free ferrous iron react with peroxides to produce free radicals (its ability to donate and accept electrons means that if iron is free within the cell, it can catalyze the conversion of hydrogen peroxide into free radicals), which are highly reactive and can damage DNA, proteins, lipids, and other cellular components. Thus, iron toxicity occurs when there is free iron in the cell, which generally occurs when iron levels exceed the capacity of transferrin to bind the iron. Damage to the cells of the gastrointestinal tract can also prevent them from regulating iron absorption leading to further increases in blood levels. Iron typically damages cells in the heart, liver and elsewhere, which can cause significant adverse effects, including coma, metabolic acidosis, shock, liver failure, coagulopathy, adult respiratory distress syndrome, long-term organ damage, and even death (Cheney *et al*., 1995).

To prevent that kind of damage, all life forms that use iron bind the iron atoms to proteins. This binding allows cells to benefit from iron while also limiting its ability to do harm (Andrews, 1999; Conrad and Umbreit, 2000). Humans experience iron toxicity above 20 milligrams of iron for every kilogram of mass, and 60 milligrams per kilogram is considered a lethal dose. The Dietary Reference Intake (DRI) lists the Tolerable Upper Intake Level (UL) for adults as 45 mg/day. For children under fourteen years old the UL is 40 mg/day (Conrad and Umbreit, 2000; Tenenbein , 1996).

2.1.5 Bioavailability and human iron regulation

Human iron homeostasis is regulated at two different levels. Systemic iron levels are balanced by the controlled absorption of dietary iron by enterocytes, the cells that line the interior of the intestines, and the uncontrolled loss of iron from epithelial sloughing, sweat, injuries and blood loss. In addition, systemic iron is continuously recycled. Cellular iron levels are controlled differently by different cell types due to the expression of particular iron regulatory and transport proteins.

2.1.5.1 Systemic iron regulation - Dietary iron uptake

Iron uptake is tightly regulated by the human body, which has no regulated physiological means of excreting iron. The absorption of dietary iron is a variable and dynamic process. The amount of iron absorbed compared to the amount ingested is typically low, but may range from 5% to as much as 35% depending on circumstances and type of iron.

The efficiency with which iron is absorbed varies depending on the source. Generally the best-absorbed forms of iron come from animal products. Absorption of dietary iron in iron salt form (as in most supplements) varies somewhat according to the body’s need for iron, and is usually between 10% and 20% of iron intake. Absorption of iron from animal products, and some plant products, is in the form of heme iron, and is more efficient, allowing absorption of from 15% to 35% of intake. Heme iron in animals is from blood and heme-containing proteins in meat and mitochondria, whereas in plants, heme iron is present in mitochondria in all cells that use oxygen for respiration. Non-heme iron from plant based food have poor absorption ranging from 2 to 20% which is strongly influenced by other food components ingested at the same time (increased by meat and ascorbic acid and inhibited by phytates, polyphenols and calcium) (Abbaspour *et al*., 2014; Geoffrey, 2010; Hurrell and Egli, 2010; Nanami *et al*., 2005).

Like most mineral nutrients, the majority of the iron absorbed from digested food or supplements is absorbed in the duodenum by enterocytes of the duodenal lining. These cells have special molecules that allow them to move iron into the body. To be absorbed, dietary iron can be absorbed as part of a protein such as heme protein or iron must be in its ferrous Fe^{2+} form. A ferric reductase enzyme on the enterocytes' brush border, duodenal cytochrome B (Dcytb), reduces ferric Fe^{3+} to Fe^{2+} (McKie *et al*., 2001). A protein called divalent metal transporter 1 (DMT1), which can transport several divalent metals across the plasma membrane, then transports iron across the enterocyte's cell membrane into the cell. These intestinal lining cells can then either store the iron as ferritin, which is accomplished by Fe^{3+} binding to apoferritin (in which case the iron will leave the body when the cell dies and is sloughed off into feces), or the cell can release it into the body via the only known iron exporter in mammals, ferroportin. Hephaestin, a ferroxidase that can oxidize Fe^{2+} to Fe^{3+} and is found mainly in the small intestine, helps ferroportin transfer iron across the basolateral end of the intestine cells.

In contrast, ferroportin is post-translationally repressed by hepcidin, a 25-amino acid peptide hormone. The body regulates iron levels by regulating each of these steps. For instance, enterocytes synthesize more Dcytb, DMT1 and ferroportin in response to iron deficiency anemia (Fleming and Bacon, 2005). The human body's rate of iron absorption appears to respond to a variety of interdependent factors, including total iron stores, the extent to which the bone marrow is producing new red blood cells, the concentration of hemoglobin in the blood, and the oxygen content of the blood. The body also absorbs less iron during times of inflammation (Ganz, 2005).

2.1.5.2 Storage

Most well-nourished people have 4 to 5 grams of iron in their bodies. Of this, about 2.5 g is contained in the hemoglobin needed to carry oxygen through the blood, and most of the rest (approximately 2 grams in adult men, and somewhat less in women of childbearing age) is contained in ferritin complexes that are present in all cells, but most common in bone marrow, liver, and spleen. The liver's stores of ferritin are the primary physiologic source of reserve iron in the body. The reserves of iron in industrialized countries tend to be lower in children and women of child-bearing age than in men and in the elderly. Women who must use their stores to compensate for iron lost through menstruation, pregnancy or lactation have lower non-hemoglobin body stores, which may consist of 500 mg, or even less. Of the body's total iron content, about 400 mg is devoted to cellular proteins that use iron for important cellular processes like storing oxygen (myoglobin) or performing energy-producing redox reactions (cytochromes). A relatively small amount (3–4 mg) circulates through the plasma, bound to transferring (Camaschella and Schrier , 2011).

2.1.5.3 Recycling and loss

Most of the iron in the body is hoarded and recycled by the reticuloendothelial system, which breaks down aged red blood cells. In contrast to iron uptake and recycling, there is no physiologic regulatory mechanism for excreting iron. People lose a small but steady amount by gastrointestinal blood loss, sweating and by shedding cells of the skin and the mucosal lining of the gastrointestinal tract. The total amount of loss for healthy people amounts to an estimated average of 1 mg a day for men, and 1.5–2 mg a day for women with regular menstrual periods. People with gastrointestinal parasitic infections, more commonly found in developing countries, often lose more (Conrad and Umbreit, 2000). Those who can’t regulate absorption well enough get disorders of iron overload. In these diseases, the toxicity of iron starts overwhelming the body's ability to bind and store it (Schrier and Bacon, 2011).

Macrophages of the reticuloendothelial system store iron as part of the process of breaking down and processing hemoglobin from engulfed red blood cells. Iron is also stored as a pigment called hemosiderin which is an ill defined deposit of protein and iron, created by macrophages where excess iron is present, either locally or systemically for example among people with iron overload due to frequent blood cell destruction and transfusions. If the systemic iron overload is corrected, over time the hemosiderin is slowly reasorbed by macrophages (Schrier and Bacon, 2011).

2.1.6 Iron deficiency

Iron deficiency first affects the storage iron in the body, and depletion of these stores is thought to be relatively non-symptomatic, although some vague and non-specific symptoms have been associated with it. Since iron is primarily required for hemoglobin, iron deficiency anemia is the primary clinical manifestation of iron deficiency. Iron-deficient people will suffer or die from organ damage well before cells run out of the iron needed for intracellular processes like electron transport (FAO, 2006). Functional or actual iron deficiency can result from a variety of causes. These causes can be grouped into several categories:

2.1.9.3 **Nutritional deficiency**

This can result due to a lack of dietary iron (consumption low bioavailable iron) or consumption of foods that inhibit iron absorption, including calcium, phytates and tannins. It can also resulted from other nutrients deficiency like vitamin A and B12, riboflavin, folic acid. This cause is believed to be a primary cause and responsible for more than 50 to 60% of anemia (Allen *et al*., 2006; Ganz, 2003).

2.1.9.4 **Increased demand**

An increased demand for iron , which the diet can’t accommodate, due to some natural circumstances like pregnancy, rapid growth, menstruation are also an important cause for iron deficiency (Abbaspour *et al*., 2014; Schrier and Bacon, 2011; Zimmermann and Hurrell, 2007).

2.1.9.5 **Increased loss**

An increased loss of iron usually through blood loss leads to iron deficiency as it depletes the iron store (0.5 mg iron loss for every 1ml of blood loss) (Zimmermann and Hurrell, 2007).

2.1.9.6 **Impaired absorption**

Inability to absorb iron: This could be arising from use of acid reducing medications, the strongest are the proton pump inhibitors (PPIs) such as omeprazole. The use of this class of medication is causing an increase in iron deficiency and is almost an epidemic

(Camaschella, 2005). Damage to the intestinal lining: Examples of causes of this kind of damage include surgery involving the duodenum, or diseases like Crohn's or celiac sprue which severely reduce the surface area available for absorption, and Inflammation: leading to hepcidin-induced restriction on iron release from enterocytes are also the common causes to impair the absorption (Frazer and Anderson , 2005).

2.1.10 **Groups at risk of iron deficiency**

Worldwide, the highest prevalence of iron deficiency is found in infants, children, adolescents, and women of childbearing age, especially pregnant women. The weaning period in infants is especially critical because of the very high iron requirements in relation to energy requirements. The highest probability of suffering iron deficiency is found in those parts of a population that have inadequate access to foods rich in absorbable iron during stages of high iron demand. These groups correspond to children, adolescents, and women of reproductive age, in particular during pregnancy.

In the case of infants and adolescents, the increased iron demand is the result of rapid growth. For women of reproductive age the principle reason is the excessive blood loss during menstruation. During pregnancy, there is a significant increase in iron requirement due to the rapid growth of the placenta and the fetus and the expansion of the globular mass. In contrast, adult men and postmenopausal women are at low risk of iron deficiency and the amount of iron in a normal diet is usually sufficient to cover their physiological requirements.(Dallman, 1990; Björn-Rasmussen and Hallberg, 1979; FAO, 2006; Taylor, 1995).

2.1.11 **Methods of measuring human iron status**

Scholl (2011) defines iron deficiency by three stages of increasing severity: depletion of iron store (stage I) which can be diagnosed by showing that there is no stainable iron in the reticuloendothelial cells in bone marrow smears or more easily by a low concentration of ferritin in serum (£15 μg/l), iron deficiency without anemia (stage 2) and iron deficiency anemia (stage 3). Iron deficiency and eventually anemia develop in stages and can be assessed by measuring various biochemical indices.

Although some iron enzymes are sensitive to iron deficiency, their activity has not been used as a successful routine measure of iron status (Dallman, 1990; Wood *et al*., 2005). Therefore, laboratory measurements are essential for a proper diagnosis of iron deficiency. The plasma or serum pool of iron is the fraction of all iron in the body that circulates bound primarily to transferrin. Three ways of estimating the level of iron in the plasma or serum include: measuring the total iron content per unit volume in μg/dL; measuring the total number of binding sites for iron atoms on transferrin, known as total iron-binding capacity in μg/dL; and estimating the percentage of the two bindings sites on all transferrin molecules that are occupied called the percentage transferrin saturation. However, marked biologic variation can occur in these values as a result of diurnal variation, the presence of infection or inflammatory conditions and recent dietary iron intake (WHO, 2004).

Serum ferritin is a good indicator of body iron stores under most circumstances. When the concentration of serum ferritin is ≥15 μg/L iron stores are present; higher concentrations reflect the size of the iron store; when the concentration is low (<12 μg/L for <5 years of age and <15 μg/L for >5 years of age) iron stores are depleted. However, ferritin is an acute phase reactant protein and its serum concentrations can be elevated, irrespective of a change in iron stores, by infection or inflammation. This means that it might be difficult to interpret the concentration of ferritin where infectious diseases are common (WHO, 2004; Wood *et al*., 2005).

Zinc protoporphyrin reflects the shortage of iron supply in the last stages of hemoglobin synthesis so that zinc is inserted into the protoporphyrin molecule in the place of iron. Zinc protoporphyrin can be detected in RBCs by fluorimetry and is a measure of the severity of iron deficiency (WHO, 2004).

Another indicator of iron status is the concentration of serum transferring receptor (TfR) in serum. Since TfR is mostly derived from developing RBCs, it reflects the intensity of erythropoiesis and the demand for iron. As iron stores are exhausted, the concentration rises in iron deficiency anemia indicating severe iron insufficiency. This is provided that there are no other causes of abnormal erythropoiesis (WHO, 2004). Clinical studies indicate that the serum TfR is less affected by inflammation than serum ferritin (Beguin, 2003). The major advantage of TfR as an indicator is the possibility of estimating the magnitude of the functional iron deficit once iron stores are depleted (Baynes, 1991). However, the high cost and the lack of standardization of the TfR assay so far have limited the applicability of the method (Yang *et al.*, 2008).

2.1.12 **Bioaccessibility and bioavailability assessment of iron**

Bioaccessibility of iron cans defined as the proportion of iron released from food matrix into the lumen of gastrointestinal tract and the fraction of iron potentially available for absorption mostly relies on food consumption. Bioaccessibility can be measured using *in vitro* methods. Bioavailability of iron can be defined as the portion of ingested iron that can be used for normal body function and it can be measured using *in vivo* methods (Fairweather-Tait *et al*., 1995; Bokhari *et al*., 2012).

