Addis Ababa University

School of Graduate Studies

College of Natural Sciences

Centre for Food Science and Nutrition

Nutritional composition, phytochemical screening, processing methods and Sensory attributes of a brew made from infusions of matured leaves of Arabica coffee tree consumed in Sidama, Kambata and Harar communities, Ethiopia

BY

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Advisors

Dr. Tarekegn Birhanu

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A thesis submitted to the School of Graduate studies of Addis Ababa University in partial fulfillment of the requirement for the Degree of Master of Science in Food Science and Nutrition.

June, 2015

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Declaration

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

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TABLE OF CONTENTS

LIST OF TABLES

List of figures

List of abbreviations

BHA	butylated hydroxyl anisol
BHT	butylated hydroxyl tolune
C. Arabica	coffee Arabica
CNB	carbon nitrogen balance
CRD	complete randomized design
DNA	Deoxy ribo nuclic acid
DPPH	1,1-Diphenyl-2-picrylhydrazyl
ET	Electron transfer
FAO	Food and agricultural organization
GAE	Galic acid equivalent
HCEs	Easters of hydroxycinnamic acid
HPLC	High performance liquid chromatography
ICO	International coffee organization
N	The normality of the HCl used
RNA	Ribo nuclic acid
SNNPR	southern nations nationalities and peoples
Spss	Statistical package of social scientists
TBHQ	Tert butyl hydroquinone
TPC	Total phenolic content
UK	United king dom
Uv	ultra-violet
V	volume of HCl consumed (ml) to the endpoint of titration
Wo	Sample weight on dry matter basis
Wt	Weight

Abstract

Coffee leaf tea is a traditional drink for which many consumers in the rural area of Ethiopia are familiar since the early times. In the present study proximate and trace metal composition, selected phyto chemical screening, sensory attribute analysis, and antioxidant level determination of six coffee leaf samples from different sites were determined. Proximate composition in the study samples ranges from (14.43±0.12)% to (18.98±0.55) for protein, (8.821±0.31)% to (12.41±0.23)% for ash,(4.5±0.7 to12.5±2.12)% for crude fat,(17.15±0.69 to 20.01±0.70) % for fiber. The trace element level ranged from (124.16±4.47 to 174.5 ±2.58) mg/100g for calcium, (2.76±0.24 to 93.42±3.41)mg/100g for iron and (9.5±0.5 to79.98±0.94) mg/100g for magnesium. The total phenolic content ranged from (642.74±7.95) mg GAE/g to (301.72±11.32) mg GAE /g of dry weight of extract. Antioxidant activity of extracts was expressed as percentage of DPPH free radicals inhibition levels in percentage ranged from (78.55±0.55 to 98.27±0.028) %. An ant nutritional factor ranges from 63.39±9.70 to 125.70±20.68mg/g for tannin and 29.71±1.77 to 36.02±0.50mg/g for phytate, respectively. Caffeine content ranges from (691.26 to 1443) ppm. Methanolic extracts of coffee leaf samples from different sites have showed that the highest phenolic concentration and strong antioxidant activity. The high contents of phenolic compounds indicated that these compounds contribute to the antioxidant activity and this indicates that coffee leaves can be regarded as promising candidates for natural antioxidants.

Key words: Coffee tree leaves, antioxidant, proximate analysis, methanolic extract.

Chapter one

1. Introduction

Coffee is a major agricultural commodity in the world, and its production is economically important to several tropical countries, including Brazil, Vietnam, Colombia, Indonesia, Mexico, Ethiopia and Kenya, among others (Hein and Gatzweiler, 2006).*Coffea Arabica* L. (Rubiaceae) is the most cultivated and consumed coffee species in the world, yielding approximately 70 billion dollars per annum (DaMatta, 2004).It is the second most traded commodity after oil, accounting for exports worth an estimated US$15.4 billion in 2009/10, when some 93.4 million bags were shipped [International Coffee Organization (ICO), 2011], and has an estimated annual retail value exceeding US$70 billion (Vega *et al*., 2003; Lewin *et al.*, 2004).

Coffee plant is categorized among the medicinal plants because studies on beans and fleshy organs of the coffee plant including the leaves reveal that these organs were found to have generous amounts of secondary metabolites such as phenolic compounds,esters of hydroxy cinamic acids,mangiferin, etc, which have high level of antioxidant properties and anti-inflammatory effects on humans (Camp *et al*., 2012).

Coffee has been used traditionally in the treatment of asthma, atropine-poisoning, fever, headache, jaundice, malaria, migraine, narcosis, sores and vertigo. Coffee enemas have been used for cancer (Camp *et al*., 2012). As stated by Davis (2006), the leave of coffee plant play a significant role for human use as it was found to contain high amounts of secondary metabolites including mangiferin which is well known for its numerous pharmacological properties such as anti-inflammatory,anti-diabetic,anti-hyperlipideamic and neuroprotective activities (Muruganandan *et al*.,2005; Campos-Esparza *et al*., 2009).

Coffee leaves are the actual leaves from the coffee plant (either Coffee Robusta or Coffee Arabica). The leaves in coffee tree produce secondary metabolites or phenolic compounds such as maginiferin and esters of hydroxycinnamic acids (HCEs). In plants mangiferin provides antioxidant and antimicrobial protection upon biotic stress (Franklin *et al.,* 2009). More generally, phenolics are involved in the response to biotic and abiotic stresses (Santiago *et al.,* 2009), mainly due to their antioxidant properties (Moglia *et al*., 2008). In this way, they may

play a role in adaptation to environmental change (Boudet, 2007) and in co evolution (adaptation) with pests and diseases (Eyles *et al*., 2010).

An herbal tea (brew) from coffee tree leaves has a high medicinal value as good sources of natural antioxidants. After being roasted, the leaves are ground or crumpled, then brewed in hot water similar to normal tea. A brew from coffee leaves (coffee-leaf tea) is so far not a common drink, but has been popular in some regions such as Sumatra, Ethiopia and South Sudan .There was even an attempt to sell it in the UK during the 1800s. In 1851, people were touting it as the next tea and part of its advertising slogan was to "clear the brain of its cobwebs (tiredness) (Davies, 2006).

In Ethiopia, a drink made from the infusion of coffee leaves is locally called k*uti,* and it was drunk in some areas of the country, centuries before coffee bean roasting was invented. The locals there believe that the drink stems hunger and tends to energize both the body and mind (Davis, 2006).

Old coffee leaves and barks which are often considered as agricultural and factory wastes can also be used as potential source of caffeine and natural antioxidants (Campa *et al.,* 2012). This is because it contains high levels of the phenolic compounds, mangiferin and hydroxycinnamic acid esters credited with lowering the risk of heart disease and diabetes, reducing cholesterol levels and protecting neurons in the brain (Zang *et al.*, 2003). Mangiferin is a natural chemical normally found in mangoes while hydroxycinnamic acid esters have been isolated from blackcurrants and a range of vegetables. Interestingly,mangiferin appears to be found only in the leaves of the coffee plant, not the beans (Lopes and Monaco, 1979).

The latest contender in the hot beverage market is coffee leaf tea. This unusual tea, made from the coffee plant is healthier than its two caffeine-based rivals (compotators). The leaves were also found to contain more antioxidants than normal tea, as well as natural chemicals (Davis, 2006).

Therefore the present study was initiated to study the nutritional composition, phytochemical screening, processing methods and sensory attributes of a brew made from matured leaves of coffee tree consumed in Sidama, Kambata and Harar communities, Ethiopia.

1.1. Statement of the problem

Coffee leaves have been consumed in different parts of Ethiopia for different reasons since the early times. However, the users may not understand why they drink it and what benefits does the drink have. In contrast to the considerable amount of research on green beans, there are relatively few studies concerned with the metabolite content of the other parts of the coffee plant, such as the leaves, the outer fleshy layers of the fruit and vascularized organs.

The matured leaves from the coffee plant which are suspected to have high medical effects are being thrown as unwanted waste. Even though some communities are preparing a beverage called *kuti* from the coffee leaves in some parts of the country, its preparation methods are different from place to place and there are no common documented procedures to prepare the delicious and useful beverage *(kuti)* from these cheap but most valuable leaves of the coffee plant.

The nutritional compositions and attributes and processing of the beverage from coffee leaves are not well characterized. There is very limited information about these attributes of coffee tree leaves beverage in the literature. Therefore, in this thesis work an attempt have been made to produce comprehensive information about the nutritional compositions, selected phytochemica levels, antioxidant components and scavenging potential of leaves from commonly available Arabica coffee tree employing multiple assay methods.

1.2. Objectives of the study

1.2.1. General objective

To characterize and evaluate coffee tree leaves in terms of their nutritional compositions, phyto-constituents, antioxidant properties and sensory attributes and to promote it for food security and economical imputes

1.2.2. Specific objective

- To study mineral compositions in matured coffee tree leaves
- To determine caffeine level of matured coffee tree leaves
- To study anti nutritional levels in matured coffee tree leaves
- To determine the total phenolic compositions and antioxidant levels in methanolic extracts of coffee leaves by DPPH method.
- To study traditional methods of preparing the *kuti* beverage from infusions of coffee tree leaves in different parts of the country.

Chapter two literature Review

2.1. The Coffee plant

2.1.1. General description of the species

Several species of shrub of the genus Coffee produce the berries from which coffee is extracted. The two main species commercially cultivated are Coffea canephora (predominantly a form known as 'robusta') and C. Arabica (ICO, 2011). C. Arabica, the most highly regarded species, is native to the southwestern highlands of Ethiopia and the Boma Plateau in southeastern Sudan and possibly Mount Marsabit in northern Kenya (Davis *et al.,* 2006).C. canephora is native to western and central Sub-Saharan Africa, from Guinea to Uganda and southern Sudan (Maurin *et al*., 2007). Less popular species are C. liberica, C. stenophylla, C. mauritiana, and C. racemosa. All coffee plants are classified in the large family Rubiaceae. They are evergreen shrubs or small trees that may grow 5 m (15 ft) tall when unpruned (Anthony *et al.,* 2010).

2.1.2. The Leaves of the coffee plant

The leaves are dark green and glossy (shine &smooth), usually 10–15 cm (4–6 in) long and 6 cm (2.4 in) wide, simple, entire (smooth edges), and opposite. Petioles of opposite leaves fuse at base to form interpetiolar stipules, characteristic of Rubiaceae. The developmental stages of the leaves are categorized as (a) buds and young leaves, (b) mature leaves, and (c) aged leaves. Young leaves are the most recently emerged, and they weighed approximately 25 mg (fresh weight) and are approximately 20 mm long and 7 mm wide. Mature leaves comprised the fully expanded, second and third leave below the apex (weight approximately 1.2 g), whereas aged leaves are dark green from near the base of the shoot and weighed approximately 1.3 g. (Ashihara *et al*., 1996).

The flowers are axillary (between branch and steam), and clusters of fragrant white flowers bloom simultaneously. Gynoecium consists of inferior ovary, also characteristic of Rubiaceae. Flowers followed by oval berries of about 1.5 cm (0.6 in).Green when immature, they ripen to yellow, then crimson, before turning black on drying. Each berry usually contains two seeds, but 5–10% of the berries have only one; these are called pea berries. Arabica berries ripen in six to eight months, while Robusta take nine to eleven months.

2.2. Constituents of coffee plant

Natural coffee constituents that are common in other food products are water, carbohydrates, proteins, peptides and free amino acids, carboxylic acids, minerals, and lipids (e.g. triacylglycerols, sterols, tocopherols and wax).

2.2.1. Proteins

Proteins are polymers of α-amino acids. Twenty different types of amino acids occur naturally in proteins. Proteins differ from each other according to the type, number and sequence of amino acids that make up the polypeptide backbone. As a result they have different molecular structures, nutritional attributes and physiochemical properties. Proteins are important constituents of foods for a number of different reasons. They are a major source of energy, as well as containing essential amino-acids which are essential to human health, but which the body cannot synthesize. Typically, proteins are used as gelling agents, emulsifiers, foaming agents, stabilizers, and thickeners. Therefore, knowing the total concentration, type, molecular structure and functional properties of the proteins in foods is very important.

Proteins, peptides, and free amino acids are vital for coffee flavor since they are needed for the Maillard reaction. They serve as precursors for the formation of volatile compounds such as furans, pyridines, pyrazines, pyrrols, aldehydes, and melanoidins. The melanoidins are responsible for coffee's color and to some extent, its antioxidant activity. The total nitrogenous compounds (excluding caffeine and trigonelline) account for 9%–16% of the green coffee chemical composition, with a slightly higher content in *C. canephora* than *C. arabica.* However, coffee is not a good nutritional source of protein because it lacks essential amino acids (Variyar *et al.,* 2003).