2.1.12.1 ***In vitro* iron bioaccessibility assessment methods**

The *in vitro* iron bioaccessiblity methods are the dominant method in iron bioavailability test since it is relatively simple, rapid and cost effective. It has been used to analyze the minerals including iron for more than half a century (Ismail, 1999). These methods are, generally, advantageous if *in vivo* bioavailability test could be based on preliminary *in vitro* screening methods for efficient identification of the promising food substances of interest (Beiseigel *et al*., 2007).

V. **Dialysis or solubility technique** - This is a two step digestion at simulated physiological conditions: gastric phase having pepsin and hydrochloric acid at pH 2 and intestinal phase having pancreatic enzymes, bile acids and $NaHCO_3$ at pH 7 measure the soluble or dialysable minerals (Frontela et al., 2011; Luten et al., 1996). This technique can be useful to identify enhancers , inhibitors, (phytate and degradation products, polyphenols, ascorbic acid) but does not predict same magnitude of response as in humans; since A) small polyphenolic compounds and organic acid complexes is dialysable but not bioavailable, B) large molecules like ferritin can be absorbed but is not dialyzable.compounds and organic acid complexes is dialysable but not bioavailable, B) large molecules like ferritin can be absorbed but is not dialyzable.

VI. **Caco 2 cell model** – This method involve two-step *invitro* digestion simulating gastric phase, intestinal phase, uptake/absorption measured as ferritin formation in Caco-2 cells after 22 hours and partial transport into circulation (Boato *et al*., 2002; Yun *et al*., 2004). The model is quit better as compared to the dialysis/solubility technique but not far enough compatible with the magnitude of response in humans as the model assume ferritin formation proportional to iron uptake and does not include hepcidin controlled transport, uptake is only considered for cell but not blood. Duizer *et al.*,(1997) also indicate that caco-2 cells are colon cells – transport rates of hydrophilic compounds paracellular lower, less leaky, less discrimination on the basis of molecular size of compounds transported parallellary compared to duodenum cells. Therefore, the model can be an excellent predictor of direction of response but not magnitude of response in human.

VII. **Absorption prediction algorithm** – which involves the prediction of iron bioavailability in the meal based on the calculation of heme and non-heme iron composition and their bioavailability, balanced factors that enhance or inhibit the iron absorption. According to Reddy, (2005) the model is inaccurate for quantitative measurement since all factors effect are assumed to be independent and additive rather than interactive. Knowledge about the exact amount of contaminant iron and its bioavailability in soil contaminated foods may be a prerequisite for proper interpretation of algorithm to predict absorption unless the prediction only apply to intrinsic iron (Baye *et al*., 2013).

VIII. **Phytic acid/Mineral molar ratio –** which involve the calculation of molar ratio of phytic acid to iron. The model is not the sufficient indicator of iron bioavailability since it only based on the inhibition effect of phytic acid while there are other factors like tannin, calcium, fiber etc which can possibly inhibit iron absorption. According to Baye *et al*., (2013) knowing the exact amount of contaminant iron and its bioavailability in soil contaminated cereal foods may be a prerequisite for use of phytic acid/iron molar ratio to correctly predict bioavailability in food containing contaminant iron.

2.1.12.2 ***In vivo* iron bioavailability assessment methods**

Human and animal trial are used to evaluate the iron bioavailability and considered as a golden standards. Determining the iron bioavailability in human is complicated by the fact that once a food is consumed, it mixes with in the gastrointestinal tract with the other foods that are consumed at about the same time or may be because the iron is present in a mixture of sources available in the diet. Moreover, human iron bioavailability study is cumbersome, complicated, costly and time consuming to perform (Aragon *et al*., 2012; Ismail, 1999). Despite human trial, animal hemoglobin repletion bioassay is good for efficacy study because their environment condition can be controlled properly and the most common method in use (Weink *et al*., 1999). However, animal bioavailability study have limitations because of different iron requirements, metabolism, digestive capacity, sensitivity to iron absorption inhibitors and promoters as compared to human (Aragon *et al*., 2012). Despite this fact rat hemoglobin repletion efficiency has been used for determining the relative biological values of iron by numerous scholars for the last three decades (Aragon *et al.,* 2012; Habtamu, 2015; Lucia *et al*., 2013; Rohner *et al*., 2007; Swain *et al.,* 2003; Urga *et al*., 1999; Weber *et al*., 2004). Moreover, the study which compared *invirto*, animal and clinical determination of iron bioavailability suggest that rat hemoglobin depletion-repletion model serves as the most reliable predictor of iron bioavailability in human (Forbes *et al.*, 1989).

2.2 Soil

Soil is a natural body composed of solids such as minerals and organic matter, liquid and gases that occurs on the land surface, occupies space and is characterized by one or both of the following horizon, or layers , that are identified from the initial material as a result of additions, losses, transfer and transformation of energy and matter or the ability to support rooted plants in natural environment (Soil Survey Staff, 2010). The mineral matter is the result of weathered rock and consisits of particles of different size, ranging from clay(the smallest) to silt, sand, gravel and stons (White, 2006). Soil water contains dissolved organic and inorganic solutes and called the soil solution (Frank and Tolgyessy, 1993). The soil air consists primarily of nitrogen and oxygen; it usually containing higher concentration of carbon dioxide and traces of other gases that are byproduct of microbial metabolism (White, 2006).

Soil contains some amounts of minerals. Iron in soil is weathered from minerals exists as a divalent cations in solution form and can be available for plants. Acidic soil have sufficient iron in solution to meet plants needs. In some very acidic soils, iron in a lesser extent is found to be toxic because of the high amount in solution but in alkaline soils iron is deficient where oxidized forms of iron exists as insoluble oxides and hydroxides (Forth, 1990).

The high potential cereal crop zone of Ethiopia is covered predominantly by vertisol which accounts 20% of its area, followed by nitisol and cambisol soils (FAO, 1986). In recent study the concentrations of iron available in soil in flat plain areas of Abaya Chamo Lake Basine was found ranged from 6.14 to 40.25ppm (Ayele *et al*., 2014). The available iron in the soil was also negatively correlated with soil pH and clay content and positively correlated with silt and sand content of the soil. Another study also reported that the available iron content of soil sample collected from Northern Tigray was found 42.93ppm (Abayu, 2012).

2.3 Teff (Eragrostisis tef(Zucc) Trotter

Teff which belongs to the family *poaceae* is one of the major and indigenous cereal crop in Ethiopia, where it is believed to have originated. The name ‘teff’ is derived from Amharic word ‘tef’, which means lost which refers to the grain’s small size (Saturni *et al*., 2010; Temesgen, 2013; Vinning and McMahon, 2006). History states that Ethiopia was the only country in the world that uses teff as a cereal crop (Vavilov, 1957). However, now a days there are many countries(USA, Australia, Sweden…) that uses teff for different products like juice, chocolate etc and there is a growing interest in teff utilization due to its nutritional importance as it is a gluten free grain (Boka *et al*., 2013). Teff is self pollinated, warm season grass and its production occurs in a wide range of environments, from drought-stressed to water-logged land. It prefers altitudes between 1700 and 2200m and relatively dry condition 450 to 550 millimeter per year and its preferred temperature is 10 to 27^{O}C with 12 hours of sunlight (Vinning and McMahon, 2006).

Even though there are few different varieties of teff that vary in color from light to dark, there is no significant difference in their calories, protein, carbohydrate, moisture content of their product called injera (EHNRI, 1997). However, due to its darker color, red variety of teff contains higher level of pigmented material such as tannins and polyphenols which in turn contains higher level of iron (Alauntye, 2013; Umeta and Parker, 1996; Vinning and McMahon, 2006).

Enjera is a thin fermented Ethiopian traditional bread made from teff flour, water and starter (ersho), which is a fluid saved from previously fermented dough. Thought there are other grains to make enjera like sorghum, maize, barley, and wheat, teff is the most popular grain for making enjera. The process of enjera making involves a series of steps like grounding teff into the flour, mixing it with water and starter, fermentation, cooking a part of dough and cooling and then mixing with the rest of dough, finally baking to get fermented, sour dough type flat bread (Ashgrie and Abate 2012; Boka *et al*., 2013; Temesgen, 2013).

Since early 1960th different scholars suggests that the higher iron content of teff is due to the soil contamination as a result of uncontrolled traditional threshing (Almgard , 1963; ENS, 1959). Thought these works had not been confidential as some scholars blame that there was no control/reference, they were giving an important insight to the area for many scholars. According to Saturni *et al*, (2010) and Umeta *et al*., (2005) teff is an excellent source of iron for most of the population. However, some authors argue that teff’s highest content of iron is due to soil contamination as a result of threshing the crop on the ground (Abebe *et al*., 2007; Ambaw , 2013; Bokhari *et al*., 2012; Harvey *et al*., 2000). A significant proportion of the iron in the teff diets of Ethiopia is extrinsic to food, usually referred to as contaminant iron and the complete removal of extrinsic iron from teff is not possible (Baye *et al*., 2013; Umeta *et al*., 2005). No matter the iron source whether extrinsic or not, the question is on the impact of this iron on iron status of an individual which is left controversial yet.

A study conducted in Benin published in 2010 assesses the impact of different processing method and contaminant iron boiaccessibility on different maize based meal. Evaluation of iron bioaccessibility was performed by *invitro* enzymatic digestion followed by dialysis. Finally the researchers conclude that iron bioaccessibility in mawè and owo products showed that only a small part of the contaminant iron originating from the mill was available for absorption. However it appears that fermentation can greatly increase the amount of bioaccessible iron. Thus, combined effect of contamination and fermentation resulted in enhanced levels of iron potentially accessible for absorption and further invivo study were suggested by the authors (Greffeuille *et al*., 2010). However, another study which follows the same iron bioaccessibilty test from Burkina Faso published in 2013 disagrees with the previous authors. The researcher's intention was to measure iron contamination levels in millet and sorghum after decortications and milling and to assess the bioaccessibility of contaminant iron. The bioaccessibility of contaminant iron was measured using *invitro* digestion followed by measurement of dialyzable, soluble and insoluble iron. At the end of the research phytate/iron molar ratios were lower in iron contaminated flours, suggesting an improvement in iron bioavailability. However, measurement of bioaccessibility in iron contaminated tô (traditional food) showed that contaminant iron was mainly insoluble and thus not available for absorption and has poor nutritional interest (Icard-Verniere *et al*., 2013).

In vitro digestion/Caco-2 cell model in which ferritin formation was used as an index of iron bioavailability were used in a study conducted in Zanzibar, Tanzania, and Uganda and published in 2013. The researcher aimed at assessing the *invitro* iron bioavailability and the capacity of geophagic earths and clay minerals to inhibit dietary iron absorption using methods that better approximate human physiology. Authors, then, concluded that although geophagic earths have high iron concentrations compared to iron-rich foods, this iron is not bioavailable, and that some geophagic earths inhibit dietary iron absorption (Seim *et al*., 2013). However, some authors, in contrast to the above authors, suggests that the fraction of a iron mineral that is soluble in the gastrointestinal environment and available for absorption, after conducting more sophisticated studies of the iron content of geophagic earth using the *invitro* methods (Hooda *et al*., 2004; Kikouama *et al*., 2009; Smith *et al*., 2000).

Hallberg and Bjrn-Rasmussen (1981) also found that amount of iron absorbed increased from 0.14 to 0.18 mg or about 30% due to the contamination as it is calculated from the iron exchangeability of contaminated food. The results show that contamination iron can be a good dietary source of iron.

Invivo studies which have used human as a study subjects have investigated the effect of geophagy on iron absorption using Turkish, Texan, and South African geophagic earth . Minnich *et al*., (1968) were studied the effect of geophagy on iron absorption on 31 healthy and iron deficient human subjects. Even thought the researchers investigated that Turkish geophagic earth effectively blocks iron absorption, they suggest that the effect of clay and soil on iron absorption may not be the sole factor in time production of anemia. Nutritional and parasitic factors are usually involved as well. Another study from the same country conducted an investigation on 12 patients between 8 and 21 years of age, with iron deficiency anemia and geophagia. Then, decreased iron absorption was detected respectively in patients against the elevated absorption curves in control subjects (Arcasoy *et al*., 1978). However, a study from USA, Texas conducted on 27 healthy subjects and 5 patients deficient in iron concludes that pica does not impair iron absorption (Talkington *et al*., 1970). Although these studies have a number of limitations, including outdated methods, very small sample sizes, unblocked confounding factors, and inadequate statistical analyses, these studies represent the only available *invivo* data (Seim *et al*., 2013; Young, 2010). Their findings suggest that geophagy does not enhance iron status and may even lower it, but the mechanisms and degree to which geophagic substances adsorb or inhibit iron absorption remain unclear and with these circumstances it would be difficult to conclude that geophagy interferes with iron absorption.

Chapter Three

Materials and Methods

3. Materials and Methods

3.1 Study area, sample collection and preparation

Teff sample was collected from a farmers land Debre Birhan wereda, North Shewa zone, Amhara region, which is 130 km far from the capital city Addis Ababa in North East at 9^{O} 42’ and 9^{O} 45’ latitude North and 39^{O} 35’ and 39^{O} 39’ longitude East with the altitude ranging from 2840 to 2943m above the sea level and has an average annual rainfall of 966mm (Molla, 2013). The teff sample were placed in polyethylene bag immediately after harvest to avoid any contact with the soil to prevent soil contamination and transported to Addis Ababa University food science and nutrition laboratory. After arrival the teff sample were threshed, sifted, ground using stainless steel laboratory miller (FW 100, Ohaus, Bejing, China) and sieved using 0.42mm mesh size sieve and finally the flour was packed in air tight dry polyethylene plastic bag until analysis.

Vertisol soil sample was collected from Ginchi Agricultural Research Sub-center and cambisol soil sample was collected from Debre Birhan Mush district. Ginchi Agricultural Research Sub-center is located at 75km from Addis Ababa on the way to Ambo at 9^{O} 02’ North latitude and 30^{O} 12’ East longitude with an altitude of 2200m above sea level and having an average annual rainfall of 1095mm, average relative humidity of 58.2%, average maximum and minimum air temperatures of 24.6^{O}C and 8.4^{O}C respectively (Kebede *et al.,* 2013).The samples were packed in polyethylene bag and transported to Addis Ababa University Food Science and Nutrition Laboratory. After arrival the soil sample were finely crushed using mortar and pestle until the sample passes through 0.42mm mesh size sieve.

3.2 Moisture content analysis

Moisture content of teff sample was analyzed according to AOAC (2000) using official method 925.09. Moisture crucibles were cleaned, dried in an oven at 105^{O}C and then taken out of the oven and left in the desiccator to cool. Weight of crucible were measured and recorded **(W_1)** in a data collection form. Approximately 5gm of samples were measured in each dry crucible (**W_2**) and placed in an oven to dry at 105 ^{O}C for 3 hours. The crucibles were taken out of the oven after 3 hrs and cooled in a desiccators for 30 minutes. The weight of the crucibles and the samples were measured after cooling and then crucibles containing the sample were returned again in to the dry oven to dry and their weight were measured until it became constant. Finally, the last constant measurement (**W_3**) were taken and the moisture content of the samples were determined using the following formula

$$\% \textit{Moisture} = \left(\frac{W2-W3}{W2-W1}\right) X\ 100 \qquad \textbf{Eq. 1}$$

3.3 Proximate composition analysis

3.3.1 Crude protein analysis

Protein content of teff sample were analyzed according to AOAC, (2000) using official method 979.09.

Digestion - Approximately 0.5gm of teff flour were measured in three tecator tubes and placed in the tecator rack (**W**). A blank were used in order to avoid overestimation of the results due to nitrogen from reagents. Six ml of concentrated sulfuric acid were added in to tubes containing the sample using a pipette and then 3.5ml of hydrogen peroxide were added step by step in to each sample tube after careful mixing. The tubes will shake a few times after violent reaction has cease and put back in to the rack. Then 3gm of copper sulfate and potassium sulfate catalytic mixture were added in to the sample tubes and the tubes were let to stand for 15 minutes before digestion. The sample tubes were placed in a digester and let the working temperature reach 370 ^{O}C and the digestion process was continue until clear solution were observed. Finally, the sample tubes were taken out, placed in the rack and allowed to cool in fume hood and 50ml of distilled water were added into sample tubes in order to avoid precipitation of sulfate

$$\textbf{Protein (sample)} \xrightarrow[\textbf{Heat, catalyst}]{\textbf{Sulfuric acid}} \mathbf{(NH_4)_2SO_4}$$

Distillation – After digestion 25ml of 35% sodium hydroxide solution were added in to the digested and diluted solution and 250 ml conical flask containing 25ml of 2% of boric acid, 25 ml of distilled water and an indicator solution under the condenser of the distiller with its tip immersed in to the solution and let distillation process continue until a total volume become between 200 ml and 250 ml. The tips were rinsed with few ml of water before the receiver was remove.

$$\mathbf{(NH_4)_2SO_4 + 2NaOH \longrightarrow 2NH_3 + Na_2SO_4 + 2H_2O}$$

$$\mathbf{NH_3 + H_3BO_3 \longrightarrow NH_4 + H_2BO_3^-} \quad \textbf{Borate ion}$$

Titration - The solution containing an indicator, ammonium ion and borate ion were titrated using 0.1N HCl till the color of the solution changes to reddish and the total volume of the HCl required to reach the end point of the titration were recorded. The volume of the HCl consumed during titration were adjusted by subtracting the average volume of HCl consumed by the blank from HCl consumed by each sample. In addition, the weight of the sample were adjusted by subtracting the moisture content from initial weight of the samples

$$\mathbf{H_2BO_3^- + H^+ \longrightarrow H_3BO_3}$$

- Protein in the sample were calculated:

$$\%\ \mathbf{Nitrogen} = \frac{\mathbf{V\ x\ N\ x\ 14\ x\ 100}}{\mathbf{1000\ x\ Wo}} \qquad \mathbf{Eq.\ 2}$$

$$\mathbf{\%\ Protein = 6.25\ x\ \%\ Nitrogen} \qquad \mathbf{Eq.\ 3}$$

Where; V- Volume of HCl consumed to the end point of titration,

N- The normality of the HCl used,

Wo- Sample weight on dry matter basis,

14- The molecular weight of the atomic nitrogen

3.3.2 Crude fat analysis

Fat content of teff sample were analyzed according to AOAC, (2000) using official method 4.5.01. Extraction cylinders were cleaned and dried in oven at a 105 ^{O}C for 1 hr and then cooled in a desiccators. The weight of the cylinders were measured and recorded ($\mathbf{W_1}$). The bottom of the extraction thimbles were covered with a layer of fat free cotton and approximately 2gm of teff flour were measured in thimbles and covered with cotton layer (**W**). The thimbles were put in the extraction chamber. Extraction cylinders were taken out of the desiccator and put on the bracket and 50ml of ether were added into the extraction cylinders and moved into the heating plank. The extraction was run for about 4 hours and then the extraction cylinders were disconnected and put in a drying oven at 70 °c for about 30 min. The cylinders were taken out of the oven and cooled in a desiccator for 30 minutes and the weight of cylinders were measured immediately after they taken out of desiccators **(W2)** and the fat content of the samples were determined using the following formula

$$\%\boldsymbol{Fat} = \frac{\mathbf{W2-W1}}{\mathbf{W}}\,\mathbf{X\ 100} \qquad \textbf{Eq. 4}$$

Where, W1 = weight of the extraction cylinder,

W2 = weight of the extraction cylinder plus dried crude fat and

W= weight of sample.

3.3.3 Crude fiber analysis

Fiber content of teff sample were analyzed according to AOAC, (2000) using official method 962.09. Two crucible were cleaned and dried with 1gm celite in an oven at 105 °c for 1hr and about 1gm of teff flour samples were weighted in to pre-dried crucible **(W1).** The crucibles were inserted to holder then to the digester and 1.25% H_2SO_4 solution were added in to each column by pressing the button for R1. The time was set for 37 minutes and the temperature weretween 6 and 8. The acid were drained by using a vacuum pump after 37 minutes and the samples were cooled for 5 minutes, washed with distilled water and 1.25% NaOH solution were added into each column by pressing the button R2. The time and temperature was as previously settled, the base were drained by using a vacuum pump after the set time. Crucibles containing residue were dried at 130 ^{O}C for 2 hrs after washed again and cooled in a desiccator and weighted (**W2**). The residues were ashed in a muffle furnace at 550 ^{O}C for 3 hours and let to cool down to below 250 ^{O}C before removing from the furnace. The crucibles were cooled in a desiccator to room temperature and their weight were measured using analytical balance (**W3).** Finally, the ash content in the samples were calculated using the following formula

$$\%\,\boldsymbol{Crude\ fiber} = \frac{\mathbf{W2-W3}}{\mathbf{W1}}\ \mathbf{x\ 100} \qquad \mathbf{Eq.5}$$

Where:

W1= sample weight,

W2= crucible weight after drying,

W3 = crucible weight after ashing.

3.3.4 Crude ash analysis

Ash content of teff sample were analyzed according to AOAC, (2000) using official method 923.03. Four crucibles were cleaned, dried in muffle furnace at 550 ^{O}C for 30 minutes and their weight were measured and recorded (**M_1**) after cooled in a desiccator for 30 min. Approximately 2.5 gm of teff flour sample were measured in each crucible (**M_2**) and charred on a hot plate under a fume hood until the smoke ceased down. Then the samples were ashed in muffle furnace at 550 ^{o}c for five hours and crucibles were cooled in a desiccators. Finally, their weight were recorded (**M_3**) and the ash content were determined by using the following formula

$$\% \boldsymbol{Ash} = \frac{\mathbf{M3-M1}}{\mathbf{M2-M1}} \mathbf{X\ 100} \qquad \textbf{Eq.6}$$

3.4 Iron content analysis

Iron content was determined by atomic absorption spectrophotometer (AAS) using AOAC, (2000) official method 985.35. The ash which was obtained from dry ashing of teff flour was completely wetted with 5ml of 6N , HCl and dried on low temperature hot plate. About 7ml of 3N HCl were added to the dried ash and heated of the hot plate until the solution were boiled. The ash solution were cooled to room temperature in a hood and filtered in to a 50ml graduated flask using 125mm filter paper. Then, about 5ml of 3N HCl were added to each crucibles dishes, heated until the solution boiled, cooled and filtered in to the flask. The crucible dishes were washed three times with deionized water and the washing were filtered in to a flask. Then, the solution were cooled and diluted to 50ml volumetric flask and were prepared for flame atomic absorption spectrometry (FAAS) reading.

A standard solution were prepared as follows: Four series of working standard metal solutions (0.5, 1, 3, 4) were prepared by appropriate dilution of metal stock solution (nitrate of the metal) with deionized water containing 2.4ml 3N HCl in 10 ml volumetric flask. Then, calibration curve were constructed after the standard solution absorbance analyzed by FAAS.

A sample iron concentration were analyzed from the calibration curve after the absorbance of sample solution were analyzed using FAAS. Sample blank solution absorbance was also run with the sample solution.

$$\boldsymbol{Metal\ content}\ \left(\frac{\boldsymbol{mg}}{\boldsymbol{100g}}\right) = \frac{[(\mathbf{a} - \mathbf{b}) * \mathbf{V}]}{\mathbf{10} * \mathbf{W}} \qquad \mathbf{Eq.\,7}$$

Where:

W = Weight (g) of sample,

V = 50ml= Volume of extract,

a = Concentration ($\mu g/ml$) of sample solution,

b = Concentration ($\mu g/ml$) of blank solution

3.5 Rat hemoglobin repletion method

Rat hemoglobin repletion assay AOAC, (2006) official method 973.31 were used to analyze the relative bioavailability of iron by following two phase known as depletion and repletion phases.

3.5.1 Study animals collection and screening

Forty one male Wistar rats (*Rattus novergicus*, albino variety, rodent class) of 21 to 27 days old with body weight ranging from 27 to 35 gram were obtained from biology department, Addis Ababa University and Ethiopian public health institute Addis Ababa/Ethiopia and acclimatized under laboratory conditions for a week by keeping them on standard rat ration and deionized water *ad libitum*. Animals were housed in an individual cage at light and temperature controlled room for 12 hours light and 12 hours darkness and 24±3^{O}c after undertaking a test for C-reactive protein for exclusion of infected animals from the experiment (Weber *et al.*, 2010).

3.5.2 Study animals handling and ethical consideration

Animals were handled as per the National Research Council of National Academies Guide for the care and use of laboratory animals (2011). During the whole experimental periods, all rats were received *ad libitum* deionized water, standard ration and experimental diets. All rats were handled in a way that minimizes stress during weight measurement and blood sample collection. Blood sample were collected using tail vein. For CRP test non-heparinized needle were used instead of centrifuging the blood sample. Tail vein blood sample collection total avoids the stress resulted from tail incision and convenient for frequent blood sample collection from rats. At the end of the experimental period, all rats were sacrificed under light diethyl ether anesthesia. The experiment was carried out after the approval/ethical clearance from the Ethical Clearance Review Board of the College of natural science s of Addis Ababa University on its meeting on December 31/2015 (Reference No: CNSDO/178/08/2016).

3.5.3 Rat hemoglobin depletion- repletion method

Rat hemoglobin repletion assay according to AOAC, (1997) using official method 973.31 was used to determine the relative bioavailability of iron of the experimental diets. Rats were acclimatized under laboratory condition for six days in order to get them acclimatized with the new environment of individual cage and new experimental diet (Miyada *et al.*, 2011). The experiment has two phases: depletion phase in which rats were fed iron deficient AIN-93G rodent diet for approximately one month followed by repletion phase in which rats were grouped in to four groups and fed the experimental and control diets for fourteen days.