2.2.2. Lipids

Lipids are one of the major components of coffee, and their total content varies considerably between *C. Arabica* and *C. canephora* species. The lipid fraction of coffee is composed mainly of triacylglycerols (approximately 75%), free fatty acids (1%), sterols (2.2% un esterified and 3.2% esterified with fatty acids), and tocopherols (0.05%), which are typically found in edible vegetable oils (k¨olling and speer, 2005*)*.

Other recently identified components are coffeadiol and arabiol I, which have structures similar to the diterpenes cafestol and kahweol, respectively, but with different substitutions in the furan ring (Speer and K¨olling-Speer,2006).The total lipid content in Arabica seeds (approximately 14 g/100 g dry matter) is approximately two times that of Robusta seeds. Fatty acids in coffee are found primarily in combined forms; most are esterified with glycerol in the triacylglycerol fraction, 20% esterified with diterpenes, and a small proportion in sterol esters. Most fatty acids in coffee are unsaturated. Linoleic acid (18:2(*n*-6)), oleic acid (18:1(*n*-9)), and linolenic acid (18:3(*n*-3))account for approximately 43%–54%, 7%–14%, and 1%–2.6% of the triacylglycerol fraction, respectively, and approximately 46%, 11%, and 1% of the free fatty-acid fraction, respectively (Nikolova *et al.,* 1998, Lercker *et al.,* 1996). Fatty acids are not only important for health, but their integrity is important to keep coffee fresh and avoid the staleness caused by hydrolysis and oxidation of triacylglycerols (Toci *et al*., 2008).

The major categories of sterols in coffee are 4-desmethylsterols (accounting for approximately 93% of total sterols), 4-methylsterols (2%), and 4,4-dimethyl-sterols (5%). Sitosterol belongs to the first category and accounts for up to 54% of the sterol fraction; stigmasterol and campesterol each account for approximately 20% (Speer, K and K¨olling-Speer,2006).The average amount of tocopherols in coffee has been reported as 11.9 mg/100 g green coffee(Ogawa, *etal*), but varies considerably depending on the methodology used. The α ,β and γ forms of tocopherols are present in coffee. The analytical separation of the β and γ forms is difficult, but a few studies have reported the predominance of β tocopherol, followed by _α and γ_. (Folstar *et al*, 1977) found concentrations of 8.9–18.8 mg α -tocopherol and 25–53 mg β - + γ -tocopherol/100 g coffee oil. (Ogawa *et al., 1989*) reported the maximum total tocopherol content as 15.7 mg/100 g green coffee, with α -tocopherol accounting for 2.3–4.5 mg and β -tocopherol accounting for 3.2–11.4 mg/100 g green coffee; γ-tocopherol was not detected, possibly because of separation difficulties.

Although most lipids are located in the endosperm of green coffee seeds, the coffee wax is located in the outer layer. This fraction accounts for 0.2%–0.3% of the coffee seed's weight. The main components of coffee wax are carboxylic acid-5-hydroxytryptamides, which are amides of serotonin and fatty acids of varying chain lengths (Speer, 2006).

The principle physicochemical characteristics of lipids (the "analyte") used to distinguish them from the other components in foods (the "matrix") are their solubility in organic solvents, immiscibility with water, physical characteristics (e.g., relatively low density) and spectroscopic properties.

2.2.3. Soluble dietary fiber

Soluble dietary fiber in coffee consists of high-molecular-weight polysaccharides that increase the brew's viscosity (Nunes and Coimbra, 2001). Galactomannans and type II arabinogalactans are the most important types of soluble fiber in coffee. Galactomannans are polymers of 1,4-linked mannans with a single galactose unit side chain at C6, and type II arabinogalactans consist of a main chain of 1,3-linked galactose branched at C6, with side chains containing arabinose and galactose residues.The hot water-soluble green coffee type II arabinogalactans are highly branched and covalently linked to proteins in which 10% of the amino acid chains are 4-hydroxyproline residues. These polysaccharides are extremely complex. In addition to galactose and arabinose, they also contain raminose and glucuronic acid residues. Rhamnoarabinosyl and rhamnoarabinoarabinosyl side chains have recently been reported (Nunes *et al*., 2008).

In the last decade, these compounds have received special attention because they cannot be digested by humans; therefore, they reach the colon intact, potentially serving as substrates for beneficial colonic microbiota fermentation (Gntechwitz *et al*., 2007). A high intake of dietary fiber is positively associated with several beneficial physiologic and metabolic effects such as lowering blood cholesterol and modulating the blood glucose and insulin responses. Fermentable polysaccharides are degraded by colonic microbiota to short-chain fatty acids Coffee (e.g., acetate, propionate, and butyrate). This process lowers the colonic pH, impeding the growth of certain pathogenic species and supporting the growth of *Bifidobacterium* speciesand other beneficial lactic acid bacteria (Gntechwitz *et al.*, 2007).

2.2.4. Essential minerals in coffee plant leaves

Nutrients are very essential for plant growth. In total, seventeen nutrients (or chemical elements) are known in relation to tree growth. These are divided in two groups: non- mineral and mineral. Non-mineral nutrients are: CO_2 (part of the air) and H_2O (water). Under normal conditions these are freely available and are absorbed by the tree through leaves and roots. Although water

availability might be insufficient during dry spells. Fourteen mineral nutrients can be divided in three groups (Table 2.1).

Table: 1 Mineral nutrients of coffee plant

Primary nutrients	**Secondary nutrients**	**Micronutrients**	
Nitrogen (N)	Calcium (Ca)	Boron (B)	Manganese (Mn)
Phosphorus (P)	Magnesium (Mg)	Chloride (Cl)	Molybdenum (Mo)
Potassium (K)	Sulfur (S)	Copper (Cu)	Nickel (Ni)
		Iron (Fe)	Zinc (Zn)

Source :manual for Arabica coffee (2004)

Primary nutrients are called such because they are present in the tree in larger quantities. The secondary and micronutrients are present in much lower concentrations. Usually, the tree will show deficiencies of primary nutrients, although after the harvest Calcium and Magnesium deficiencies can be observed in coffee trees. In general the tree uses large amounts of primary nutrients and only smaller amounts of secondary- and micronutrients. Still the second and third Groups are just as important to the tree.

2.2.5. Nutrient up take of coffee tree

The nutrient up take of coffee plat varies from organ to organ. The level of nutrient distribution in coffee plant is summarized as shown in the table below. Three elements (carbon, hydrogen and oxygen) make up 94% of the plant tissues and are obtained from air and water. The other 13 elements are obtained from the soil and are divided into two broad categories: 'macro' and 'micro'. These terms do not refer to the importance of the elements; macronutrients are required in greater amounts than micronutrients for normal plant growth.

Table 2: Nutrient up takes of coffee tree

	Elements (kg)					
Parts of tree	N	P	K	Ca	Mg	S
Roots	15	2	25	9	2	2
Branches	14	2	20	6	3	1
Leaves	53	11	45	18	7	3
Fruits	30	3	35	3	3	3
Total	112	18	125	36	15	9

Source: www.fao.org

It is obvious from this table that leaves need the major part of the uptake - more than the flowers or fruits. However, nutrients are returned to the soil when the leaves drop. The early years of root development are very important as branches and roots store nutrients for a long time. Nutrients accumulated in the fruits will be removed when cherries are harvested. This loss needs to be compensated by the addition of fertilizers, organic manures; leaf fall or pruning's and leaves from shade trees. Recycling of pulp to the soil after composting can help to reduce the additional (chemical) fertilizer needed.

In humans minerals are also essential for health normal body functioning. Micronutrients are vitamins and minerals needed (but not synthesized) by the body in small amounts for a wide range of functions and processes. Micronutrients are essential for optimal human growth and development, and healthy maintenance of the body over life span. Micro nutrient deficiencies affect more than 2 billion people globally. Although less prevalent in higher-income populations, these deficiencies do occur in such groups, especially among premature infants, infants, children, and the elderly (Siekmann *et al*.,2003).Micronutrients are needed to maintain strong bodies and mental sharpness, to fight against disease, and bear healthy children. Micronutrient deficiencies can cause learning disabilities, mental retardation, decreased immunity, low work capacity, blindness, and premature death. They affect child survival, women's health, educational achievement, adult productivity, and overall resistance to illness. They may impair immune function; increase the risk of opportunistic infections; and the severity of diseases (Gibson and Ferguson, 1998).

Iron

Iron is the fourth most abundant and one of the cheapest elements in the Earth's crust but iron deficiency is still the most prevalent nutritional disorder in the world (Lind, 2004).

The main causative factors of iron deficiency are poor iron content of the diet, low bioavailability of iron in the diet, or both. Food components such as phytate, tannins, and selected dietary fibers, which bind iron in the intestinal lumen, can impair iron absorption. Phytate has the greatest effect on iron status because many plant foods have high phytate content that can severely impair iron absorption (Mendoza *et al.,* 2001).The absorption of iron in food depends on: iron status of the body; the presence of iron absorption inhibitors and enhancers; types of iron (haem and non-haem); vitamin A status of the body and the status of other

micronutrients. Weaning foods made from cereals is often low in Iron content and contains significant quantities of iron absorption inhibitors, phytic acid and condensed tannins (Pynaert *et al*, 2006). Age, gender and physiological status determine iron requirements. Rapid growth of infants during the first year of life requires an adequate supply of iron for synthesis of blood, muscle, and other tissues.

Iron requirements are especially high in infants from the age of 6 months, in young children, and in pregnant and menstruating women. The increased iron requirement of infants and pregnant women is due to rapid growth and new tissue formation. Menstruation and parasitic diseases cause excessive iron loss, and iron utilization is impaired during chronic infection (WHO, 2000, Domellof *et al.*, 2002).Iron deficiency is one of the major nutritional problems in the developing world, affecting primarily women of childbearing age, infants, and children. Infants of age between 6 and 24 months are especially vulnerable to the development of iron deficiency. Iron deficiency ranges in severity from iron depletion, which causes no physiological impairment, to iron deficiency anemia. Iron deficiency anemia (IDA) affects about 30% of the global population; 43% of the world's infants and children under the age of 4 years; and 20–38% of schoolchildren in West and North Africa. In sub-Saharan Africa, the prevalence of iron deficiency among pregnant women is estimated about 44%, and 42% to 53% pre-school African children suffer from anemia (WHO, 2000, Domellof *et al.*, 2002, Iannoti *et al*.,2006,Zimmerman *et al*.,2004).Consequences of IDA are: physical growth retardation, perinatal mortality, compromised mental development, lowered physical activity and labor productivity, and increased maternal morbidity. It can also affect mental and motor development. IDA impairs thyroid metabolism and reduces the efficacy of iodine prophylaxis in areas of endemic goiter (WHO, 2000, Domellof *et al*.,2002,Iannoti *et al*.,2006, Zimmerman, *et a*l.,2004).IDA in infancy is associated with significant loss of cognitive abilities, decreased physical activity, and reduced resistance to diseases. Iron deficiency in women of childbearing age increases hazards associated with complications of pregnancy, premature birth and low birth weight, and leads to newborns with sub-optimal iron reserves and it is responsible for a large share of maternal deaths. In school-age children, IDA may affect school performance; in adulthood, it can cause fatigue and reduced work capacity (slinger land *et al*, 2006).

Zinc

Zinc is the fourth important micronutrient after vitamin A, iron and iodine. It is found in all organs, tissues and body fluids, especially the bones and skeletal muscles. Zinc plays vital role in cell division, protein synthesis and growth which makes infants, children, adolescents, and pregnant women at risk for an inadequate zinc intake. Zinc contributes to reproduction, growth, taste, night vision, appetite, and the immune system functioning. It also contributes to stabilize the structure of membranes and cellular components (Hambidge, 1987).The total body zinc content has been estimated to be 30 mmol (2 g). Skeletal muscle accounts for about 60% and bone mass for approximately 30% of the total body zinc content. Zinc is an essential component of a large number (>300) of enzymes involved in the synthesis and degradation of carbohydrates, lipids, proteins, and nucleic acids and in the metabolism of other micronutrients. Zinc has an essential role in polynucleotide transcription and thus in the process of genetic expression (Shankar and Prasad, 1998).The main causes of zinc deficiency are inadequate dietary zinc intake, inhibitors of zinc absorption, high zinc losses due to diarrhea or both.