3.5.3.1 Experimental diets preparation and composition

The experimental diets were prepared based on nutritional requirements of laboratory rats in accordance with Reeves *et al*., (1993). Table 3.1 summarizes the nutritional composition of the experimental diets. The vitamin and mineral content of all treatment diets were adjusted equal by the addition of vitamin and mineral mix, since the vitamin A, B2, B12, folate known to affect the hemoglobin level (Brabin *et al.*, 2001) and vitamin C which is known for iron absorption enhancer and some mineral elements can compete with the iron absorption like Zinc and Calcium (Abbaspour *et al*., 2014; Zimmermann and Hurrell, 2007).

The total energy of the experimental and control diets were adjusted by the addition of approximately 9mg soya bean oil to each experimental diet to make the diet's energy supply isocratic in accordance with Webere *et al.,* (2010).

Table 3.1 Nutritional composition of experimental diets (g/kg of diet)

Components	Depletion phase AIN 93G	Repletion phase			
		TV+AIN93G	TC+AIN93G	T+AIN93G	$FeSo_4$+AIN93G
Total iron (mg/kg)	-	35(mg/kg)	35(mg/kg)	35(mg/kg)	35(mg/kg)
Total fat	70	56*	56*	56*	70
Total protein	179	143	143	143	179
Total Carbohydrate	591	641	641	641	591
Dietary fiber	48	46	46	46	48
Fe-free mineral mix	35	35	35	35	35
Vitamin mix	10	10	10	10	10
Total energy (Kcal/kg)	3800	3784	3784	3784	3800

* Addition of soybean oil. AIN 93G: American Institute of Nutrition formula for growing rats. TV: teff mixed with vertisol soil. TC: teff mixed with cambisol soil. Kcal: kilocalories

The teff flour was then divided in to three groups for pellet formation. The first group flour was mixed with 0.78g of vertisol soil and the second group flour was mixed with 0.55g of cambisol soil to come up with 35mg of extrinsic iron per kg of diet. Then after pellets were formed through agglomerating the flour by adding 250 to 300 ml of deionized water for 1kg of flour, and made in to paste with the right consistent manually in stainless steel bowl. Then after the formed clumps forced to pass through a glass tube with 5cm radius to give the pellets a uniform cylindrical shape and the extruded strip were then cut in to pieces having 5cm length using stainless steel knife. Finally, the strip was placed in an oven at 45 ^{O}C for 24hrs. After 24 hrs drying the pellets were allowed to cool and refrigerated until consumption after placed in zip locked polyethylene plastic bag (Green and Turner, 1974).

3.5.3.2 Depletion phase (induction of iron deficiency-anemia)

The purpose of the depletion phase was to induce iron deficiency-anemia to the experimental animals. Accordingly all experimental animals were fed iron deficient diet, AIN 93G, (Dytes Inc, USA) and deionized water *ad libitum* from three to four weeks, until they become anemic with their hemoglobin level less than 6 g/dL which is recommended for depletion-repletion assey implementation (Forbs *et al.*, 1989; Weber *et al.*, 2010). Every animal's daily dietary intake and their weight per three days were measured and recorded. Finally, at the end of this phase experimental animal's blood sample were collected in a duplicate using tail vein with a great care to avoid hemodilution.

For blood sample collection using tail vein, rat was placed in restrainer and needle was injected in most visible tail vein and then removed, blood was dripped on glass slide and immediately taken up by microcuvate of HemCue (Hb 301, Lot No:1408389, Angelholm, Sweeden). Then hemoglobin measurement were recorded in a duplicate after placing the wiped microcuvate containing blood sample in HemoCue machine (Sari *et al.*, 2001). If there were a wide variation between two readings of duplicate blood sample, the third measurement were carried out to avoid extreme values arising from operational errors and hemodilution with extracellular fluids (Rohner *et al.*, 2007).

Rats having the hemoglobin level less than 6g/dL were considered as depleted and kept ready for the repletion phase of the experiment.

3.5.3.3 Repletion phase

Depleted experimental rats having the hemoglobin level less than 6 g/df were randomly assigned to four groups each with eight rats. The hemoglobin level among four groups were kept similar ($P<0.05$) Group one rats were fed pellet made from teff sample contaminated with Vertisol soil variety mixed with AIN-93G iron free rat diet and deionized water *ad libitum*. Group two rats were fed with pellet made from teff sample contaminated with Cambisol soil variety mixed with AIN-93G iron free rat diet and deionized water *ad libitum* . Group three rats were fed with pellet made from teff sample mixed with AIN-93G iron free rat diet and deionized water *ad libitum*. Group four rats were fed pellet made from ferrous sulfate mixed with AIN-93G iron free rat diet and deionized water *ad libitum* and used as a control. All treatment groups were provided with the experimental diets and deionized water *ad libitum* for 14 days, since dietary restriction in rat were shown to be associated with decreased in total leukocyte, segmented neutrophil, lymphocyte, platelet count and serum biochemistry including decrease in total protein and albumin (Hubert *et al*., 2000).

Each experimental rats daily consumption which was calculated by difference and their body weight gain per every three days were measured and recorded. Finally, at the end of this phase each experimental rat's blood sample were collected in a duplicate using tail vein with a great care to avoid hemodilution and hemoglobin level were analyzed using HemoCue machine as it was described in section 4.5.3.2 .

3.5.3.4 Measurements of iron bioavailability from rat hemoglobin repletion assay

The estimation of iron bioavailability and utilization were carried out by calculating the percentage of iron bioavailability as hemoglobin regeneration efficiency. Hemoglobin regeneration efficiency is a measure of dietary iron which is incorporated in to hemoglobin (Onabanjo *et al.*, 2008). The calculation of hemoglobin iron (Hb-Fe in mg) was based on the assumption that 6.7% of rat's body weight is composed of blood and hemoglobin contains 3.35mg of iron per a gram of blood (Lucia *et al.,* 2013; Miyada *et al.,* 2011). The relative biological values was determined by dividing the individual hemoglobin regeneration efficiency values of test iron sources by the mean hemoglobin regeneration efficiency of ferrous sulphate control group (Urga *et al.,* 1998).

V. Iron consumption

The calculation of iron consumption was carried out by considering the total amount of the diet consumed and the iron content of the specific diet, which was calculated for each experimental rats according to the following formula (Lobo *et al.,* 2011):

$$\text{Fe consumption} = \frac{[\text{Repletion phase total diet consumed(g)} * \text{Iron content in the diet(mg/kg)}]}{1000} \quad \textbf{Eq.8}$$

VI. Hemoglobin iron pool

The hemoglobin iron pool (Hb-Fe pool in mg) was calculated using the result obtained from iron consumption (Equation 8), rat body weight and initial and final hemoglobin concentrations of each rat during repletion phase of the experiment. The hemoglobin iron pool was calculated using the following formula (Lobo *et al.,* 2011):

$$\text{Hb-Fe pool initial} = \frac{[\text{Initial weight(g)} * \text{Initial hemoglobin(g/dL)} * 6.7 * 0.335]}{1000} \qquad \textbf{Eq. 9}$$

$$\text{Hb-Fe pool final} = \frac{[\text{Final weight(g)} * \text{Final hemoglobin(g/dL)} * 6.7 * 0.335]}{1000} \qquad \textbf{Eq. 10}$$

VII. Hemoglobin regeneration efficiency

The hemoglobin regeneration efficiency (HRE) was calculated using the result obtained from initial and final hemoglobin iron pool (Equation 9 and 10). The hemoglobin regeneration efficiency was calculated using the following formula (Lobo *et al.,* 2011):

$$\text{HER\%} = \frac{[(\text{Hb-Fe pool final} - \text{Hb-Fe pool initial}) * 100]}{\text{Fe intake (mg)}} \qquad \textbf{Eq.11}$$

VIII. Relative biological value

The relative biological value (RBV) was determined using the mean ratio of the response from test experiment to the standard ferrous sulfate response, since this study used a single iron concentration among the treatment groups to measure response. The relative biological value was calculated using the following formula:

$$\text{RBV} = \frac{[\text{HER\% of test group}] \quad * 100}{\text{HER\% of ferrous sulfate group}} \qquad \textbf{Eq. 12}$$

3.5.4 Statistical analysis

Data were analyzed using the Software Statistical Package for Social sciences (SPSS) version 20. Descriptive statistics were used and the results were presented as mean and standard deviation. The three treatment groups teff mixed with cambisol and vertisol and teff and one control group were compared for hemoglobin levels, weight, iron consumption, hemoglobin regeneration efficiency using one way analysis of variance (ANOVA) and the statistical differences among means were tested by Turky's *post-hoc* test at 5% significant level ($p<0.05$).

Chapter Four

Results & Discussions

4. Results and discussions

The teff sample was milled and the flour was analyzed for moisture content, proximate composition and total iron content. The *invivo* bioavailability of the teff, teff and vertisol soil mix, and teff and cambisol soil mix were analyzed using hemoglobin repletion efficiency of anemic rats and compared them against the standard ferrous sulfate hemoglobin repletion efficiency of anemic rats of control group.

4.1 Proximate composition and total iron content of laboratory threshed teff flour

The result of proximate composition and total iron content of laboratory threshed teff flour were presented in Table 4.1. The current results indicate higher dry matter, protein, ash, fiber and carbohydrate contents than reported by Habtamu (2015) on laboratory threshed red teff flour. This could be due to the variety difference between our samples (red and white teff) though the data from Ethiopian food composition table shows no significant difference in proximate composition between teff varieties (ENHRI, 1997).

The current finding of ash and carbohydrate content is in line with Ethiopian food composition table. However, the protein, fat and fiber contents are a bit higher than that of white teff flour in Ethiopian food composition table (ENHRI, 1997). The dry matter, protein and ash content of current result agree with the finding of Meseret (2015) on non-contaminated white teff flour.

The total iron content of laboratory threshed teff flour in the current study was four times lower than that of the report from Ethiopian food composition table (ENHRI, 1997) and twenty five times lower than the result reported by Abebe *et al.*, (2007). This difference could be attributed to the contamination of teff with soil during harvest since both the Ethiopian Nutrition Survey and Abebe *et al.*, (2007) analyzed teff seeds collected from ordinary market places; Hence, there is a good possibility that their seeds were contaminated with soil. The traditional teff trashing process in Ethiopia using cattle hooves contaminates teff with soil iron in high range (Baye *et al.*,2013). However, the current result of total iron content was in line with the result of cleaned white teff iron content (5.9 mg/100g) reported by Almgard (1963), laboratory threshed red teff flour (6.48g/100g) and white teff flour (6.52g/100g) reported by Meseret (2015) and laboratory threshed red teff flour (6.65g/100g) reported by Habtamu (2015).

The difference in iron content between laboratory threshed/washed teff flour and field threshed teff flour is the result of soil contamination. This soil is usually surface soil with exchangeable cations and potentially bioavailable (Atengo, 2005).

Table 4.1 Proximate composition and total iron content of laboratory threshed teff flour on dry basis

Parameters analyzed (/100g)	**Teff flour**
Dry matter (g)	89.02
Crude Protein (g)	13.2
Crude fat (g)	3.53
Crude fiber (g)	5.01
Crude ash (g)	2.26
Total carbohydrate (g) by difference	76.01
Iron (mg)	6

4.2 Physicochemical property and iron content of vertisol and cambisol soil

The physicochemical property and iron content of vertisol and cambisol soil were presented in Table 4.2. There was a statistical significant difference in percent of sand, clay, silt and organic carbon(OC), cation exchange capacity(CEC) between the two soil varieties. However, there was no statistical significant difference in pH between the two soil types and this agrees with the previous study result reported by Yerima *et al.*,(2013). The distribution (%) of sand, clay, silt of vertisol in the current study agrees with the report from Kebede and Bekele, (2008). The cation exchange capacity of vertisol was significantly higher than cambisol, but both are categorized as a soil having high cation exchange capacity (Hazelton and Murphy, 2007). The highest cation exchange capacity of vertisol was also reported by previous study ranging from 20 to 45 meq/100g (Kebede and Yomoah, 2009). There was a statistical significant difference in the total iron content of vertisol and cambisol soil types.

Table 4.2 Physicochemical property and iron content of vertisol and cambisol soil (Meseret, 2015)

Soil type	**Vertisol**	**Cambisol**
Sand (%)	13^{a}	19^{b}
Clay (%)	69^{a}	43^{b}
Silt (%)	18^{a}	38^{b}
Dry matter (g/100g)	89.33 ± 0.21^{a}	93.46 ± 0.15^{b}
pH(H_2O)	6.17 ± 0.06^{a}	6.5 ± 0.26^{a}
OC(%)	1.2 ± 0.04^{a}	1.32 ± 0.02^{b}
CEC(meq/100g)	55.41 ± 0.11^{a}	34.65 ± 0.15^{b}
Total iron content(mg/100g of DM)	4483.4 ± 52.93^{a}	6390.49 ± 47.84^{b}

Values are mean ± standard deviation and different superscript within a row represents statistically significant difference ($P<0.05$)

4.3 Baseline characteristics of experimental rats

The baseline characteristics of experimental rats before the realization of the experiment/repletion phase were given in Table 4.3. After the end of the depletion phase the rats with hemoglobin level less than 6 g/dL were randomly assigned to three experimental (n=8 each) and one control group (n=8) for the repletion phase. There was no statistical significant difference ($P<0.05$) between groups in the measurements of initial age, body weight, and hemoglobin among experimental and control groups. Therefore, it is possible to conclude that the randomization process among groups was efficient enough. Rat hemoglobin measurement was adjusted by deducting 1.1 g/dL from each rat measurement for Addis Ababa altitude (2,300 m above sea level) (WHO, 2011).