Zinc deficiency arises to a large extent from impaired bioavailability of dietary zinc, largely attributable to the high phytic acid content of diets (Melaku *et al*, 2005; WHO, 2000). The deficiency of Zinc is associated with poor growth, depressed immune function, increased susceptibility to and severity of infection, adverse outcomes of pregnancy, and neurobehavioral abnormalities. In many developing countries, zinc deficiency is due to the low consumption of animal source foods, which are rich in zinc, and a high intake of cereals and legumes, which contain substantial amounts of phytate (Romana, *et al,* 2003).It is also related to delayed sexual and bone maturation, skin lesions, diarrhea, alopecia, impaired wound healing, impaired taste, depressed appetite, a high risk of spontaneous abortions, and the appearance of behavioral changes. In children, zinc deficiency leads to poor growth, impaired immunity, lower weight gain, and increased morbidity from common infectious diseases and increased mortality (WHO, 2000, Domellof *etal.*,2002, Ramakrishna and Rao,2005).Zinc is found in many foods, with animal products providing the most readily absorbed sources of the mineral. The best sources of highly bioavailable zinc include red meat, liver, poultry, fish, eggs, crabs, and oysters. Staple foods in developing countries including cereals and legumes are the main sources of zinc. The bioavailability of zinc in plant-based foods is reduced by the phytate, fiber, calcium, and lignin (a component of vegetable fiber) present in the plant matrix. Specific food processing techniques

such as soaking, germination, and/or fermentation also help to reduce the impact of zinc inhibitors (WHO, 2000, Sandberg, 2002, Gibson 1994).

It is possible to predict the relative bioavailability of zinc from the molar ratio of phytate to zinc in the diet and ratios >15 have been negatively associated with growth in children and optimal zinc status in adults. High level of calcium in the diet has also been shown to exacerbate the inhibitory effect of phytate on zinc absorption in humans by forming insoluble complexes with calcium and zinc in the intestine. Such complexes are even less soluble than complexes of phytate with zinc or with calcium alone (Melaku *et al.,*2005).

Calcium

Calcium is particularly important in coffee plants, being the 3rd absorbed element in *C. arabica* and the 2nd in *C. canephora*, corresponding up to 12 and 31 % of total macronutrients, respectively (Ramalho *et al.* 1995), This essential nutrient is required for normal plant growth and development being involved, namely in stabilization of cell walls and membranes and in facilitating root extension (Ramalho *et al.,* 1995).

Calcium is the most abundant mineral element in the body. it(small proportions) regulates critical functions including nerve impulses, muscle contractions and the activities of enzymes and (more than 99%) is located in the bones, plays an important role for structure and strength of bones. The body of an adult man contains about 1.2 kg calcium, accounting for about 2% of body weight. The element is present in two body parts: bone and teeth. Sufficient calcium intake is essential for obtaining optimal peak bone mass in youth and for minimizing bone loss later in life (Gurr, 1999).

Up to about a third of calcium from food is absorbed, the remainder being excreted in the faces. The proportion of calcium absorbed from food depends on how the calcium is chemically bound in the food and the presence of many substances also present in the food, which may either enhance or inhibit absorption. The efficiency of calcium absorption decreases as the amount ingested increases (Gurr, 1999). Calcium deficiency appears to occur after birth, since the ratio of calcium to nitrogen decreases. When infants are weaned and start to become mobile and their bones thus become weight bearing, rapid calcification of the bones takes place. Preterm babies are particularly at risk of not being able to absorb enough calcium for optimal bone growth.

Mineral deficiencies, such as calcium, are a world-wide problem particularly in developing countries (Boukari *et al*., 2001, Gurr, 1999).

As a conclusion an adequate availability of several minerals and their dynamics (particularly the simultaneous enhancement of N, Cu, Zn, and Mn, and the highest Fe content) would contribute to the acclimation process in Coffea spp. constituting a useful tool to assess cold tolerance. For human uses, the key sources of micro and macronutrients are plants and vegetables.

2.2.6. Phytochemicals of coffee plant

Ian Johnson and Gary Williamson (2003) have defined that Phytochemicals are biologically-active, non-nutritive secondary metabolites which provide plants with color, flavor and natural toxicity to pests. The classification of this huge range of compounds is still a matter of debate, but they fall into three main groups:

- phenolic compounds (including flavonoids and phytoestrogens);
- glucosinolates;
- carotenoids.

Many thousands of phenolic compounds have been identified. They include monophenols, the hydroxycinnamic acid group which contains caffeic and ferulic acid, flavonoids and their glycosides, phytoestrogens and tannins. Flavonoids are widely distributed in plants where they have a role in plant color, taste and smell. Some have antioxidant properties whilst others are phytoestrogens.

2.2.6.1. Total polyphenols

During coffee tree development the vegetative and reproductive phases occur at the same time and there is competition between both for photo assimilates. This is the main reason for the coffee production alternation, due to fruit development only on branches grown in previous years. The principles for an ecological management are: (first) every organism has natural enemies, (second) every plant tolerates a certain level of infestation or infection, (thirdly) control management has to be selective and (fourth) every crop can reach an equilibrium condition (National Research Council, 1996).

Based on the second principle it can be concluded that for plants such as coffee trees that show defense mechanisms, growers should interfere in the natural crop defense system only when faced with the possibility of economic losses. Generally, plants show defense mechanisms that react differently to biotic and abiotic stress agents. The nature and intensity of the reaction vary according to the age, adaptation level and the seasonal activity of the plant .The biochemical compounds involved in the defense mechanisms are produced by a secondary plant metabolism, which does not affect directly plant growth and development, but has an essential role in plant metabolism (Strack, 1997).Some of the most likely occurring secondary metabolic products in coffee plant are:

2.2.6.2. Caffeine

Caffeine, an alkaloid of the methylxanthine family, is a naturally occurring substance found in the leaves, seeds or fruits of over 63 plants species worldwide. The most commonly known sources of caffeine are coffee, cocoa beans, cola nuts and tea leaves. In its pure state, it is an intensely bitter white powder. In tea and coffee plants, it is predominantly produced in the young buds of leaves and in immature fruits. Caffeine is the major low-molecular-weight nitrogenous compound in coffee plants, and at times, it functions as a chemical defense for new bud leaves (Marcelo *et al*., 2013). Its chemical formula is $C_8H_{10}N4O_2$, its systematic name is 1, 3, 5-trimethylxanthine . Its structural formula is as shown below.

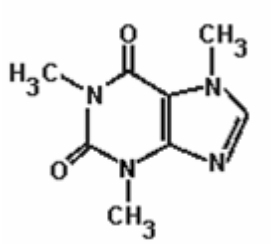

Figure 1: Structure of caffeine
(Camp *et al.,* 2012)

Caffeine is a pharmacologically active substance and depending on the dose, can be a mild central nervous system stimulant. Caffeine does not accumulate in the body over the course of time and is normally excreted within several hours of consumption. The capacity for caffeine biosynthesis, especially from guanine and xanthosine, was reduced markedly in both fully

developed mature and aged leaves. The levels of endogenous (produced inside the leaves) caffeine and theobromine are much higher in buds and young leaves of Coffea *arabica* L. than in fully developed leaves (Ashihara *et al*., 1996).

Uses of caffeine

Caffeine which is found in tea and coffee imparts bitterness and also acts as a flavor constituent (Leo, 1992). It is a mild nervous stimulant towards drowsiness and fatigue. In this respect, is used by athletes to enhance performance since it mobilizes fats from stores a process that normally does not become maximal until intense activity is underway (Eva, 1988).Caffeine is used as a drug on the basis of its effect on respiratory, cardiovascular and the central nervous system. It is included with aspirin in some preparations for treatment of headaches as it decreases cerebral eye blood flow. It is included with ergotamine in some antimigrane preparations, the object being to produce a mildly agreeable sense of alertness (Lawrence, 1986).

2.2.6.3. Tannins

The word tannin is very old and reflects a traditional technology. Tanning was the word used in the scientific literature to describe the process of transforming raw animal hides or skins into durable, non-putrescible leathers by using plant extracts from different plant parts (Aletor, 2005).They are phenolic compounds of high molecular weight. Tannins are soluble in water and alcohol and are found in the root, bark, stem and outer layers of plant tissue. Tannins have a characteristic feature to tan, i.e. to convert things into leather. They are acidic in reaction and the acidic reaction is attributed to the presence of phenolics or carboxylic group. Tannin is an astringent, bitter plant polyphenolic compound that either binds or precipitates proteins and various other organic compounds including amino acids and alkaloids (Redden *et al.*, 2005).The term tannin refers to the use of tannins in tanning animal hides into leather; however, the term is widely applied to any large polyphenolic compound containing sufficient hydroxyls and other suitable groups to form strong complexes with proteins and other macromolecules.

Tannins have molecular weights ranging from 500 to over 3000(Muzquiz *et al.*, 2000).Tannins are heat stable and they decreased protein digestibility in animals and humans, probably by either

making protein partially unavailable or inhibiting digestive enzymes and increasing fecal nitrogen. Tannins are known to be present in food products and to inhibit the activities of trypsin, chemotrypsin, amylase and lipase, decrease the protein quality of foods and interfere with dietary iron absorption (Felix and Mello,2000).Tannins are known to be responsible for decreased feed intake, growth rate, feed efficiency and protein digestibility in experimental animals. If tannin concentration in the diet becomes too high, microbial enzyme activities including cellulose and intestinal digestion may be depressed .Tannins also forms insoluble complexes with proteins and the tannin-protein complexes may be responsible for the anti-nutritional effects of tannin containing foods (Panhwar, 2005).

2.2.6.4. Phytate

Phytate (is also known as Inositol hexakisphosphate (InsP6)) is the salt form of phytic acid, are found in plants, animals and soil. It is primarily present as a salt of the mono- and divalent cations K+, Mg2+, and Ca2+ and accumulates in the seeds during the ripening period. Phytate is regarded as the primary storage form of both phosphate and inositol in plant seeds and grains. In addition, phytate has been suggested to serve as a store of cations, of high energy phosphoryl groups, and, by chelating free iron, as a potent natural anti-oxidant (Mueller I, 2001).Phytate is ubiquitous among plant seeds and grains, comprising 0.5 to 5 percent (w/w) (Loewus, 2002).

The phosphorus bound to phytate is not typically bio-available to any animal that is non-ruminant. Ruminant animals, such as cows and sheep, chew, swallow and then regurgitate their food. This regurgitated food is known as cud and is chewed a second time. Due to an enzyme located in their first stomach chamber, the rumen, these animals are able to separate, and process the phosphorus in phytates. Humans and other non-ruminant animals are unable to do so (Harold, 2004). Phytate works in a broad pH-region as a highly negatively charged ion, and therefore its presence in the diet has a negative impact on the bioavailability of divalent, and trivalent mineral ions such as $Zn^{2+,}$ $Fe^{2+/3+,}$ Ca^{2}+, Mg^{2}+,Mn^{2}+, and Cu^{2}+. Whether or not high levels of consumption of phytate-containing foods will result in mineral deficiency will depend on what else is being consumed.

In areas of the world where cereal proteins are a major and predominant dietary factor, the associated phytate intake is a cause for concern (Mueller I., 2001).

2.2.6.5. Antioxidants

Antioxidants are substances that have the ability to delay the oxidation of a substrate by inhibiting the initiation or propagation of oxidizing chain reactions caused by free radicals. They play important roles to prevent fats and oils from becoming rancid (cause attack) and protects human body from detrimental effects of free radicals. Antioxidants protect cells against the damaging effects of reactive oxygen species otherwise called, free radicals such as singlet oxygen, super oxide, peroxyl radicals, hydroxyl radicals and peroxynite which results in oxidative stress leading to cellular damage (Mattson , 2008). Natural antioxidants play a key role in health maintenance and prevention of the chronic and degenerative diseases, such as atherosclerosis, cardiac and cerebral ischema, carcinogenesis, neurodegenerative disorders, diabetic pregnancy, rheumatic disorder, DNA damage and ageing (Uddin *et al*., 2008).