Table 4.3 Baseline rats profile at the end of depletion phase

Indices	**Groups**			
	Teff + Vertisol	**Teff + Cambisol**	**Teff**	**Ferrous sulfate**
Initial Hemoglobin(g/dL)	4.563 ± 0.794^{a}	4.85 ± 0.823^{a}	4.863 ± 0.776^{a}	4.813 ± 0.554^{a}
Age in days	48.5 ±3.024^{a}	49.1 ±3.5229^{a}	50.1 ±1.458^{a}	48.9 ±2.3566^{a}
Initial weight	84.7 ±3.04^{a}	81.7 ±3.42^{a}	86.1 ±4.35^{a}	83.8 ±3.17^{a}
CRP	Negative	Negative	Negative	Negative

Values are mean ± standard deviation and different superscript within a row represents statistically significant difference ($P<0.05$)

4.4 Food and iron intake, body weight gain and feed efficiency ratio during repletion phase

The total food and iron intake, body weight gain and feed efficiency ratio of rats during repletion phase were given in Table 4.4. There were no statistical significant deference in total food intake, total iron intake, body weight gain and feed efficiency ratio between and among treatment and control groups. These possibly could be due to the similarity in initial age, body weight, CRP status and hemoglobin measurement between groups, since total food intake strongly correlated to the rat's age, weight and health status (infection occurrence) (Kim and Atallah, 1993; Shiga *et al.*,1987; Weber *et al.,* 2010). Hence, weight gain and iron intake are directly related to the total food intake. It could also be due to the isocaloric formulation of the experimental rat diet.

Table 4.4 Food and iron intake, body weight gain and feed efficiency ratio during repletion phase

Indices	**Groups**			
	Teff + Vertisol	**Teff + Cambisol**	**Teff**	**Ferrous sulfate**
Total food intake (g)	148.7±8.237 ^{a}	152.9±10.707 ^{a}	153.3±6.808 ^{a}	152.2±8.9011 ^{a}
Iron intake (mg)	5.209±0.291 ^{a}	5.352±0.375 ^{a}	5.366±0.238 ^{a}	5.327±0.312 ^{a}
Weight gain (g)	31.69±2.67 ^{a}	33.38±2.039 ^{a}	32.53±1.538 ^{a}	31.7±1.835 ^{a}
Feed efficiency ratio	0.213±0.012 ^{a}	0.212±0.012 ^{a}	0.21±0.009 ^{a}	0.209±0.019 ^{a}

Values are mean ± standard deviation and different superscript accros a row represents statistically significant difference (P<0.05)

The total food intake, total iron intake and body weight gain of rats in the present study is lower than similar experiment conducted on rats as reported by Habtamu (2015). These could be due to the difference in the experimental rats initial age in days which ranged from 51±3.2 to 52.5±3.96, weight in gram ranges from 146.5 ± 14.2 to 160.25± 25.39 and hemoglobin measurement in g/dL ranged from 5.6±0.21 to 5.73± 0.17 since the older rats consume more than younger and higher hemoglobin associated with an improved appetite (Kim and Atallah, 1993; Khan *et al.,* 2014). The rats feed efficiency ratio of current experiment revealed similarity with other similar researches that are reported by Habtamu (2015) and Urga *et al*., (1998) on teff.

4.5 Hemoglobin regeneration efficiency and relative biological value

The final hemoglobin, hemoglobin gain and hemoglobin regeneration efficiency ratio of the rats fed with teff, teff mixed with cambisol, vertisol, and AIN-93G mixed with ferrous sulfate are summarized under Table 4.5. There was no statistically significant difference in final hemoglobin measurement and final hemoglobin-iron among treatment groups ($P<0.05$). However, there was a significant difference in final hemoglobin measurement and final hemoglobin iron between teff and ferrous sulfate group. There was also no statistical significant difference in hemoglobin gain among treatment groups ($P<0.05$); however, there was a significant difference in hemoglobin gain between teff and ferrous sulfate group. There was no statistical significant difference in an initial hemoglobin measurement and initial hemoglobin iron between and among treatment and control groups ($P<0.05$). With regard to hemoglobin regeneration efficiency there was no statistical significant difference among treatment groups ($P<0.05$); however, there was a significant difference in hemoglobin regeneration efficiency between the teff and the ferrous sulfate group. The hemoglobin regeneration efficiency of ferrous sulfate group was about 12% higher than the teff group.

The final hemoglobin concentration of laboratory threshed teff flour in the current study was similar to the result from teff injera (10.45 $\pm$ 0.63) reported by Urga *et al.*, (1998) and result from laboratory threshed red teff flour (10.16 $\pm$ 0.8) reported by Habtamu (2015). The final hemoglobin concentration of ferrous sulfate in the current study was similar to the result reported by Habtamu (2015) which was 13.4 ± 1.28 and by Dostal *et al.*, (2012) which was 14 ± 0.21. The 58% of the iron in the control group diet which was composed of ferrous sulfate was converted to hemoglobin which shows higher conversion rate than the previous results 43 ± 9.46 reported by Weber *et al.*, (2010) and 44.96 ± 5.01 reported by Habtamu (2015).

Table 4.5 The final hemoglobin, hemoglobin gain and hemoglobin regeneration efficiency of rats

Indices	**Groups**			
	Teff + Vertisol	**Teff + Cambisol**	**Teff**	**Ferrous sulfate**
Final Hb (g/dL)	13.65 ± 1.135^{ab}	14.03 ± 1.505^{ab}	12.8 ± 1.154^{a}	15.51 ± 1.701^{b}
Hb gain (g/dL)	8.775 ± 0.87^{a}	9.175 ± 1.868^{ab}	7.938 ± 0.798^{a}	10.7 ± 1.594^{b}
Initial Hb iron (mg)	19.01 ± 0.681^{a}	18.34 ± 0.768^{a}	19.77 ± 0.975^{a}	18.82 ± 0.712^{a}
Final Hb iron (mg)	26.12 ± 1.091^{ab}	25.83 ± 1.07^{ab}	27.07 ± 0.76^{a}	25.93 ± 0.859^{b}
Hb regeneration efficiency (HRE)	50.66 ± 4.602^{ab}	51.06 ± 8.297^{ab}	46.69 ± 4.263^{a}	58.75 ± 9.134^{b}

Values are mean ± standard deviation and different superscript across a row represents statistically significant difference ($P<0.05$)

The relatve biological value (RBV) for the three treatment groups of teff, teff mixed with cambisol and vertisol were ilustrated in Figure 4.1. The RBV of teff flour, teff flour mixed with vertisol and cambisol were 79.47%, 86.91% and 86.24% respectively($P<0.05$). The RBV of this study for all treatment groups were lower than the result reported by Urga et al., (1998) for fermented teff injera. This might be due to two possible reasons: the first reason is that the combined effect of contamination and processing specialy fermentation since such combined effects were already reported to increase iron bioavailability (Greffeuille *et al.*, 2010); and the second reason is that there could be extra additional contaminant iron from the processing equipment (Adish *et al.*, 1999). However, the RBV of teff flour mixed with vertisol and cambisol were in line with the result of field threshed red teff flour (88.4 %) reported by Habtamu (2015) which suggest significant portion of contamiant iron was bioavailable.

There was no statstical significant difference in the total intrisic iron intake of rats among treatment groups ($P<0.05$). However, teff mixed with vertisol and cambisol groups were given extra 35mg/kg of diet extrinsic iron. Thus, this additional extrinsic iron from mixed soil supply 6.77% and 7.44% enhancements of RBV and 3.97% and 4.31% enhancements of HRE for vertisol and cambisol groups, respectively.

The present studies confirm that some portion of extrinsic iron originating from contaminant cambisol and vertisol is bioavailable as suggested by the few previous *in vitro* studies. Greffeuille *et al*., (2010) in their *in vitro* iron bioaccessibilty test reported that a small portion (4%) of the contaminant iron was available for absorption in mawè and owo products; Abrahams *et al.*, (2006) suggested that geophagical soils consumed by ethnic Bengali communities were found to be a significant source of bioaccessible iron. Kikouama *et al*., (2009) and Smith *et al*., (2000) also suggested that the fraction of iron that is soluble in the gastrointestinal environment and available for absorption, after conducting *in vitro* studies on geophagic earth. Another study on the fractionation of iron of laboratory and field threshed teff suggested that the exchangeable fraction and acid soluble fraction were significantly higher in the field threshed teff flour than the laboratory threshed teff flour, thus, indicating that contaminant iron could be bioavailabile (Ambaw, 2013).

Incontrast, Icard-Verniere *et al*., (2013) conducted an *in vitro* study on the bioaccessibility of contaminant iron in iron contaminated tô (traditional food) showed that contaminant iron was mainly insoluble and thus not available for absorption and has poor nutritional interest . Similarliy, the i*n vitro* digestion/Caco-2 cell model in which ferritin formation was used as an index of iron bioavailability was suggested that although geophagic earths have high iron concentrations compared to iron-rich foods, this iron was not bioavailable, and that some geophagic earths inhibit dietary iron absorption (Seim *et al*., 2013).

Hooda *et al.,* (2002) also reported that despite being rich in minerals, geophagic soils can potentially reduce the absorption of micronutrients such as iron and zinc, which may exacerbate their deficiency in geophagic individuals regardless of their dietary iron and zinc intake. However, the mechanisms and degree to which geophagic substances adsorb or inhibit iron absorption remain unclear. Thus, with these circumstances it would be difficult to conclude that geophagy interferes with iron absorption (Demeke, 2016) and also the studies did not consider the interaction effect since the study was conducted on isolated effect of soil, since soil after ingestion may bound be to mucosal layer in the intestine for longer period (Gonzalez *et al.,* 2004). Another methodological limitation of those studies were that the model assumes ferritin formation proportional to iron uptake and does not include hepcidin controlled transport, uptake was only considered for cell but not for blood (Beiseigel *et al.,* 2007).

Duizer *et al.,* (1997) also indicate that Caco-2 cells are colon cells – transport rates of hydrophilic compounds paracellular lower, less leaky, less discrimination on the basis of molecular size of compounds transported parallellary compared to duodenum cells.

Therefore, the model can be an excellent predictor of direction of response but not the exact magnitude of response in human.

A previous *in vitro* study caried out on the bioaccessibility of iron originated from different soil types suggested that from 0.1 to 2.9% of the total iron was potentially bioavailable (Smith *et al.*, 2000). More recent *in vitro* study conducted on different soil type to determine iron bioaccessibility using iron fractionation suggested that only cambisol and vertisol soil types had a relatively small portion (0.001% and 0.004% of the total iron) of potentially bioaccessible iron. Contamination of vertisol to white teff flour enhances the exchangeable fraction from10.6% to 11.7% and contamination of cambisol to white teff flour enhances the exchangeable fraction from10.6% to 12.6% (Meseret, 2015). Even though vertisol had the highest potential of bioavailable iron in the previous *invitro* study, the current finding reveals that there was no statstically significant difference in the relative biological values of the two soil varieties.

Every results from *in vitro* studies on contaminant iron must always be confirmed by *in vivo* studies since it depends on multiple effects that could not be entirely addressed under any of the *in vitro* iron bioavailability/bioaccessibility tests (Demeke, 2016). Minnich *et al*., (1968) have studied the effect of geophagy on iron absorption on human subjects and concluded that geophagic earth effectively blocks iron absorption. They also suggest that the effect of clay and soil on iron absorption may not be the sole factor in the development of anemia (nutritional and parasitic factors are usually involved as well). Another study on geophagia of human subjects shows decreased iron absorption from intrinsic sources than control subjects (Arcasoy *et al*., 1978). However, an *in vivo* study in the human trial in USA, concluded that pica does not impair iron absorption

(Talkington *et al*., 1970). In contrast to Hooda's claim that extrinsic iron negatively affects the absorption of intrinsic iron, we have seen no such negative effect in this study. Indeed soil contaminated treatments had more bioavailable iron. Although these *invivo* studies have a number of limitations, including outdated methods, very small sample sizes, unblocked confounding factors, and inadequate statistical analyses, they represent the only available *in vivo* data (Seim *et al*., 2013; Young, 2010).

It is vital to consider multiple parameters in the *in vivo* iron bioavailability test before drawing any conclusion. Thus, it was suggested that particle size of soil and their element have a considerable impact on the bioavailability of elements (Abrahams *et al*., 2006). Arredond *et al.*,(2006) found an increment of relative iron bioavailability from 31.1% to 68.2% in fortified bread by reducing the particle size of hydrogen-reduced iron from 45 to 8 micro meters. Therefore, it is imperative to further investigate the relationship between soil particle size and iron

The current finding agrees with more recent *in vivo* study conducted on the rat hemoglobin regeneration efficiency of anemic rat fed with laboratory and field threshed red teff flour. An increment of 9.33% HRE and 20.76% RBV was observed in field threshed teff flour with 29mg of extrinsic iron (Habtamu, 2015). However, the result was slightly higher than the current findings which were 6-8% RBV and 4-4.5% HRE enhancements in teff contaminated with vertisol and cambisol. These might be due to multiple reasons: the differences in the total food intake, total iron intake and body weight gain of rats between studies may have resulted in the difference in HRE and RBV, The more the iron intake the more the HRE and RBV. A difference in soil types may lead to different results since in previous study the soil variety was not specified.