Antioxidants exert their activity by scavenging the 'free-oxygen radicals' thereby giving rise to a fairly 'stable radical'. The free radicals are metastable chemical species, which tend to trap electrons from the molecules in the immediate surroundings. These radicals if not scavenged effectively in time, they may damage crucial bio molecules like lipids, proteins including those present in all membranes, mitochondria and, the DNA resulting in abnormalities leading to disease conditions (Uddin *et al*. 2008). Thus, free radicals are involved in a number of diseases including: tumour inflammation, hemorrhagic shock, atherosclerosis, diabetes, infertility, gastrointestinal ulcerogenesis, asthma, rheumatoid arthritis, cardiovascular disorders, cystic fibrosis, neurodegenerative diseases (e.g. Parkinsonism, Alzheimer's diseases), AIDS and even early senescence (Chen *et al*., 2006; Uddin *et al*., 2008).

The human body produces insufficient amount of antioxidants which are essential for preventing oxidative stress. Free radicals generated in the body can be removed by the body's own natural antioxidant defenses such as glutathione or catalases (Sen, 1995)**.** Therefore this deficiency had to be compensated by making use of natural exogenous antioxidants, such as vitamin C, vitamin E, flavones, β-carotene and natural products in plants (Madsen & Bertelsen, 1995; Rice- Evans, *et al*., 1997; Diplockn, *et al*., 1998).

Plants contain a wide variety of free radicals scavenging molecules including phenols, flavonoids, vitamins, terpenoids that are rich in antioxidant activity (Madsen & Bertelsen, 1995; Cai & Sun, 2003). Many plants, citrus fruits and leafy vegetables are the source of ascorbic acid, vitamin E, caratenoids, flavanols and phenolics which possess the ability to scavenge the free radicals in human body. Arabica coffee leaves were found to contain the highest levels of mangiferin, which has been found to have anti-inflammatory effects while also reduce the risk of diabetes, blood cholesterol, and protecting neurons in the brain (Claudine *et al.*,2008).

2.3. Factors that affect phenolic compound synthesis in coffee leaves.

The coffee plant is adapted to the understory of tropical rainforests in its native habitat. Caffeine and other phenolic compound concentrations vary in plant tissues and throughout the plant's organs. The caffeine content of seeds derived from different Coffea species varies within the range of 0.4% to 2.4% (Ashihara *et al.*, 2008). In C. arabica seedlings, caffeine resides predominantly in the leaves and cotyledons at concentrations varying from 0.8% to 1.9% dry weight (Zheng and shihara, 2004).

Currently, few environmental/physiological features have been shown to affect caffeine biosynthesis in the leaves or coffee seeds. Particularly, nitrogen (N) availability (Gonthier, 2011) and tissue age (Ashihara *et al.*, 2008) and references therein) have little impact on caffeine production. Nevertheless, little is known about the influence of a biotic factor on the biosynthesis of caffeine and its degradation in leaves. Studies suggests that, in coffee, caffeine is strongly regulated by genetic factors, and the environment is likely less important to the caffeine phenotype (Gonthier, 2011).

Recent studies have shown that shaded or dark growth conditions generally increase the caffeine levels in tea leaves (Ashihara *et al*., 2008) and references therein)or coffee cell cultures (Schulthess,1995). In contrast, light strongly stimulates the methylation steps of caffeine biosynthesis in the pericarp of coffee (Coffea arabica) (Frischknecht, 1985). Independent of this effect, light provides the energy to maintain elevated growth rates through photosynthesis and appears to provide a fixed carbon source for the increased production of primary metabolites, such as D-ribose 5-phosphate, which serve as substrates for caffeine biosynthesis via their conversion to purine nucleotides. Coffee plants growing in full sunlight exhibit an increase in their absolute and relative growth rates and process a higher leaf fraction (Cavatte, 2012) i.e., a

high carbon and nitrogen investment to build those leaves. On this favorable condition, high caffeine content helps protect the plant against herbivores (Kim and Sano, 2008).

According to the carbon/nutrient balance (CNB) hypothesis (Bryant, *et al*, 1983) the excess carbohydrates, especially starch, that accumulate in nutrient-rich plants (Pompelli, 2012) supply the energy and carbon skeletons necessary for secondary metabolism. Because alkaloids are nitrogen-containing compounds, lower caffeine content might be expected in nutrient-deficient plants, even for those with high carbon availability. Therefore, the production of nitrogen-based secondary metabolites (e.g., alkaloids) should increase as nitrogen (N) is acquired to supply primary metabolism and growth requirements. Additional N should then stimulate downstream metabolite biosynthesis (Hamilton *et al.*, 2001). Although there are a small number of exceptions, several reports have shown an increase in alkaloid content due to N fertilization (Gonthier *et al.*, 2011).

2.4. Coffee leaf brew

Coffee leaf brew has a long history in different countries. Davies (2006), a coffee expert and botanist at Kew Gardens, said coffee leaf tea was popular among some locals in places like Ethiopia and South Sudan and there had even been an attempt to market it in Britain in the 1800s. He said: "In 1851 people were touting it as the next tea and there were all these reports at the time about its qualities "What was surprising was how many antioxidants are in the coffee leaves. They are much higher than those in green tea and normal black tea". Davies found samples of coffee leaf tea in the Kew collections that date back nearly 100 years.

Figure .2.Samples of coffee-leaf tea in Kew's botanic garden.

(Campa *et al* ., 2012).

In Ethiopia coffee leaf brew is not yet widely available, but has long history. At the beginning people started preparing the brew because of different reasons. When the production of coffee bean is reduced because of coffee related diseases, high drought, high rain fall, etc, people were obligated to prefer coffee leaf brew to meet their coffee(stimulant needs).Those economically lower groups also prefer to prepare coffee leaf brew to satisfy their coffee thrust. Some communities believe that coffee leaf brew steams hunger and thrust of their workers in a better way than normal coffee or tea. In different rural areas of the country mothers provide coffee leaf brew for lactating women believing that it generates much milk for the baby. Those who have kidney, gastric and sinus related health problems and normal coffee sensitive people also prefer coffee leaf brew than the normal coffee or tea.

2.4.1. Health benefits of a brew from the infusions of coffee plant leaves

Even though it is not a widely imbibed substance, researchers states that a tea made from coffee leaves packs even more antioxidants and healthful compounds than either regular tea or coffee. (Johnston *et al*., 2003) However, it has been recorded that "unlike tea, no nervous disorders arise from its inordinate use. In some regions, such as Sumatra and Ethiopia, only the leaves are taken from the coffee plant and the berries left on the bush.

The natives of these places and other experimental drinkers find that the concoction (mixture) stems hunger and tends to energize both the body and mind while actually having less caffeine than the roasted bean brew. Research also demonstrates high antioxidant potential for chlorogenic acids (Johnston *et al*., 2003; Stalmach *et al*., 2006; Parras *et al*., 2007), which can prevent oxidative damage, cerebrovascular diseases, brain performance and mental health, ageing and cancer. The potential health benefits of coffee-leaf tea, and beverages and masticatory products made from the fleshy parts of Coffea fruits and leaves are supported by phenolic quantification from these parts (Claudine Campa *et al.,* 2012).

The phenolic composition of coffee (*Coffea*) leaves which has a vital role in life sustainability has been studied in detail (Claudine Campa *et al*.,2012) and in certain study a Concentrations of hydroxycinnamic acid esters (HCEs) and mangiferin were measured for 23 species native to Africa or Madagascar. HCEs are commonly grouped under the generic name of chlorogenic

acids; mangiferin, a *C*-glucosylxanthone, which was first, isolated from mangoes (*Mangifera indica*), was found in high concentrations in seven of these species.

Coffee leaf brew contains high levels of compounds credited with lowering the risk of heart disease and diabetes. The potential health benefits of coffee-leaf tea (a 'tea' made from coffee leaves), and beverages and masticatory (medicine to be chewed) products made from the fleshy parts of coffee fruits, are supported by the phenolic quantification. Chlorogenic acids are powerful antioxidants and mangiferin has numerous pharmacological properties (Annals Bot., 2012).

Research also demonstrates that high antioxidant potential for chlorogenic acids which can prevent oxidative damage, cerebrovascular diseases, brain performance and mental health, ageing and cancer (Zang *et al.,* 2003) and have potential for chemotherapy (Belkaid *etal*., 2006).

2.4.2. Sensory attributes of coffee leaf brew

Sensory evaluation is a scientific method used to evoke measure, analyze and interpret responses to products as perceived through the senses of sight, smell, touch, taste, and hearing. It is an irreplaceable tool in food industry while interacting with the key sectors in food production. When consumers buy a food product, they can buy nutrition, convenience and image. In sensory evaluation, judges are asked to score the products for appearance, color, flavor, taste and overall acceptability using a scorecard of Hedonic Rating Scale (Ahmad *et al.*, 2011). This test relies on people's ability to communicate their feelings of like or dislike.

Hedonic testing is popular because it may be used with untrained people as well as with experienced panel members. A minimum amount of verbal ability is necessary for reliable results. Coffee leaf brew users states that it had a very fresh flavor, a bit like cut grass that is similar to what you would expect from a green tea. There is not any hint of coffee in there and most people would struggle to identify it from other leaves.

3. Materials and Methods

3.1. Description of the study sites

Sidama

Sidama Zone is northeast of Lake Abaya and southeast of Lake Hwassa. The zone is bordered by the Arsi Oromo in the north and west, Gedeo, Burji, Guji Oromo people groups in the south, Guji Oromo in the west, and Wolayta and Kambata groups to the east. Sidama has geographic coordinates of latitude, Nnorth: 5′ 45″ and 6′ 45″ and longitude, East, 38′ and 39′. It has a total area of 10,000 km^2, of which 97.71% is land and 2.29% is covered by water. Hawassa Lake and Logita falls are water bodies that attract tourists.

Sidama has a variety of climatic conditions. Warm conditions cover 54% of the area. The mean annual rainfall of the area varies between 1200 mm and 1599 mm, with 15 °C to 19.9 °C average annual temperature. A hot climatic zone, Kolla, covers 30% of the total area. Its elevation ranges from 500 m to 1500 m above sea level. It has a mean annual rainfall of 400 mm to 799 mm, and the mean annual temperature ranges from 20 °C to 24.9 °C. Cool climatic conditions known as Aliicho or Dega exist in the mountainous highlands. This covers 16% of the total area with an elevation between 2500 m and 3500 m above sea level. This part gets the highest amount of rainfall, ranging from 1600 mm to 1999 mm. It has a mean annual temperature of 15 °C to 19.9 °C.

Kembata Tembaro

Kembata Tembaro zone is a zone in the Ethiopian Southern Nations, Nationalities and Peoples Region (SNNPR). It was formerly known as Kembata, Alaba and Tembaro, until Alaba became a special worede in 2002.This zone is named after sub-groups of Kambaata people. The zone is bordered on the south by Wolayita, on the southwest by Dawro, on the northwest by Hadiya, on the north by Gurage, on the east by the Alaba special worede and on the southeast by an exclave of the Hadiya Zone. The administrative center is Durame. Other local landmarks include the three mountains of Ambaricho, Kataa and Datoo, and the hot springs at Motokoma. The longest river in the area is the Lagabora which means in Kambatinga the "river of Bora". The Central Statistical Agency (CSA) reported that 8,364 tons of coffee was produced in

Gurage, Hadiya and Kembata Tembaro in the year ending in 2005, based on inspection records from the Ethiopian Coffee and Tea authority. This represents 8.33% of the SNNPR's output and 3.36% of Ethiopia's total output.

Hararge

Geographically, Harari Regional State is located in the eastern part of Ethiopia. The total geographical area of the region is about 343.21 km^2. It is geographically located between 42.03 – 42.16 north of latitude and 9.110-9.240 last of longitude. The region shares common boundaries with Easter zone of oromia Woredas Jarsso Woreda in the north and Babile Woreda in the east; Fedis wareda in the south and Haromaya wareda in the west. The city of Harar is the capital of Harari People's Regional State; which is located in east at a distance of 510 Km from Addis Ababa. The elevation above sea level of the city varies from 1600 to 1900 meter above sea level. Its urban morphology represents two main parts, the old city (Jugal) and the modern one.

In Harari Region temperature various from 10^{0c} in highland and 26^{0c} in low lands and it has little variation among seasons. The duration and intensity of rainfall in the region various and decreases from West to North between 700mm and 900mm respectively. And regarding to reliability of rainfall for crop production and duration of growth periods; the pattern of rainfall in the region aerated in three seasons that is kirimt, bega and bulge. In the region about 46.7 % of land is cultivable and the rest is not suitable for agriculture. Among cultivable land around 42.3% were cultivated land.

3.2. Collection of coffee leaf samples

One(1) Kg of fresh mature leaf samples of the coffee Arabica were collected from selected areas of Shambadino and yirgalem woredas in Sidama zone, Hadero-Tunto zuriya and kechebira woredas in Kambate -Tembaro zone and West and Eastv Hararge sites in Harar region. The samples were collected between first March to twenty fifth March2015.

3.3. Reagents and chemicals

K_2SO_4,$CuSO_4$,$NaSO_4$,H_3BO_3,Na_2SO_4,NaOH,H_2O_2,Ethanol, Methanol, Acetonitrile, Dichloro methane, petroleum ether, Chloroform,1%HCl,vanillin- HCl reagent,D-catechin,8%HCl,4%

vanillin and DPPH, caffeine, BHT, ascorbic acid and double distilled water were used as solvents, reagents and standards (some chemicals with HPLC grade).

3.4. Sample preparation

After collection, the samples were washed with distilled water and chopped into small pieces (2 to3 mm in size). All the six types of chopped samples were placed under shade for two to three days until a constant weight was attained. The dried samples were ground to powder form to pass through 250μm(1mm) sieve to get a uniform texture and were stored in sealed polyethylene bags at ambient conditions until further analyses.

3.5 .Traditional *Kuti* preparation procedures in Kambata and Harar communities

3.5.1. Target group discussion

As mentioned earlier *kuti* (coffee leaf brew) is a traditional drink which has been consumed in many places of Ethiopia since the early times. The communities living around Harar Regional States, the Kambata and Hadiya communities, Wolyta societies and people around Jima area took the lion's share in consuming coffee leaf brew in Ethiopia. Although the brew is being consumed in these areas for many years, there is no common practice of preparing this brew. Therefore, the main intension of this section was to study the *kuti* preparation procedures in these selected sites and to develop common understanding in its preparation techniques. An interview containing a total of ten (10) questions was developed by the researcher and presented for selected respondents in these three sites.

3.5.2. Coffee Leaf collection and preparation

The first task in preparing a beverage from a coffee leaf is collecting the appropriate leaves from the coffee plant in the coffee farm.

Around kambata communities as it was stated by all respondents the most important part of coffee leaf which is appropriate for *kuti* preparation was the matured (aged) part of the coffee leaf which is yellowish green in color. These matured coffee tree leaves were collected from the

coffee farm using two options: the first option is collecting the leaves from the soil near the coffee tree. This is because since the leaves are matured enough they fall down near the coffee tree by themselves, without any external force. The second option is to collect these matured leaves from the bottom part of coffee tree by picking up directly by hand. The weak side of this option is that it harms the coffee tree and results in less production of coffee beans from that three. After collection the leaves were brushed by bare hand or by other soft material to remove some dirty particles and other impurities and then washed with clean water to ensure its cleanness.

3.5.3. Leaf grounding

After all unnecessary parts were removed and the leaves were cleaned, the next step was size reduction. The leaves were subjected to size reduction using mortar and pestle. The size of the grounds ranges from a fine grounds (small pieces) to a larger cuts. As the consumers states the size of the final ground has significant effect on some attributes of the final product such as color, taste, texture appearance and mouth fell. If the leaves were well ground to smaller sizes, they could be able to mix with boiling water and release the chemical constituents in the leaves easily. This in turn results in, good appearance, the most acceptable or red color which was most preferable by the consumers, appropriate product thickness and mouth fell of the final product. In contrast if the leaves were not well ground and simply left in small cuts, the quality of the final product will be affected significantly. For instance, as the consumer's states in this site, if the coffee leaves for kuti preparation were not well ground, they could not be able to mix with the boiling water easily and this results in un acceptable properties of the final product. For example the final product will be very thin or watery, the color will not be red instead it will develop un-pleasant color and grassy taste.

3.5.4. Drying

The next task after size reduction was drying the grounds of the coffee leaves. The main aim of this step was to improve the quality of the final product as well as the shelf life of the coffee leaf grounds. Some users state that sometimes they boil the coffee leaf grounds immediately after grounding, without drying .But they face a problem with quality of the final products. The quality parameters such as color, taste, appearance and odor of the product is highly affected if it was prepared skipping the drying step. Basically drying can be performed by two methods, sun

and heat (griddle) drying. The first method is most preferable and used by most people because it is the natural method and cheaper than the griddle method. In griddle method the grounds of coffee leaves were roasted on a flat griddle using artificial heat source. This method was preferable when the users want to prepare the brew in a short period of time. Besides of this when the season is rainy and if it is not suitable for sun dry, the users prefer this method also. But this method has its own side effects. It uses artificial energy sources and hence it is cost. The other problem of this method is product quality.

3.5.5. Boiling or making the infusions

As stated by consumers (local communities), coffee leaf brew boiling process was more or less similar to normal tea boiling process. Appropriate amount of clean water is boiled in metal spot for about 15 to 20 minutes. Then enough amount of the dry coffee leaf powder was added to the boiling water and it was waiting for 5-10 minutes until the color of the extract become red enough.

3.5.6. Filtration of the infusion

Filtration was to separate the extracts from the residues. After boiling step was completed the infusion was subjected to filtration using different traditional equipments. Traditional sieve and any other locally available traditional materials were used to separate the hot water extracts from the residue.

3.5.7. Boiling the filtrates with different ingredients

This is the step where different ingredients were blended with filtrates of coffee leaf extracts. Here different ingredients such as garlic, ginger, onion, chills, etc which were mainly used to improve the quality parameters of test, color, odor, smell, etc of the final product were prepared. Then these ingredients were mixed together and put in to the bottom of coffee boiling pot and the filtrates were also poured in to the boiling pot and heated for a few minutes until the ingredients were well mixed with the filtrates.

3.5.8. Serving the brew

The brew was served with different sweeteners such as garlic, ginger, and chills. But this was not common in all places. It has been indicated that, in Hararge communities garlic was never added in this product. In contray to this, in Kambata communities *kuti* is not preferable without garlic.

Sweeteners such as salt, sugar, milk and butter were also be added in a final product which was ready to be served.

3.6. Chemical analysis

3.6.1. Proximate Composition

The proximate composition (ash, moisture, lipid, fiber and protein) of fresh leaf samples was determined using AOAC protocol (2000) methods.

3.6.1.1. Moisture analysis

For each sample analysis, two crucibles were cleaned and dried in an oven at 105 ^{oC}. The dried crucibles were taken out of the oven and allowed to cool desiccators. The weight of the crucibles was measured using an analytical balance (**W_1**) and recorded in a data collection form. Five (5) g of coffee leaf powder samples were measured in each dry crucible (**W_2**) and placed in an oven to dry at 105 ^{oC} for 3 hours. The crucibles were taken out of the oven after 3 hours and cooled in desiccators. The weight of the crucibles and the sample was measured after cooling. Then, the crucibles containing the sample were returned again in to the oven to dry and their weight was measured until it became constant. Finally, the last constant measurement (**W_3**) was taken and the moisture content of the samples was determined using the following formula.

$$\%\text{Moisture} = \frac{W_2 - W_3}{W_2 - W_1} \times 100$$

Where W_1 = weight of crucible, W_2 = weight of crucible and sample before drying, W_3 =weight of crucible and sample after drying.

3.6.1.2. Ash analysis

Total ash content of the coffee leaf samples were determined according to AOAC (2000), using the official method 941.12.Three porcelain crucibles were cleaned and dried in a muffle furnace for 30 minute at 550 ^{o}C. The crucibles were let to cool in desiccators for 30 minutes to room temperature and their weight were measured (**M_1**). 2.5 gm coffee leaf powder sample were measured in each crucible. The samples were charred on a hot plate under a fume hood until the smoke ceased. The samples were ashed in a muffle furnace at 550^{o}C for 5 hours. Crucibles were cooled in a desiccators and their weight were measured (**M_3**). Finally the ash content was determined by using the following equation.

$$\%\text{Ash content for samples} = \left[\frac{W3-W1}{W2-W1}\right] \times 100$$

Where: M_2-M_1=sample mass in gram on dry base and (M_3-M_1) =mass of ash in gram

3.6.1.3. Protein analysis

Protein content was determined according to AOAC (2000) using the official method 979.09 for each sample half (0.5) g of coffee leaf powder was measured in three Tecator tubes and they were placed in the Tecator rack (W). Three blanks were used in order to avoid overestimations of the results due to nitrogen from reagents. Six (6 ml) of concentrated sulfuric acid was added in to the tubes containing the sample using a pipette and then, mixed carefully. Three and half (3.5) ml of hydrogen peroxide was added step by step in to each sample tube. The tubes were shaken a few times after the violent reaction has ceased and put back in to the rack. Three (3) g of copper sulfate and potassium sulfate catalytic mixture was 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 after the working temperature (370 $^{o}_{C}$) has reached and the digestion process has continued until clear solution was observed. The sample tubes were taken out, placed in the rack and allowed to cool in fume hood. Fifty (50) ml of distilled water was added into the sample tubes in order to avoid precipitation of sulphate.

$$\text{Protein (sample)} \xrightarrow[\text{Heat, catalyst}]{\text{Sulfuric acid}} (NH_4)_2\,SO_4$$

25 ml of 40% sodium hydroxide solution was added in to the digested and diluted solution. 250 ml conical flask containing 25 ml of boric acid, 25 ml of distilled water and an indicator solution was placed under the condenser of the distiller with its tip immersed into the solution. The distillation step is continued until the volume become between 200 ml and 250 ml. The tip of the distiller was rinsed with a few milliliters of water before the receiver was removed.

$$(NH4)_2\,SO_4 + 2NaOH \rightarrow 2NH_3 + Na_2SO_4 + 2H_2O$$

$$NH_3 + H_3BO_3 \text{ (boric acid)} \rightarrow NH_4 + H_2BO_3\text{- (borate ion)}$$

The solution containing an indicator, ammonium ion and borate ion titrated using 0.1 N HCl till the color of the solution changes to reddish and the total volume of the HCl required to reach the

endpoint of the titration was recorded. The volume of the HCl consumed during titration was 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 was adjusted by subtracting the moisture content from initial weight of the samples.

$$H_2BO_3^- + H^+ \rightarrow H_3BO_3$$

Lastly, the amount of protein in the coffee leaf sample was calculated by using the following equations.

$$\%\text{Nitrogen} = \frac{\text{V HCl} \times \text{N HCl} \times 14.0 \times 100}{1000 \times \text{Wo}}$$

$$\%\textbf{Protein} = 6.25 \times \%\textbf{Nitrogen}$$

Where**;** V-volume of HCl consumed (ml) to the endpoint of titration, N-the normality of the HCl used, W_o-Sample weight on dry matter basis, 14-the molecular weight of atomic nitrogen, 6.25-conversion factor

3.6.1.4. Fat analysis

Crude fat content of the raw capsicum flour samples were determined according to AOAC (2000), using the official method 4.5.01. Extraction cylinders were washed to remove impurities and put in an oven for about an hour at a temperature of 105^{o}C.They were taken out of the oven and cooled in desiccators. The weight of the cylinders was measured and recorded ($\mathbf{W_1}$) and they were return in to the desiccators. The bottom of the extraction thimbles were covered with a layer of fat free cotton. 2 gm of the powder of the sample were measured in the thimbles and was covered with a layer of fat free cotton (**W**).The thimbles were put in the extraction chamber. Extraction cylinders were taken out of the desiccators and put on the bracket. Fifty (50) ml of ether was added into the extraction cylinders and moved into the heating plank. The extraction was let to go on for about 4 hours. The extraction cylinders were disconnected and were put in a drying oven at 70^{o}C for about 30 minutes. The cylinders were taken out of the oven and cooled in desiccators for 30 minutes. The weight of the cylinders was measured immediately after they were taken out of the desiccators ($\mathbf{W_2}$).The percentage of fat in the sample was calculated using the following formula.

$$\%\text{Fat} = \frac{W3 - W2}{W1} \quad 100$$

Where;

W_1 = Weight of the sample
W2 = Weight of the extraction cylinder
W3 = Weight of the extraction cylinder plus the dried crude fat

3.6.1.5. Determination of crude fiber:

Crude fiber content of the raw capsicum flour samples were determined according to AOAC (2000), using the official method 920.169. For each sample two crucibles were cleaned and dried with 0.001 g celite in an oven at 105^{oC} for 1 hour. The crucibles were taken out and cooled in desiccators. About 1 gm of coffee leaf samples was weighed in to pre-dried crucibles (**W_1**). The crucibles were placed in the digester using a holder. 1.25% H_2SO4 solution was added in to each column by pressing the button for R1. The time was set for 37 minutes and the temperature between 6 and 8. 3-5 drops of n-octanol antifoaming agent was dropped into each column to prevent loss of sample. The acid was drained by using a vacuum pump after 37 minutes. The samples were cooled for 5 minutes and washed with distilled water thrice.