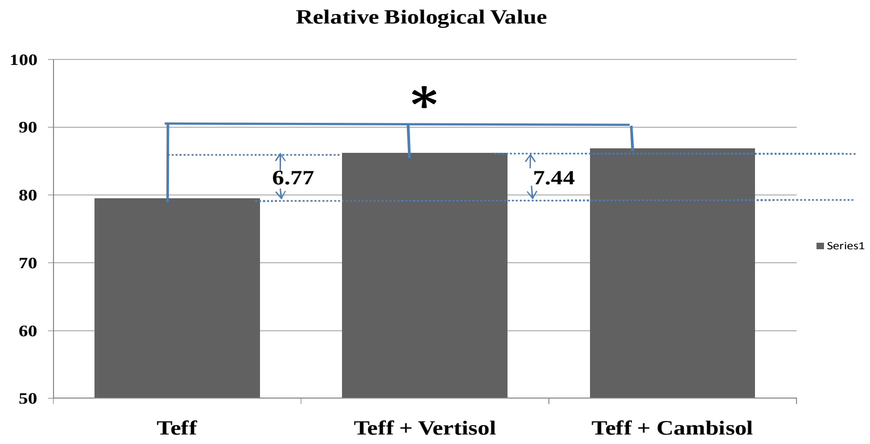

Figure 4.1. The relative biological value of treatment groups.

* Contribution of contaminant iron.

4.6 Bioavailability of teff’s intrinsic iron

Since the Ethiopian government is making an effort to control micronutrient deficiency (hidden hunger), iron fortification of staple food flours was considered as a strategy to control iron deficiency. Iron fortification is considered as a cost effective to combat iron deficiency anemia worldwide (Allen *et al*., 2006; Hurrell, 2002).

The RBV of laboratory threshed teff flour of the current study was compared to the previous results obtained from different iron sources that were fortified in Table 4.6. The current result revealed that laboratory threshed teff flour containing 35mg/kg iron level shows that higher RBV than the previous result obtained from 12mg/kg of Electrolytic Iron Powder (EPI) fortification and 20mg/kg of iron phosphate fortification.

Table 4.6 Comparison of relative biological value of current study with other studies

Iron source	**Iron content**	**RBV**	**References**	**Statistical methods used**
Electrolytic Iron Powder (EPI)	12(mg/kg)	64.5	Swain *et al*.,(2013)	Slope ratio
Electrolytic Iron Powder (EPI)	12(mg/kg)	66	Forbes *et al*., (1989)	Slope ratio
Electrolytic Iron Powder (EPI)	20(mg/kg)	78	Forbes *et al*., (1989)	Mean ratio
NaFeEDTA	20(mg/kg)	86	Lysionek *et al*., (2001)	Mean ratio
$FePO_4$	20(mg/kg)	61	Forbes *et al*., (1989)	Slope ratio
Teff flour (lab. threshed)	35(mg/kg)	67.6	Habtamu, (2015)	Mean ratio
Teff flour (field threshed)	35(mg/kg)*	88.4	Habtamu, (2015)	Mean ratio
Teff flour (lab. threshed)	35(mg/kg)	79.5	Present result	Mean ratio
Teff flour + vertisol soil	35(mg/kg)#	86.2	Present result	Mean ratio
Teff flour + cambisol soil	35(mg/kg)#	86.9	Present result	Mean ratio

*Contains extra 29mg/kg of extrinsic iron originated from unspecified soil type contamination and # contains extra 35mg/kg of extrinsic iron originated from vertisol and cambisol soil contamination

However, similarities in RBV had been shown between laboratory threshed teff flour and 20mg/kg of Electrolytic Iron Powder (EPI) fortification, and between teff flour mixed with vertisol and cambisol soils and 20mg/kg sodium iron EDTA fortification.

Chapter Five

Conclusion & Recommendation

5. Conclusions and Recommendations

In summary, the current study demonstrates that intrinsic iron originated from teff has a good relative biological value, even better as compared to certain iron fortified foods, which could be potentially bioavailable. Even though, there was no statistical significant differences in the hemoglobin regeneration efficiency of anemic rat between iron originated from cambisol and vertisol, from this paper it is possible to conclude that contamination of teff with both varieties of soil considerably improves the iron bioavailability. Thus, contaminant iron has a good nutritional interest. Unlike, the previous studies, it also demonstrated that soil contamination do not interfere with the intrinsic iron bioavailability in the case of vertisol and cambisol.

Promoting the production, acceptability and consumption of teff could contribute to efforts undertaken to control iron deficiency anemia, since high iron biological value was observed in the current study that cannot be achieved from other cereals.

Further research covering more varieties of soil, larger sample size, assessing the potential hazard from heavy metals and others due to soil contamination, iron desorption potentials of varies soil types, human trail to test teff iron bioavailability/utilizability, and effect of mixed soil on iron bioavailability are strongly suggested. It is also recommended that the reconsideration of iron fortification program to different cereal flours which takes the contaminant iron bioavailability in to account.

References

Abebe Y., Bogale A., Hambidge k., Stoecker B., Bailey K., and Gibson R., (2007). Phytate. Zink, iron and calcium content of selected raw and prepared foods consumed in rural Sidamo, Southern Ethiopia, and implication for bioavailability. *Journal of food composition and analysis*, 20, 161 -168.

Abayu B., (2012). Agronomic and Economic Effects of Blended Fertilizers Under Planting Method on Yield and Yield Components of Tef in *Wereda* Laelay Maychew, Central. Master thesis, Mekelle University, Mekelle, Ethiopia.

Abbaspour N., Hurrell R., and Kelishadi R., (2014). Review on iron and its importance for human health. *J Res Med Sci.*, 19(2) 164–174.

Abrahams W., Follansbee H., Hunt A., Smith B., and Wragg J., (2006). Iron nutrition and possible lead toxicity: an appraisal of geophagy undertaken by pregnant women of UK Asian communities. *Appl Geochem*., 21, 98–108.

Adish A., Esrey S., Gyorkos T., Jean-Baptiste J., Rojhani A., (1999). Effect of consumption of food cooked in iron pots on iron status and growth of young children: a randomized trial. *J.-Lancet* 353, 712−716.

Alaunyte I., (2013). Development of nutrient rich teff bread and its effect on iron status and exercise performance of female runners. Docteral dissertation, Manchester Metropolitan University. Retrieved on June 27/2015 from http://www.e-space.mmu.ac.uk/e-space/bits tream/2173/313142/1/ THESIS-%20definitive% 20copy.pdf.

Allen L., Benoist B., Dary O., and Hurrel R., (2006). Guidelines on food fortification with micronutrients. World Health Organizations and Food and Agricultural Organization of United nations. Retrieved on June 26/2015 from http://www.who.int/nutrition/publication /guid_food_ fortification_micronutrients.pdf.

Almgard G., (1963). High content of iron in teff, *Eragrostis abyssinica* Link., and some other crop species from Ethiopia-a result of contamination. *Lantbrukshogskolans Annaler* 29: 215-220.

Ambaw A., (2013). Determination of iron fractions of laboratory and field threshed teff flour, fermented past and enjera: implication to iron bioavailability (Master's Thesis). Addis Ababa University, Center for food science and nutrition.

Andrews N., (1999). "Disorders of iron metabolism". *The New England Journal of Medicine* 341 (26): 1986–1995.DOI:10.1056/NEJM199912233412607.

Aragon J., Ortiz D., and Pachon H., (2012). Comparison between *invitro* and *invivo* methods to screen iron bioavailability. *Journal of Food,* 10(2), 103-111.

Arcasoy A., Çavdar A., and Babacan E., (1978). Decreased Iron and Zinc Absorption in Turkish Children with Iron Deficiency and Geophagia. *Acta Haematol*., 60, 76–84.

Arosio P., and Levi S., (2002). "Ferritin, iron homeostasis, and oxidative damage". *Free Radical Biology & Medicine* 33 (4): 457–63.

Arredond M., Salvat V., Pizarro F., and Olivares M., (2006). Smaller iron particle size improves bioavailability of hydrogen-reduced iron-fortified bread. Nutr Res, 2, 235-239.

Ashgrie Z., and Abate D., (2012). Improvement of injera shelf life through the use of chemical preservatives. *AJFAND*. 12(5), 6409-6423.

Association of Official Analytical Chemists, (2000). Official method of the association of Official Analytical Chemists. 16th edition 3rd revised. *JAOAC,* Int. 80,834(1997).

Association of Official Analytical Chemists, (2006). Official methods of analysis of Association of Official Analytical Chemists. 18th edn. AOAC International, Maryland, chapter 45, pp 80–81.

Atengo E., (2005). Characteristics, genesis and classification of reddish soils from Sidamo Region of Ethiopia. Doctoral dissertation, Universiti Putra Malaysia.

Ayele T., Ayana M., Tanto T., and Assefa D., (2014). Evaluating the status of micronutrient s under irrigatd and rain fed agriculture soils in Abaya Chamo Lake Basin, South-West Ethiopia/. Journal of Scientific Researches and Reviews, 3(1), 18-27.

Baye K., Icard-Verniere C., and Mouquet-Rivier C., (2012). Nutrient intake from complimentary foods consumed by young children (aged 12-23 months) from North Wello, northern Ethiopia: the need for agro-ecologically adapted intervention. *Public health nutrition*. DOI 10.1017/S1368980012005277.

Baye K., Mouquet-Rivier C., Icard-Verniere C., Picq C., and Guyot J., (2013). Changes in mineral absorption inhibitor consequent to fermentation of Ethiopian enjera: implications for predicted iron bioavailability and bioaccessibility. *International journal of food sience and technology*. DOI: 10.1111/ijfs.12295.

Baynes R., (1996). Assessment of iron status. *Clin Biochem*., 29,209–215.

Beguin Y., (2003). Soluble transferrin receptor for the evaluation of erythropoiesis and iron status. *Clinica Chimica Acta*., 329, 9–22.

Beiseigel M., Hunt R., Glahn P., Welch M., Menkir A., and Maziya-Dixon B., (2007). Iron bioavailability from maize and beans: A comparison of human measurement with Caco-2 cell and algorithm prediction. *Am J Clin Nutr*., 86(2), 388-396.

Birhane G., Paulos Z., Tafere K., and Tamiru S., (2011). Food grain consumption and calorie intake patterns in Ethiopia. Development Strategies and Governance Division, International Food Policy Research Institute, Ethiopia Strategy Support Program II, Ethiopia.

Björn-Rasmussen E., & Hallberg L., (1979). Effect of animal proteins on the absorption of food iron in man. *Nutr. Metab.,* 23, 192-202.

Bland J., and Altman D., (1986). Statistical method for assessing agreement between two methods of clinical measurements. *Lancent*, 1, 307-310.

Boato F., Wortley G., Liu R., and Glahn R., (2002). Red grape juice inhibits iron availability: application of an *invitro* digestion/Caco-2 cell model. *Journal of agriculture and food chemistry*, 50, 6935-6938.

Boka B., Woldegiorgis A., and Haki G., (2013). Antioxidant properties of Ethiopian traditional bread (injera) as affected by processing techniques and teff grain (Eragostis tef(Zucc)) varieties. *Canadian chemical transactions*, 1(1), 7-24.

Bokhari F., Derbyshire E., Brennan C., and Stojceska V., (2012). A study to establish whether food-based approaches can improve serum iron levels in child-bearing aged women. *J Hum Nutr Diet*, 25(1), 95-100.

Brabin J., Premji Z., and Verhoeff F., (2001). An analysis of anemia and child mortality. *J Nutr*., 131:636–645.

Bruner A. (1996). Randomised study of cognitive effects of iron supplementation in non-anaemic iron-deficient adolescent girls. *Lancet*, 348: 992-96.

Camaschella C., (2005). Understanding iron homeostasis through genetic analysis of hemochromatosis and related disorders. *Blood* 106 (12): 3710–3717. DOI:10.1182/blood-2005-05-1857.

Camaschella C. and Schrier S., (2011). Regulation of iron balance. *The New England Journal of Medicine* 342 (17): 1293–4. DOI:10.1056/NEJM200004273421716.

Central Statistics Agency, CSA, (2011). Ethiopia Demographic and Health Survey, EDHS. Addis Ababa, Ethiopia and Calverton: Maryland USA Central Statistical Authority.

Central Statistics Agency of Ethiopia (CSAE), (2012). Agricultural Sample Survey 2011/2012: Report on Area and Production of Crops. Addis Ababa, Ethiopia.