1.25% NAOH solution was added in to each column by pressing the button for R_2. The time and temperature was set as previous, the base was drained by using a vacuum pump after the set time.

The samples were washed again with distilled water thrice. The crucibles containing residue was dried at 130oC for 2 hours. The crucibles were cooled in desiccators and weighed (**W_2**).

The residues were ashed in a muffle furnace at 550 for 3 hours. The crucibles were let to cool down to below 250oC before removing from the furnace. The crucibles were cooled in desiccators to room temperature and their weight was measured using analytical balance (**W_3**). Finally, the ash content in the samples was calculated using the following formula:

$$\%\textbf{Crude fiber} = \frac{\mathbf{W2 - W3}}{\mathbf{W1}} \quad \mathbf{100}$$

3.6.1.6. Mineral Analysis

The ash was dissolved by 5 ml of 6 M HCl at low temperature on hot plate for about 2 hrs. Then 7 ml of 3 M HCl was added and heated on hot plate until the solution boils. The digest was cooled and filtered through a filter paper (42 mm, whatmann) in to a fifty (50) ml volumetric flask. Then five (5)ml 3MHCL was added to the dish and heated to dissolve the residues in the dishes and then transferred to the volumetric flask. Then the filter paper was washed thoroughly and the washing was collected in the flask made to the mark. Afterwards the mineral concentration was determined by AAS. For calcium determination two and half (2.5) ml of 10% Lanthanum chloride solution was added to the flask. Then diluted to 50 ml mark with deionized water. The blank was prepared by taking the same amount of reagents through the steps all of the above without the sample. The instrument was set based on the instruction and the reagent blank solutions were measured first. Then the samples were run following the calibration values. The calibration curve was prepared for the required metal by plotting the absorption values against the metal concentration in ppm. The mineral contents of each sample were calculated using the following formulae:

$$\text{Metal content (mg/100gm)} = \frac{(a-b)xV}{10xW}$$

Where; W = weight in gm of the sample

a= concentration in ppm of sample solution.

V = volume in ml

b= concentration in ppm of blank solution

3.7. Phytochemical screening

3.7.1. Determination of total polyphenols content

Total Phenolic content of coffee samples was estimated by the method as described by (Martin et al., 1999) with some modification. Stock solutions of Gallic acid (0.05, 0.10, 0.15, 0.20 and 0.25 µg/ml) were prepared in methanol for preparation of standard solutions. Ground coffee leaf powder of 2.5 g was mixed 25 ml methanol in the flask and placed it in the mechanical shaker for 2 hr at 40 ^{0}C in order to get methanol extract. After sample preparation, 1 ml of appropriately

diluted samples and a standard solution of Gallic acid were added to a 25 ml volumetric flask containing 7 ml of distilled water. A blank reagent using distilled water was prepared. One milliliter of Folin-Ciocalteu phenol reagent was added to the mixture and shaken. After 5 min, 1 ml of a 7% sodium carbonate solution was added with mixing. The solution was then immediately diluted to a volume of 25 ml with double distilled water and mixed thoroughly. After incubation for 90 min at room temperature, the absorbance at 725 nm was measured. The total phenolic contents of the samples were expressed in milligrams per serving of Gallic acid equivalents (GAE).

In the prepared extracts total phenolic content was calculated as gallic acid equivalent (GAE) by the following equation: T=CxV/M. where, T is the total phenolic content in $mg \cdot g^{-1}$ of the extracts as GAE, C is the concentration of gallic acid established from the calibration curve in $mg \cdot ml^{-1}$, V is the volume of the extract solution in ml of coffee leaf samples and M is the weight of the ex-tract in g.

3.7.2. Determination of caffeine content

High performance liquid chromatographic (HPLC) method was employed for the analysis of caffeine content. For caffeine determination by HPLC method coffee leaf powder was extracted according to the international (ISO) method with some modifications.

An accurately weighed amount two (2g) of coffee leaf sample was placed in to tea bags. The tea bags were placed into the bottom of a clean 150 ml beaker. Thirty (30) ml of deionized water and 2.0 g of anhydrous sodium carbonate were added into the beaker. The contents were gently boiled for ten (10) minutes using a heat plate. The hot liquid was decanted into a 50 ml Erlenmeyer flask. Twenty (20) ml of deionized water was also added to the beaker and again the contents were brought to a boil. The combined extracts were transferred from the 50 ml Erlenmeyer flask to a 125 ml separatory funnel. Five (5) milliliters of dichloromethane was added to the separatory funnel. The contents of the separatory funnel were allowed to settle until two distinct mostly clear layers were observed .The dichloromethane layer was carefully drained into a 25 ml Erlenmeyer flask repeatedly. Half (0.5) g of anhydrous sodium sulfate was added to the combined dichloromethane extracts in the twenty five (25) ml Erlenmeyer flask and the

contents were swirled. The liquid was decanted from the flask into a 25 ml beaker. The beaker was placed on a hot plate and when the volume of material in the beaker was between 3 and 5 ml, petroleum ether was added by means of a Pasteur pipet. When the solution in the beaker begins to get cloudy the beaker was removed from the heat and allowed to cool. Finally crystals of caffeine were formed in the solution .The solvent containing the caffeine was evaporated to remove water from the solution .Crude caffeine (solid) was the final result and its weight was recorded.

Caffeine content in methanolic extracts was determined by reversed-phase high-performance liquid chromatography (HPLC) in the Quality Monitoring and Testing Laboratory of Ministry of Agriculture according to (Mazzafera *et al.*, 1994) with some modifications.All glass wares were thoroughly cleaned, rinsed with distilled water and dried before being used (Marcelo *et al*, 2013) and made to the mark with the mobile phase. The caffeine extracted at each round was pipette into volumetric flasks. The standards and the samples were run on the HPLC system. Ten microliter of the sample was injected into the HPLC system. The mobile phase was MeOH:H2O (6:4 v/v) at a flow rate of 1 mL min-1.The separation was achieved using a C-18 column (4.6 × 250 mm) with 5- µm packing (Zorbax, model Eclipse XDB-C18). The absorbance of the solutions was measured by diode array detector at of spectral of 273 nm against the corresponding reagent blank (Belay *et al*., 2008; Belay *et al*., 2009).A calibration curve of peak areas versus concentration of the standards was plotted. Finally the caffeine levels of the samples were calculated from the regression equation of the best line of fit of the standards. The results were reported as mean ± standard division of the two parallel measurements.

3.7.3. Estimation of antioxidant levels in coffee tree leaves

For antioxidant level analysis, 0.004%of DPPH was prepared by measuring 0.01 g of DPPH and dissolving in 250 ml of methanol in volumetric flask.0.3mg/ml of standard of ascorbic acid for comparison purpose was prepared by dissolving 0.3 mg of the standard in 1ml of methanol (3mg in 10 ml of methanol).

Antioxidant level analysis was conducted according to procedures developed by Kirby and Schmidt (2004). Four (4ml) of 0.004%solution of DPPH radical solution in methanol was mixed with 1ml of various concentrations (2-12mg/ml of the extracts in methanol with a vortex mixer.

The samples were incubated for 30 minutes in the dark at room temperature. Scavenging capacity was read spectrophotometrically by monitoring the decrease in absorbance at 517nm using a UV spectrophotometer.Inhibition of free radical $DPPH^{\cdot}$ in percent was calculated in following way.

$$\% \text{ DPPH } (1\%) = 100 \times (A_{\text{control}} - A_{\text{sample}})/A_{\text{Control}}$$

Where: A_{control} is the absorbance of control reaction.

A_{sample} is the absorbance of the test compound

Ascorbic acid was used as the control. Estimation of the scavenging was carried in triplicate and the results were reported as mean ± standard error of the three parallel measurements.

3.7.4. Estimation of tannin

Tannin analysis was conducted following the method developed by Burns (1971) as modified by Maxson and Roony (1972). UV-Vis spectrometer was used for tannin analysis.

For Reagents preparation 1% Hydrochloric acid was prepared by adding 1ml of concentrated HCl in 100mlvolumetric flask partially field with methanol and the volume was made up with methanol. Vanillin- HCl reagent was prepared by dissolving 4gm of vanillin in 100ml of methanol(w/v)(solution A) and 8ml of HCl was added in 100ml of methanol(v/v)(solution B).Finally equal volume of solution A and B were mixed.

For Sample extraction 1gm of the sample was weighed in a screw cap test tube.10ml of 1%HCl in methanol was added to the tube containing the sample. The tubes were putted in mechanical shaker for 24 hr at room temperature. The tubes were also centrifuged at 1000G for 5minutes.1ml of the supernatant was taken and mixed with 5ml of vanillin-HCl reagent in another test tube. Then after waiting for 20minute to complete the reaction, the absorbance was read at 500nm.

For Standard solution preparation 40mg D-catechin was weighed and dissolved in 100ml of 1%HCl in methanol (stock solution). 0.0.2, 0.4, 0.6, 0.8 and 1ml of the stock solution was taken in a test tube. The volume of each tube was adjusted to 1ml with 1%HCl in methanol. 5ml of

vanillin –HCl reagent was added in each tube. After waiting for 20minute to complete the reaction, the absorbance was read at 500nm.Using SPSS, the calibration curve (absorbance Vs concentration) was plotted and the slope and the intercept was estimated. Finally tannin concentration was calculated as:

$$\text{Tannin in mg/g} = \frac{[\text{As-Ab})-\text{Intercept}]*10}{\text{Slope x d x w}}$$

Where A_s=sample absorbance

A_b = blank absorbance

d =density of the solution (0.791g/ml)

w =weight of sample in gram

The measurements were in duplicate and the results were reported as mean ± standard division of the two parallel measurements.

3.7.5. Estimation of phytic acid

Phytic acid analysis was conducted according to the method developed by Adeniyi *et al*. (2009). For reagent preparation, 1.67 ml of HCl was added in to a flask containing 100 ml of deionized water to get a 0.2 N HCl solution. 0.03 gm of FeCl3.6H2O was added in a flask containing 100 ml of distilled water to get a 0.03% FeCl3.6H2O solution (solution B). O.3 gm of sulfosalcilic acid was dissolved in 100 ml of deionized water to get a 0.3% sulfosalcilic solution (solution A). Equal amount of solution A and solution B was mixed to get Wade reagent.

A series of five standard solutions (5ppm, 9ppm, 18ppm, 27 ppm and 36ppm) were prepared from 90 ppm stock solution. 0.2 N HCl solution was used to get the final volume of 50 ml. 3ml of each standard solution were pipetted in to 15 ml centrifuge tubes and 3 ml of 0.2 N HCl was used as a blank. 2 ml of the Wade reagent was added to each tube and the solution was mixed on a vortex mixer for 5 seconds. The mixture was centrifuged at 3000 rpm for 10 minutes. The supernatant was read at 500 nm by using water as a blank. The calibration curve was plotted (absorbance Vs concentration) and the slope and intercept was determined.

Half (0.1) gm of dried coffee leaf powder was weighed in three test tubes (W). Ten (10) ml of 0.2 N HCl was added in to each test tube. The mixture was centrifuged at 3000 rpm and stored at ambient temperature for 30 minutes to extract the analyte from sample. 3 ml of the supernatant sample solution was taken and 2 ml of wade solution was added. The mixture was homogenized and centrifuged at 3000 rpm. The absorbance was measured at 500 nm using UV-Vis spectrophotometer. The phytate was calculated from the difference between the absorbance of the blank (3 ml of 0.2 N HCl + 2 ml of Wade reagent) and that of assayed sample. The amount of phytic acid was calculated using phytic acid standard curve and the result was expressed as phytic acid in µg/g fresh weight. The following formula was used to calculate the amount of phytic acid (Adeniyi *et al*., 2009).

$$\text{Phytic acid in } \mu g/g = \frac{(A_b - A_s) - \text{intercept} * 10}{\text{Slope} \times W \times 3 \times 100}$$

Where Ab = absorbance of the blank, As = absorbance of the sample, W = Fresh sample weight, Slope = Slope of the calibration curve, Intercept = Intercept of the calibration curve.

3.8. Sensory attribute analysis

The Functional drink was subjected to sensory evaluation by trained taste panel using nine point hedonic scale system (9 = like extremely; 1 = dislike extremely) as described by Meilgaard *et al.* (2007). Sensory evaluation regarding attributes like color, flavor, sweetness, sourness and overall acceptability was performed. Hedonic response was judged in Sensory Evaluation Laboratory of Ethiopian public health institute. On the day of evaluation, panelists were seated in separate booths with white fluorescent light and drinks were presented in transparent glasses labeled with random codes. Panelists were provided water to neutralize their mouth between samples testing. Samples were presented to the judges randomly and were asked to rate their acceptance by giving score for selected parameters.

3.9. Method of Data Analysis

The experiment was produced in triplicate and evaluated under a completely randomized design (CRD).The collected data was subjected to analysis of variance (ANOVA). Significant differences between treatments was analyzed using the least square significance difference method (LSD) .significance difference was defined at $p \leq 0.05$.

4. Result and discussion

4.1. Proximate composition analysis

The results of proximate analysis of Moisture, Ash, Crude protein, crude fat, crude fiber and Carbohydrate were indicated in table 3

Table 3.Proximate compositions of matured leaves of coffee tree

proximate		Moisture%	Ash (%)	Crud protein%	Crud fat%	Fiber%	Carbohydrate
type of sample	S	5.93±0.50^{ab}	8.821±0.313^{a}	16.27±0.07^{b}	5.075±0.60^{a}	19.8±0.70c	63.93±1.30^{c}
	Y	6.90±0.11^{de}	11.21±0.23^{c}	14.43±0.12^{a}	5.91±1.02^{ab}	20.01±0.70^{ac}	61.47±1.11^{c}
	M	7±0.01^{de}	10.32±1.31^{b}	17.91±0.96^{d}	12.5±2.12^{d}	17.15±0.69^{b}	51.99±2.68^{a}
	E	5.5±0.15^{a}	12.41±0.31^{c}	18.10±0.41^{cd}	11.5±2.82^{cd}	19.5±2.12^{c}	53.89±1.44^{ab}
	K_h	7.78±0.31^{e}	9.33±1.20^{ab}	18.98 ±0.55^{d}	4.5±0.71^{a}	17.05±2.12^{b}	59.26±0.36^{bc}
	K_s	5.89±0.13^{ab}	10.3±0.45^{b}	16.76±0.42^{bc}	6.25±0.35^{ab}	18.12±1.41^{bc}	60.85±0.57^{c}

Results were expressed as Mean value ± standard deviation, n=2. Means in the same column with different letters are significantly different ($P < 0.05$)
S=samples from Shambadino area, Y=samples from Yirgalem Worede , M=West Hararge (Chiro Worede) samples ,E=East Hararge Sample and K=sample from Kambata area .

4.1.1. Moisture analysis

In present study the moisture content of coffee leaf samples from different sites ranged from (7.78±0.31 to 5.5±0.15) % with highest moisture content for Hadaro samples and with the lowest moisture content for samples from East Hararge. According to results shown in table 4.1,Samples from Shinshicho, Kambata area Yirgalem and West Hararge sites have no significant difference ($p>0.05$) for their moisture content. But sample from Kambata area (Hadero –Tunto zuriya Woreda was significantly different ($p<0.05$) from samples of other sites for its moisture content.

As it can be understood from results in Table (3), it is possible to remove the water content of Arabica coffee leaves to an acceptable level using sun energy because the results are in

agreement with or even lower than moisture content of different tea brands (13.858±0.78) %, as reported by Akande *et al*.,*(*2012) ,which can be stored at room temperature and resist fungal and mold growth. High moisture content aids microbial activities, oxidation – reduction processes and fungal growth.

The significant variations observed for the moisture content of coffee leaf samples from different sites might be due to sample storage and handling problems during transportation and analysis. Moisture content can also vary from one coffee leaf to the other, depending on the drying time and nature of the coffee involved (Kumar *et al*., 2005).

The weather conditions and the moisture level of the sampling sites also might be one of the reasons for the moisture variation of the study samples. As moisture can affect the quality of a food product in different aspects, it is therefore, important for food scientists to be able to reliably measure moisture contents of a certain food item.

4.1.2. Ash analysis

Ash is the inorganic residue remaining after the water and organic matter have been removed by heating in the presence of oxidizing agents, which provides a measure of the total amount of minerals within a food. Analytical techniques for providing information about the total mineral content are based on the fact that the minerals (the analyte) can be distinguished from all the other components (the matrix) within a food in some measurable way. The most widely used methods are based on the fact that minerals are not destroyed by heating and that they have a low volatility compared to other food components.

In present study the ash content was determined by dry ashing method and the ash contents of coffee leaf samples from different sites was in the range of (12.41±0.31-8.821±0.313)%.The trend of the ash composition in the study samples was according to the following order: E>Y>M>Ks>Kh>S. East Hararge samples exhibited the highest ash content (12.41±0.31)% followed by Yirgalem samples (11.21±0.23%) and samples from Shambadino area were found to contain the least ash content (8.821±0.313)%.

The range of the ash content of present samples was higher than the range of ash content as (3.90-4.42) % for coffee bean samples as reported by (Martin and Gonzalez, 1999 and

Risoo,2007). Studies also showed that the average ash content in different coffee bean samples is in the range of (4.00-4.9) % as reported by (Santose *et al*., 2001), which is also lower than the average ash content for coffee leaf samples. In this study the average ash content in all coffee leaves samples which is in range of (12.41±0.31-8.821±0.313)% was higher than the ash content in green tea samples (4.79±0.01) which was reported by (Akande *et al.,*2011).

Statistically there was a significant difference among the ash contents of the samples from different sites. For ash content samples from Yirgalem Woreda were significantly different from samples from other sites($p<0.05$).On the other hand samples from Shambadino Woreda, Kambata area and West Hararge area were found to have no significant different($p>0.05$) for their ash content. Sample from east Hararge area was significantly different from samples from other sites for ash content.

The significant difference between the ash content of coffee leaf samples from different sites might be due to the soil type and the mineral composition in that soil. It also might be the maturity level of the coffee leaves from different sites. As the ash content in the samples is an indication of the mineral content of a sample, the significant difference in the ash content in the study samples might also be due to the level of mineral compositions of samples. According to the results from present study it is possible to conclude that coffee leafs can be a good source of minerals and may be able to play a significant role in reducing certain macro as well as micro nutrients deficiencies.

4.1.3. Protein analysis

In this study the percentage protein content ranged from (14.43±0.12 to 18.98±0.55)%. For the study samples the percentage protein content was in the order of Kh>E>M> Ks>S>Y, with significant differences among them ($p < 0.05$). Coffee leaf samples from Kambata area exhibit the highest protein content as (18.98±0.55) % followed by coffee leaf samples from East Hararge area as (18.10±0.41) %. Cofee leaf samples from Yirgalem Woreda were found to contain the least amount of protein content as compared to samples from other sites.

In this study results obtained for nitrogen composition were in coherence for protein contents of tea leaf samples as (18.85 ± 1.87), which was reported by (Dawodu *et al*., 2013). As reported by Santose *et al*.,(2001),the protein content of different coffee samples were in the range of (9.21-14.33) which is less than the present results for coffee leafs. According to Franca *et al.* (2005) the protein content of different coffee bean samples were in the range of (14.24-14.87)%,which is still less than the present results for protein content of coffee leaf .The protein content of different coffee samples in the range of (14.00-16.1)% were also reported by Awika *et al*. (2003).

The difference in protein composition in the coffee leaf samples from different sites might be due to different factors including soil type, climate condition, temperature, the type of fertilizers applied, etc. The protein composition in coffee leaf mainly depends on the absorption of nitrogen by coffee plants. Coffee plants have high N and K requirements. As it was described by researchers, there is a close relationship among nitrogen supply, number of leaves, and number of flower buds (Dierendonck, 1959). The significant variation in the protein composition of the samples from different sites might also be caused by difference in organic matter contents of the samples as appreciable amount of N in coffee leaf samples occurs in organic form (Mohammed and Suleiman, 2009). The protein level variation in samples of different sights might also be due to gradual loss of N or organic matter during transport, processing and storage conditions.

As observed from the analysis results, the composition of nitrogen for almost all samples is high and this in turn results in high protein concentrations in the study samples (Malavolta, 1986).The results of this study revealed that coffee leaves can play a crucial role to improve food and nutrition security being a better source of protein as compared to other leafy vegetables. Based on the study results it is possible to conclude that coffee leaf has high value of protein required for balanced diet and can be considered as a good source of plant protein.

4.1.4. Fiber analysis

The fiber compositions of the coffee leaf samples from different sites were presented in the following order: Y>S>E>Ks>M>Kh. Coffee leaf Samples from Yirgalem area contained high amount of fiber compared to samples from the other sites. And samples from Hadero-Tunto Zuriya worede were found to contain the least amount of fiber when compared to other sites.

The investigated samples were found to be statistically different ($p < 0.05$) and richer in fiber contents as compared to other leafy vegetables fiber composition (Odhav *et al*., 2007).

Dietary fibers are non-starch polysaccharides, which bind minerals and accelerate their passage through digestive tract, as a result bioavailability and absorption of nutrients is reduced. This whole process becomes more effective when fibers collaborate with other food constituents like phytate, tannin or oxalate (Brinch-Pedersen *et al*., 2007)

4.1.5. Carbohydrate analysis

In this study utilizable carbohydrate contents of the samples were in the range of (63.93±1.30)% to (51.99±2.68)%.The samples from Shambadino Woreda were found to contain the highest (63.93±1.30)% Content of utilizable carbohydrate and matured coffee samples from West Hararge were found to contain the least (51.99±2.68)% amount of utilizable carbohydrates.

For carbohydrate composition samples from different sites were found to be significantly different ($p<0.05$).Coffee leaf sample –M- was significantly different from coffee leaf samples of other sites. However Samples Y-, S, Ks,E and Kh were not significantly different from each other for carbohydrate contents($p>0.05$). According to results of carbohydrate concentration in coffee leaves it was possible to conclude that coffee leaves and infusions from the leaves can be used as enormous amount of energy source for consumers.

4.1.6. Lipid analysis

Estimation of lipids is considered amongst the key factors for nutritional evaluation of any food material (Ayaz, 2006). The coffee leaves in different locations exhibited significant variation in the amount of lipids and the lipid content in the study samples was in the range of (12.5±2.12-4.5±0.71) %. The trend of lipid content in leaves from all the investigated sites was observed in the following order: M > E > *M*> Ks >Y > S>Kh, with significant differences among them ($p < 0.05$). Coffee leaf samples from Chiro (West Hararge) site contained the highest percentage of lipids as (12.5±2.12)% and samples from Kambata area (Hadaro -Tunto zuriya Woreda) contained the least percentage of lipids (4.05±0.071).But the range of these values was higher than lipid contents for green tea plants as (6.09± 0.01)% which was reported by (Akande *et al*.,2012).The lipid fraction of the present samples (Arabica coffee leaf)was found to be less than

the averaged lipid fraction in Arabica coffee beans which was around (15%) as reported by (speer,2006). The range of lipid contents of coffee leaf samples was also found to be higher than the range of lipid contents as (3.25±0.06 to 5.53±0.05)% in green tea, Oolong tea and Black as reported by (Dawodu *et al.2013).*

In present study the variations observed in lipid composition of coffee leaf samples from different sites might be due to different reasons including analysis problems, sample storage or handling problems or it might also be the effect of sample locations, soil type, environmental conditions, etc. Coffee oil is composed mainly of triacylglycerols with fatty acids in proportions similar to those found in common edible vegetable oils. In this study the presence of an appreciable content of lipids demonstrates the potential of these leaves to have dietary purposes with promising nutritional attributes.

4.1.7. Mineral analysis

The determination of mineral nutrients in coffee leaf was of great interest, considering the great consumption of this new product by a number of people in the country. The results of selected mineral nutrients of coffee leaf samples from different sites were showed in Table (4).

Table 4.Selected mineral compositions of coffee leaf samples from different sites.

	Minerals concentration (mg/$_{100g}$		
Name of sample	Ca	Mg	Fe
Y	150.15±5.86^{b}	79.98±0.94c	2.76±0.24^{d}
S	135±4.24^{bc}	74.5±2.12^{cd}	59.66±0.94^{c}
Ks	171.0±2.33^{c}	11.33±4.24^{a}	93.42±3.41^{b}
Kh	124.16±4.47^{a}	56.66±3.30e	87.73±1.44^{b}
E	143.0±9.66^{b}	9.5±0.70^{a}	165.10±2.36a
M	174.5±2.58^{c}	20.44±1.18^{ab}	3.96±0.03^{d}

Results were expressed as Mean value + standard deviation, n=2.