Cheney K., Gumbiner C., Benson, B., and Tenenbein M., (1995). "Survival after a severe iron poisoning treated with intermittent infusions of deferoxamine". *J Toxicol Clin Toxicol* 33 (1): 61–6. DOI:10.3109/15563659509020217.

Conrad M., and Umbreit J., (2000). Disorders of iron metabolism. *The New England Journal of Medicine* 342 (17): 1293–4. DOI:10.1056/NEJM200004273421716.

Dallman R., and Siimes M., (1979). Percentile curves for hemoglobin and red cell volume in infancy and childhood. *J. Pediatr.*, 94, 26-31.

Dallman R., (1990). *Iron*. In: Brown ML, editor. Present Knowledge in Nutrition. 6th ed. Washington DC: Nutrition Foundation;. pp. 241–50.

Demeke T., (2016). The unresolved wonder on contaminant iron bioavailability: The way forward. **In** press- *Journal of Food Science and Human Wellness*.

Demissie Abebe (2001). Teff genetic resources in Ethiopia. *Narrowing the Rift: Tef Research and Development:27-31*.

Dostal A., Chassard C., Hilty M., Zimmermann B., Jaeggi T., Rossi S., and Lacroix C., (2012). Iron depletion and repletion with ferrous sulfate or electrolytic iron modifiers the composition and metabolic activity of the gut microbiota in rats. *J Nutr*, 142, 271-277.

Duizer E., Penninks A., Stenhuis W., and Groten J., (1997). Comparison of permeability characteristics of the human colonic Caco-2 and rat small intestinal IEC-18 cell lines. *Journal of Controlled Release*, 49(1), 39–49.

Ebihara K., Okano J., and Miyada T., (1994). Comparison of ferrous and ferric iron bioavailability following rat cecal infusion. *Nutr Res*, 14, 221–228.

Ethiopian Health and Nutrition Research Institute (EHNRI), (1997). *Food composition table for use in Ethiopia part III.* Addis Ababa/Ethiopia. Pp. 1-91.

Ethiopia Nutrition Survey (ENS)(1959). A report by the Interdepartmental Committee on Nutrition for National Defense. *CJFD* 45 (26). Washington, D. C.

European Communities**, (**1993). *Nutrient and energy intakes for the European Community*. *EG-Report*. Brussels Luxembourg: Commission of the European Communities.

Fairweather-Tait S., Fox T., Wharf G., and Eagles J., (1995). The bioavailability of iron in different weaning foods and the enhancing effects of fruit drink containing ascorbic acid. Pediatric Research, 37(4), 389-394.

Finney L., and O'Halloran T., (2003). Transition metal speciation in the cell: insights from the chemistry of metal ion receptors. *Science* 300 (5621): 931–6. DOI:10.1126/science.1085049

Fleming R., and Bacon B., (2005). "Orchestration of iron homeostasis". *The New England Journal of Medicine*, 352 (17): 1741–4. DOI:10.1056/NEJMp048363.

Food and Agricultural Organization, (1986). Ethiopian Highlands Reclamation Study. Rome, Italy. Volume 1, 39-40.

Food and Agricultural Organization/World Health Organization, (1988). *Requirements of vitamin A, iron, folate and vitamin B12*. Report of a Joint FAO/WHO Expert Consultation. Rome: FAO. (FAO Food and Nutrition Series No. 23).

Food and Agricultural Organization/World Health Organization, (2004). *Expert consultation on human vitamins and minerals requirements.* Vitamin and mineral requirements in human nutrition: Report of joint FAO/WHO expert consultation.

Food and Agricultural Organization, (2006). *Human Vitamin and Mineral Requirements*. FAO Corporate Documentary Repository. Produced by Agriculture and Consumer Protection. 1-28.

Forbes A., Adam C., Arnaud R., Chichester C., Cook J., Harrison H., Hurrell R., Khan S., Morris E., Tanner J., and Whittaker P., (1989). Comparison of invitro, animals and clinical determination of iron bioavailability: International Nutritional Anemia Consultative Group – Task force report on bioavailability. *American journal of clinical nutrition,* 49, 225-238.

Forth R., (1990). Fundamentals of soil science. 8th edition. John Wiley and Sons, Inc. New York, USA.

Frank V., and Tolgyessy J., (1993). The chemistry of soil: Chemistry and biology of water, air and soil environment aspects. *J. of Tolgyeey*, 31, 621-697.

Frazer D., and Anderson G., (2005). Iron imports. Intestinal iron absorption and its regulation. *American Journal of Physiology*. *Gastrointestinal and Liver Physiology* 289 (4), 631–635. DOI:10.1152/ajpgi.00220.2005.

Frontela C., Ros G., and Martinez C., (2011). Phytic acid content and "*invitro*" iron, calcium, and zinc bioavailability in bakery products: the effect of processing. *Journal of cereal science*, 54, 173- 179.

Ganz T., (2005). Cellular iron: ferroportin is the only way out. *Cell Metabolism* 1 (3): 155–57. DOI:10.1016/j.cmet.2005.02.005.

Gebremedhin M., Killandar A., Vahlquist B., and Wuhib E.,(1976). Rarity of anemia of pregnancy in Ethiopia. *Scandinavian Journal of Haematology* , 16(3), 168-175.

Geoffrey M., (2010). "Metals, minerals and microbes: geomicrobiology and bioremediation". *Microbiology* 156 (3): 609–643. DOI:10.1099 /mic.0.037143-0.

Gibson, R. S. (2006). Zinc: The missing link in combating micronutrient malnutrition in developing countries. *Proceedings of the Nutrition Society,* 65, 51–60.

Gonzalez R., Medina S., Martinez-Augustin O., Nieto A., Galvez J., Risco S., and ZarzueloA., (2004). Anti-inflammatory effect of diosmeetite in hepten-induced colitis in the rat. *Br J Pharmacol*., 141, 951-960.

Green S., and Turner C., (1974). The manufacture of small quantities of rat food pellet in the laboratory. *Laboratory Animals*, 8, 131-132.

Greffeuille V., Mouquet-Rivier C., Icard-Verniere C., Ouattara L., Avallone S., Hounhouigan J., Kayde P., Amoussa W., and Fatoumata B., (2010). *Traditional recipe of millet-, sorghum-, and maize-based dishes and related sause frequently consumed by young children in Burkina Faso and Benin.* INSTAPA, Europian Union's Seventh Fremwork Program, ISBN:978-90-8585-903-3.

Guggenheim K, (1995). Chlorosis: The rise and disappearance of a nutritional disease. *Journal of Nutrition*, 125, 1822–1825.

Habtamu G., (20015). Evaluating the extrinsic iron from soil contaminated teff on hemoglobin regeneration of anemic rats: Indicator of bioavailability of extrinsic iron. Masters thesis, Addis Ababa University, Center for Food Science and Nutrition.

Haidar A., and Pobocik S., (2009). Iron deficiency anemia is not a rare problem among women reproductive ages in Ethiopia: A community based cross sectional study. *BMC Blood Disorder*, 9, 7-15.

Haidar A., (2010). Prevalence of anemia , deficiency of iron and folic acid and their determinet in Ethiopian women. *J Health Popul Nutr*, 28(4), 359-368.

Hall A., Bobrow E., Brooker S., Jukes M., Nokes K., and Lambo J. (2000). Anemia in schoolchildren in eight countries in Africa and Asia. *Public health nutrition,* 4(3), 749-756.

Hallberg L., and Rossander-Hulthénm L., (1991). Iron requirements in menstruating women. *Am. J. Clin. Nutr.,* 54, 1047-1058.

Hallberg L., and Bjrn-Rasmussen E., (1981). Measurement of iron absorption from meals contaminated with iron. *The American Journal of Clinical Nutrition* 34: pp. 2808-2815.

Hallberg L., Hulthén L., and Gramatkovski E., (1997). Iron absorption from the whole diet in men: how effective is the regulation of iron absorption? *Am. J. Clin. Nutr.,* 66: 347-56.

Harvey W., Dexter B., and Darnton-Hill L., (2000). The impact of consuming iron from non-food sources on iron status in developing countries. *Public Health Nutrition*: 3(4), 375±383 375.

Hazelton P., and Murphy B., (2007). *Interpreting soil test results: What do all the numbers mean?* 2nd ed. CSIRO Publishing, Australia. Pp. 64-65.

Hentze M., Muckenthaler M., Galy B., and Camaschella C (2010). Two to tango: regulation of Mammalian iron metabolism. *Cell* 142 (1): 24–38. DOI:10.1016/j.cell.2010.06.028.

Hooda P., Henry C., Seyoum T., Armstrong L., and Fowler M., (2004). The potential impact of soil ingestion on human mineral nutrition. *Sci. Total Environ.,* 333, 1–3.

Hubert M., Larique P., Gillet J., and Keenan K., (2000). The effect of diet *ad libitum* feeding and moderate and severe dietary restriction on body weight, survival, clinical pathology parameters and cause of death in control Sprague-Dawley rats. *Toxicol Sci*, 58(1), 195-207. DOI: 10.1093/toxsci/58.1.195.

Hurrell R. and Egli I., (2010). Iron bioavailability and dietary reference values. *American Journal of Clinical Nutrition*, 91(5), 1461S–1467.

Icard-Verniere C., Hama F., Guyot J., Picq C., Diawara B., and Mouquet-Rivier C., (2013). Iron contamination during in-field milling of millet and sorghum. *J Agric Food Chem*, 30, 61(43), 10377-10383. DOI: 10.1021/jf402612k.

Iannotti L., Tielsch J., *et al.,* (2006). Iron supplementation in early childhood: Health benefits and risks. *The American Journal of Clinical Nutrition,* 84(6) 1261- 1276.

International Food Policy Research Institute, IFPRI (2016). *The economy of teff: Ethiopians biggest cash crop*. Nutritional composition and health benefits of teff. 275-287.

Ismail M., (1999). The use of Caco-2 cell as an *invitro* method to study bioavailability of iron. *Mal J Nutr,* 5, 31-45.

Kebede F., and Bekele E., (2008). Tillage effect on soil moisture storage and wheat yield on the vertisols of North-Central highlands of Ethiopia. *Ethiopian Journal of Environmental Studies and Management*, 1(2), 49-55.

Kebede F., and Yomoah C., (2009). Soil fertility status and Numass fertilizer recommendation of typic Hapluusterts in the Northern highlands of Ethiopia. *World Applied Science Journal*, 6(11), 1473-1480.

Ketema S., (1997). *Tef-Eragrostis (Zucc.).* Vol.12: Biodiversity international. Promoting the conservation and use of the underutilized and neglected crop 12. *Institute of plant genetics and crop plant research, Gatersleben/International Plant genetics resources institute, Rome, Italy*.

Kebede G., Assefa G., Mengistu A., Tekletsadik T., Feyissa F and Minta M., (2013). Evaluation of forage yield and yield component of different vetch species and their accession growth under nitsol and vertisol conditions in the Central highlands of Ethiopia. *Ethiopian Journal of Applied Science Technology,* 4, 14-38.

Khan S., Sohail M., Atif A., Akhtar N., Khan H., and Rasool F., (2014). Symptom-based evaluation of iron deficiency anemia in students of Bahawalpur correlated with their eating habits. *Trop J pharm Res*., 13(5), 769-775.

Kikouama J., Cornec F., Bouttier S., Launay A., Bald´e L., and Yagoubi N., (2009). Evaluation of trace elements released by edible clays in physicochemically simulated physiological media. *Int. J. Food Sci. Nutr.*, 60, 130–142. DOI: 10.1080/09637480701614956.

Kim M., and Atallah T., (1993). Intestinal solubility and absorption of ferrous iron in growing rats are affected by different dietary pectins. *J Nutr*, 123, 117–124.

Lobo A., Gaievski S., and Colli C., (2011). Hemoglobin Regeneration Efficiency in Anemic Rats: Effects on Bone Mineral Composition and Biomechanical Properties. *Biol Trace Elem Res*, 143:403–411. DOI 10.1007/s12011-010-8871-2.

Lonnerdal B., (2000). Dietary factors influencing zinc absorption. *Journal of Nutrition*, 130(5), 1378S–1383.

Lucia D., Toste V., Silveria M., Bordalo A., Roddrigues C., Ana P., et al., (2013). Iron bioavailability in Wistar rat fed with fortified rice with Ultra Rice ^{R} technology with or without addition of yacon flour *(Smallanthus Sonchiflolius). Archivos LatinoAmericanos De Nutricion*, 13(1), 64-73.

Luten J., Crews H., Flynn A., Dael P., Kastenmayer P., Hurrel R., Deelstra H., Shen L., Fainweather-Tait S., Hickman K., Farre R., Schlemmer U., and Frarhilich W., (1996).

Inter laboratory trial on the determination of the *invitro* iron dialysability from food. *Journal of the Science of Food and Agriculture*, 72, 415-424.

Lysionek A., Zubillaga M., Salgueiro J., Caro R., Ettlin E., and Boccio J., (2001). Bioavailability study of a new iron sources by means of the Prophylactic-Preventive Methods in rats. *Biological Trace Element Research*, 84(3), 123-128.

Majudar N., Paul P., Talib V., and Ranga S., (2003). The effect of iron therapy on the growth of iron-replete and iron deplete children. *Journal of Tropical Pediatrics*, 49(2), 84-88.

Mascotti P., Rup D., and Thach E., (1995). Regulation of iron metabolism: Translational effects medicated by iron, heme and cytokines. *Ann. Rev. Nutr.*, 15: 239-61.

McKie A., Barrow D., Latunde-Dada G., Rolfs A., Sager G., and Mudaly E., (2001). "An iron-regulated ferric reductase associated with the absorption of dietary iron". *Science* 291 (5509): 1755–9. DOI:10.1126/science.1057206.

Meseret A., (2015). Iron fractionation of cereals contaminated with different types of Ethiopian soils and its consequence on bioavailability. Masters thesis, Addis Ababa University, Center for Food Science and Nutrition.

Minnich V., Okuolu A., Tarkon Y., Arcasoy A., Cin S., Yrucoglu O., Renda F., and Demira B., (1968). Pica in Turkey II. Effect of Clay upon Iron Absorption. *American Journal of Clinical Nutrition*, 21(1), pp 75-56.

Miyada T., Nakajima A., and Ebihara K., (2011). Iron bound to pectin is utilized by rats. *British Journal of Nutrition,* 106(1), 73-78.

Molla A., (2013). Farmers' knowledge helps develop site specific fertilizer rate recommendations, Central highlands of Ethiopia. *World Applied Science Journal*, 22(4), 555-563.

Muckenthaler M., Galy B., and Hentze M., (2008). Systemic iron homeostasis and the iron-responsive element/iron-regulatory protein (IRE/IRP) regulatory network. *Annual Review of Nutrition*, 28: 197–213. DOI:10.1146/annurev.nutr.28.061807.155521.

Nanami M., Ookawara T., Otaki Y., Ito K., Moriguchi R., Miyagawa K., Hasuike Y., Izumi M., Eguchi H., Suzuki K., and Nakanishi T., (2005). Tumor necrosis factor-α-induced iron sequestration and oxidative stress in human endothelial cells. *Arteriosclerosis, thrombosis, and vascular biology* 25 (12): 2495–2501. DOI:10.1161/01.ATV.0000190610.63878.20.

Onabanjo O., MAziya-Dixon B., Oguntona B., Olayiwola O., Oguntona E., and Dixon O., (2008). Iron bioavailability and utilization in rats fed cassava-based complementary diets. *Journal of Food, Agriculture and Environment,* 6(4), 210-214.

Ramakrishnan U., Frith-Terhune A., Cogswell M., and Khan K., (2002). Dietary intake does not account for difference in low iron store among Mexican-American and non-Hepanic white women: Third national health and nutrition examination survey, 1988-1994. *The journal of Nutrition*, 132, 996-1001.

Reddy M., (2005). Algorithm to assess non-heme iron bioavailability. *Int J Vitam Nutr Res.*, 75(6) 405 – 412.

Reeves G., Nielsen H., and Fahey C., (1993). AIN-93G purified diet for laboratory rodents: Final Report of American Institute of Nutrition Ad Hoc Writing Committee on the Formulation of AIN-76A Rodent Diet. *J Nutr*., 123, 1939-1951.

Rohner F., Ernst F., Arnold M., Hilbe M., Biebinger R., and Ehrensperger F., (2007). Synthesis, characterization and bioavailability in rats of ferric phosphate nanoparticles. *J Nutr*, 137, 614-619.

Rossander-Hulthén L., and Hallberg L., (1996). Prevalence of iron deficiency in adolescents. In: Hallberg L, Asp N-G, eds. *Iron nutrition in health and diseas*. p.149-156. London, John Libby& Co.

Rouault T., and Cooperman S., (2006). "Brain iron metabolism". *Seminars in Pediatric Neurology*. 13 (3): 142–8. DOI:10.1016/j.spen.2006.08.002.

Sari M., Pee S., Martini E., Herman S., Sugiatmi W., Bloem M., and Yip R., (2001). Estimating the prevalence of anemia: a comparison of three methods. *Bulletin of World Health Organization*, 79, 506-511.

Saturni L., Giannia F., Bacchetti T., (2010). The gluten-free Diet: Safety and National Quality. *Nutrients*, 2, 16-34. DOI:10.3390/nu2010016.

Sazawal S., Black R., *et al.*, (2006). Effect of routine prophylactic supplementation with iron and folic acid on admission to hospital and mortality in preschool children in high malaria transmission setting: Community based, randomized, placebo-controlled trial. *The lancet*, 367(9505) 133-143.

Scholl T., (2011). Maternal iron status: relation to foatal growth, length of gestation and iron endowment of the neonate. *Nutrition Reviews*, 69(1), 23-29.

Schrier S. and Bacon B., (2011). Iron overload syndromes other than hereditary hemochromatosis. Up To Date. Retrieved on October 29/2015 from http://www.uptodate.com/contents/iron-overload-syndromes-other-than-hereditaryhemochr omatosis.

Seim G., Ahn C., Bodi M., Luwedde F., Miller D., and Hillier S., (2013). Bioavailability of iron in geophagic earth and clay minerals and their effect on dietary iron absorption using in virto digestion/Caco-2 cell model. *Food Funct*, 4(8), 1263-1270. DOI: 10.1039/c3fo30380b.

Shiga A., Sakai T., and Horii N., (1987). Correlations among pH and Mg, Ca, Na, K, Cl2 and HCO3 2 contents of digesta in the gastro-intestinal tract in rats. *Jpn J Vet Sci*, 49, 973–979.

Smith B., Rawlins B., Cordiero M., and Hutchins M., (2000). The bioaccessibility of essential and potentially toxic trace elements in tropical soils from geophagic earths**.** *J. Geol. Soc.*, 157, 884–892.

Soil Survey Staff, (2010). *Keys to soil taxonomy*. 11th edition, U.S Department of Agriculture: Washington, DC. 1-2.

Suttle N., (2010). *Mineral Nutrition of Livestick.* 4th edition. Wallingford: CAB International, Oxfordshire OX10 8DE., UK. Retrieved on September 25/2015 from http://www.ucv.ve/fileadmin/user_upload/facultad-agronomia/Production_animal/Minirals _in_Animal_Nutrition.pdf.

Swain H., Newman M., and Hunt R., (2001). Bioavailability of elemental iron powders to rat is less than Bakery-grade ferrous sulfate and predicted by iron solubility and particle surface area. *J Nutr*., 133, 3546-3552.

Talkington K., Gant N., E. Scott D., and Pritchard A., (1970). Effect of ingestion of starch and some clays on iron absorption. *Am. J. Obstet. Gynecol*., 108, 262–267.

Taylor G., **(**1995). Iron bio-availability from diets consumed by different socioeconomic strata of the Venezuelan population. *J. Nutr*., 25, 1860-1868.

Temesgen M., (2013). Nutritional status of Ethiopian weaning and complementary foods. A review. *Open access scientific reports,* 2(2), 1-9. DOI:10.4172/scientificreports.621.

Tenenbein M., (1996). Benefits of parenteral deferoxamine for acute iron poisoning. *J Toxicol Clin Toxicol* 34 (5): 485–489. DOI:10.3109/15563659609028005.

Umeta M., and Parker M., (1996). Microscopic studies of macro-components of seeds, dough and injera from teff (eragrotis tef). *An Ethiopian journal of science*, 19, 141-148.

Umeta M., West C., and Fufa H., (2005). Content of Zinc, Iron, Calcium and their absorption inhibitors in foods commonly consumed in Ethiopia. *Journal of food composition and analysis*, 18, 803-817.

Umeta M., Haidar A., Demissie T., Akalu G., and Ayana G., (2008). Iron deficiency anemia among women of reproductive age in Nile Administrative regions of Ethiopia. *Ethiopian J Health Dev*, 22(3), 252-258.

Urga K., Narasimha V., Sasikala V., and Vishwanatha S., (1998). Bioavailability of iron and zinc from in rats. *Bull Chem Soc Ethiop*., 12(2), 95-103.

Vavilov N., (1957). The origin, variation, immunity and breeding of cultivated plants. The Translated Version by K. Starr Chester. The Ronald Press Co., New York. pp. 37-38.

Vinning G., and McMahon G., (2006). Gluten free grains. A demand and supply analysis of prospect for Australian health gain industry. A report for the Rural Industries Research and Development Corporation.

Weber T., Freitas C., Amanco O., and Mmorais M., (2010). Effect of dietary fiber mixture on growth and intestinal iron absorption in rats recovering from iron-deficiency anemia. *British journal of nutrition*. 104(10), 1471-1476.

Wessling-Resnick m., (2014). *Iron*. In Rose, A.C., Caballero, B cousins, r., Turker, K.L., Ziegler, T.R. (Eds.) *Modern nutrition in health and disease*. 11th edition. Pp. 176-186. Philadelphia: Lippincott Williams and Wilkins, 2001 Market street. PA 19103. Retrieved on December 11/2015 from http://www.nutnet.ir/dl/Modern%202013.pdf.

White R., (2006). *Principles and practice of soil science: The soil as a natural resources*. 4th edition. Blackwell Science Ltd. UK.

Wienk J., Marx J., and Beynen C., (1999). The concept of iron bioavailability and its assessment. *Eur J Nutr*, 38(2), 51-57.

Wood J., and Ronnenberg A., (2005). *Iron*. In: Shils ME, Shike M, Ross AC, Caballero B, Cousins RJ, editors. *Modern Nutrition in Health and Disease*. 10th ed. Baltimore: Lippincott Williams & Wilkins, pp. 248–70.

World Health Organization, (2004). WHO/CDC. *Expert consultation agrees on best indicators to assess iron deficiency, a major cause of anaemia*. Retrieved on May 2/2016 from: http://www.who.int/mediacentre /news/notes/2004/ anaemia/en.

World Health Organization, (2007). *Assessing the iron status of populations*. 2nd ed. World Health Organization, Geneva. Pp. 3-22.

World Health Organization, (2011). *Hemoglobin concentration for the diagnosis of anemia and assessment of severity*: Vitamin and Mineral Nutrition Information System. Geneva, Austria. Retrieved on May 11/2016 from http://www.who.int/vmnis/indicators/hemoglobin. pdf.

Yang Z, Dewey G, Lonnerdal B, Hernell O, Chaparro C, Adu-Afarwuah S, *et a.,l* (2008). Comparison of plasma ferritin concentration with the ratio of plasma transferrin receptor to ferritin in estimating body iron stores: Results of 4 intervention trials. *Am J Clin Nutr*, 87, 1892–1898.

Yerima P., Ranst V., Sertsu S., and Verdoodt V., (2013). Pedogenic impact on the distribution of total and available Fe, Mn, Cu, Zn, Cd, Pb and Co content of vertisols and vertic inceptisol of the Bale Mountain area of Ethiopia. *African Journal of Agricultural Research*, 8(44), 5429-5439.

Young S., (2010). Pica in Pregnancy: New Ideas About an Old Condition. *Annual Review of Nutrition,* 30: 403-422. DOI: 10.1146/annurev.nutr.012809.104713.

Yun S., Habicht J., Miller D., and Glahn R., (2004). An *invitro* digestion/Caco-2 cell culture system accurately predict the effect of ascorbic acid and polyphenolic compounds on iron bioavailability in human. *The Journal of Nutrition,* 134, 2717-2721.

Zimmermann M., and Hurrell F., (2007). Nutritional iron deficiency. *Lancet,* 370(9586), 511–520.

ANNEXS

Annex I: Sample collection site, teff type and growing condition

Sample collection site: Debre Birhan, Asagret Kebele, Amhara region

Teff sample type: White teff

Water source: Rain water

Type and amount of fertilizer used: DAP 80kg and UREA 40kg used for 1 Hectar land

Condition of crop prior to harvest: very good

Harvest method: traditional with great care to avoid soil contamination and no contact with ground surface

Soil type: Vertisol (according to Debre Birhan agricultural research center, there is no scientific proof exists)

Annex II: Ethical clearance letter from the Ethical Clearance Review Board of the College of natural sciences of Addis Ababa University

COLLEGE OF NATURAL SCIENCES
Addis Ababa University
OFFICE OF THE DEAN
የዲን ጽ/ቤት

የተፈጥሮ ሣይንስ ኮሌጅ
አዲስ አበባ ዩንቨርሲቲ

Ref.
ቁጥር CNSDO/178/08/2016
Date.
ቀን January 12, 2016

To Whom It may Concern

College of Natural Science Institutional Review Board (CNS-IRB) has reviewed an MSc thesis project proposal entitled “Rat hemoglobin repletion efficiency of teff contaminated with various type of soils” by Demeke Teklu from Center for Food Science and Nutrition.

The proposal was approved for implementation.

With regards,

Dr. Shibru Temesgen
Dean College of Natural Science

ስልክ/Tel: +251-11-123 94 72
ፋክስ/Fax: +251- 11- 123 94 69
Please quote our reference number in your correspondence.
ፖ.ሣ.ቁ/P.O.Box: 1176Addis Ababa, Ethiopia
ኢሜል/Email: dean_cns@aau.edu.et
“Exmine all things; hold fast that which is good”
“ሁሉን መርምሩ፥ መልካሙን ያዙ”