Means in the same column with different letters are significantly different ($P < 0.05$)

4.1.7.1. Calcium analysis

The total contents of the elements determined in a set of coffee leaves were presented in Table (4.2).Calcium concentration in the coffee leaf samples was found to be the highest in coffee leaf sample from West Hararge area (174.5±2.58),followed by coffee leaf samples from Kambata area ,Shinshicho woreda ,as (171.0±2.33mg/g). Which was statistically similar to coffee leaf samples from Yirgalem Woreda).The least calcium contents were recorded in coffee leaf samples from Kambata area , Hadero –Tunto zuriya worede as (124.16±4.47) ,which was statistically varied from samples of other sites.

For calcium content samples from Shambadino Woreda, Kambata area and East Hararge area were found to be significantly different from other sites ($p<0.05$).Santos and Oliveira (2001), reported the concentration of Ca in different Brazilian soluble coffee samples in the range of (106-167 mg/100g), which was less than the results of present study.

Calcium is particularly important in coffee plants, being the 3rd absorbed element in *C. arabica* and the 2nd in *C. canephora*, corresponding up to 12 and 31 % of total macronutrients, respectively (Ramalho *et al.,* 1995). This essential nutrient is required for normal cell growth and bone strength.

4.1.7.2. Magnesium analysis

The magnesium contents of different coffee leaf samples were in the range of (9.5±0.70 mg/100g) to (150.15±5.86 mg/100g) .As it was observed from the mean values of the data in (Table 4.1), highest magnesium contents as (150.15±5.86 mg/100g) were found in coffee leaf samples from Yirgalem worede followed by samples from Shambadino worede as (135±4.24 mg/100g), while the least magnesium contents were recorded in samples from East Hararge area as (9.5±0.70 mg/100g).The present results were agreed to the findings reported by the other scientists such as Santos and Oliveira, (2012). Samples from East Hararge area were found to be significantly different from other sites ($p<0.05$).

Magnesium affects growth and development, namely through the activation of enzymes (*e.g.*, ATPases, RNA polymerase, protein kinases, and Rubisco), Chl synthesis (its deficiency

promotes interveinal leaf chlorosis). Also low Mg content reduces phloem export of photosynthates to sink organs causing sugar accumulation in leaves and, therefore, restriction of photosynthetic C-metabolism (Marschner, 1995, Waraich *et al.,*2011).

4.1.7.3. Iron analysis

The iron content of coffee leaf samples was in the range of (2.76±0.24 to 165.10±3.6 mg/100g). Coffee leaf sample from East Hararge area was found to contain the highest level of iron .While Coffee leaf sample from Yirgalem Woreda showed the least iron content as 2.76±0.24 mg/100gm).Least iron contents were also recorded in coffee leaf samples from west Hararge area. For iron content samples from Yirgalem Woreda and West Hararge area were not significantly different($p>0.05$),and samples from Kambata area were not significantly different with each other($p>0.05$) while samples from East Hararge area was significantly different from other sites ($p<0.05$).

In this study the lowest limit of iron content was supported by the findings of Santos and Oliveira (2002) for coffee bean iron content. As reported by Dawodu *et al*., (2013), the trace metal content of different tea samples was found to be lower than the present results.

The mineral concentrations in coffee leave depend on the ability of coffee plants to accumulate metals, particularly Mg and Fe, and to a lesser extent Zn and Cu. The differences in the total mineral contents could be influenced by many aspects: primarily the age of the coffee leaves, but also the genetic makeup of the plant, soil conditions, rainfall, and altitude also has significant effect (Saud and Oud, 2003).The variation in mineral composition of coffee leaves from different sites might also be due to differences in fertilization practices used by various growers.

4.2. Phytochemical screening.

Studies reveal that Coffee leaves have several phytochemicals, such as terpenoids, bavonoids, alkaloids (caffeine), hydrocarbonates, phenylpropanoids (chlorogenic acids, caffeic acids, and neochlorogenic acids), esters, pirazins, etc (Raju and Gopal 1979, Smith 1985), Some studies have suggested that the presence of these compounds may be related to plants resistance and tolerance to pest insects (Guerreiro Filho and Mazzafera, 2003, Magalha˜es, *et al*., 2008). Methanolic extracts were prepared to examine the total phenolic content and antioxidant activity.

The yield of extracts obtained from 2.5 g of dry coffee leaf was measured for each extract and presented in table 1below:

Table 5: The yields of solid residue after extraction and evaporation from 2.5 g dried plant parts

Sample Name	extraction volume(ml)	extract yields (g)
Y	**22.6**	**1.13**
S	**22**	**1.05**
E	**18.2**	**0.54**
M	**23**	**1.18**
Ks	**24**	**1.21**
Kh	**18.5**	**0.85**

In present study the preliminary phytochemical analysis revealed the presence of appreciable amount of phenolic compounds, caffeine, tannins, phytic acids and antioxidant levels in matured leaves of Arabica coffee.

Table 6. Selected phytochemical concentrations of matured Arabica coffee leaves

Samples	Total phenol mgGAE-^{1}g	Tannin mg-^{1}g	Phytic acid mg-^{1}g	Antioxidant Level % at 240µg/l
Y	642.74±7.95^{c}	66.51±4.98^{a}	31.56±0.61^{ab}	98.27±0.028c
S	596.42±2.95^{c}	122.45±6.85^{b}	36.02±0.50^{b}	91.23±0.028^{b}
ks	623.35±29.52^{c}	125.70±20.68^{b}	35.131±0.53^{ab}	92.71±0.06^{b}
kh	301.72±11.32^{a}	119.43±4.06^{b}	29.705±1.77^{a}	78.55±0.55^{a}
E	563.63±30.31^{bc}	94.21±9.97^{ab}	34.792±0.16^{ab}	93.84±0.14^{b}
M	501.16±44.40^{b}	63.39±9.79^{a}	34.847±3.00^{ab}	92.31±0.28^{b}

Results were expressed as mean± standard error of the duplicate analysis for each sample (n=2) Means with different letters with in the same column were significantly different.

4.2.1. Total poly phenol analysis (TPC)

The polyphenol composition in the study samples was found to be in the order of: Y>Ks>S>E> M>Kh. Matured coffee leaves from Yirgalem Woreda contained the highest content of phenolic compounds (642.74±7.95 mg·g^{-1}), followed by coffee leaf samples from Kambata area(Ks) which contained (623.35 ± 29.52 mg GAEg–1).On the other hand, matured coffee leaf samples from Kambata area Hadero-Tunto zuriya Woreda contained the least amount(301.72±11.32 mgGAE-1g) of phenolic compound. Statistically there was a significance difference (variation)

among the samples from different sites. Sample- Y- was significantly different from samples Kh, M and E samples ($p<0.05$). Sample Kh was significantly differ rent from the rest samples ($p<0.05$) for its phenolic content.

In current study the level of phenolic compounds in coffee leaf samples was found to be much higher than the results reported for similar leafy vegetables contents of phenolic compounds .it was indicated that, the range of phenolic contents in coffee leaf samples (301.72±11.32 to 642.74±7.95) was much higher than the range of (104 ± 2.0 mg·g–1 to 14.9 ± 0.6) of phenolic contents of callistemon species leaf samples as reported by Mohamad *et al*. (2011). It was also much higher than the phenolic contents in green tea leafs and black tea leafs as reported by (Afify *et a*l., 2011) and phenolic contents in normal coffee beans (Ivana, 2011). The total phenolic contents of the present samples were also higher than the total phenolic contents (174.0 ± 9.82 mg g-1) of the same species (matured Arabica coffee leafs as reported by (Salgado *et al.*, 2008). The significance differences between the phenolic composition results among the samples from the six sites might be due to the factors such as temperature, weather condition, and stage of maturity which may significantly influence the synthesis of total phenols in coffee leafs (Salgado *et al.*, 2008).

Polyphenolic compounds constitute the main class of natural antioxidants present in plants and may contribute directly to ant oxidative action (Awika *et al*., 2003).The level of polyhenol content in a food item is used as a good indicator of food quality.

According to the results obtained in this study, coffee leaf samples from all sites can serve as an excellent source of phenolic compositions for dietary requirements in comparison to normal tea or coffee beans from other sources. In general the high contents of phenolic compounds in the study samples indicated that these compounds contribute to the antioxidant activity and the coffee Arabica leaves can be regarded as promising candidates for natural plant sources of antioxidants with high value of phenolic compounds and hence appreciable amounts of antioxidans.

4.2.2. Antioxidant level analysis

Epidemiological studies have proved that the role of oxidative stress in generation and propagation of many chronic diseases such as cancer, cardiovascular, Alzheimer's diseases as well as neurodegenerative disorders (Zia-ul-haq *et al*., 2008). Table 7 below showed that the antioxidant level of coffee tree leaves sampled from various regions of coffee growing areas.

Table 7. Antioxidant content of coffee leaves from different areas.

sample	Y		S		K_h		Ks		E		M	
µg/ml	Abso	%inh	Ab	%inh	Ab	%inh	Ab	%inh	Ab	%inh	Ab	%inh
40	0.6041	52.39	0.8286	22.41	1.2166	10.66	1.2372	9.15	0.9942	29.98	0.7232	37.41
40	0.5861	53.81	0.8051	24.60	1.1917	12.49	1.2156	10.73	0.9827	30.79	0.6838	40.81
80	0.1697	86.62	0.5317	50.21	1.0467	23.14	1.0191	25.16	0.6630	53.30	0.3646	68.44
80	0.1635	87.11	0.5940	44.38	1.0006	26.52	1.0010	26.49	0.6432	54.70	0.3722	67.78
120	0.0944	92.56	0.4647	56.48	0.8619	36.71	0.8127	40.32	0.3160	77.74	0.1158	89.977
120	0.0733	94.22	0.4435	58.46	0.8501	37.57	0.8158	40.09	0.3163	77.72	0.0981	91.50
160	0.0671	94.71	0.1835	82.82	0.6824	49.88	0.6348	53.38	0.1214	91.45	0.0820	92.90
160	0.0569	95.51	0.1699	84.09	0.7183	47.25	0.6448	52.65	0.1093	92.30	0.0876	92.41
200	0.0416	96.72	0.1001	90.62	0.5486	59.71	0.4419	67.55	0.0904	93.63	0.0720	93.76
200	0.0494	96.11	0.0966	90.95	0.5153	62.16	0.4217	69.03	0.0917	93.54	0.0816	92.93
240	0.0222	98.25	0.0934	91.25	0.3814	71.99	0.1002	92.23	0.0857	93.95	0.0813	92.96
240	0.0216	98.29	0.0939	91.21	0.3802	72.08	0.1031	93.19	0.0889	93.74	0.0859	92.56

The coffee leaf extracts were assessed for their capacity to scavenge DDPH free radical along with ascorbic acid and alpha tocopherol as a positive control. As it can seen from table 7, the antioxidant activity data were presented as percent of free radical inhibition.

Furthermore, on the basis of conducted investigations, a comparison was made among Arabica coffee leaves of different sites regarding their antioxidant activity.

At a concentration of 240µg·ml–[1], methanolic extracts of Arabica coffee leaves from Yirgalem Woreda exhibited the highest (98%) radical scavenging potential followed by East Hararge zone coffee leaf extracts (93%), West Hararge, Shambadino Woreda, Shinshicho Woreda and Hadero-Tunto zuriya Woreda inKambata zone respectively. For some sites differences being significant ($p < 0.05$) among the sites.

The methanolic (70%) extracts of the leaves of Coffee Arabica from Yirgalem wored exhibited pronounced antioxidant activity as (98.27% ±0.028 %) at a concentration of 240µg·ml–[1].Which is higher than ascorbic acid at the same concentration. On the other hand at a concentration of 240µg·ml–[1], the extracts of the leafs of coffee Arabica from Hadero-Tunto- zuriya Woreda exhibited the least antioxidant activity as (72.035±0.063)% .Samples from Yirgalem woreda and East Hararge area were significantly different ($P<0.05$)from other sites.

The extracts of Arabica coffee leaves posses highest antioxidant potential at a concentration of 240µg·ml–[1] which ranges from (90.8% ± 1.5%) to (72.035±0.063) when compared with previous studies conducted on methanolic extracts of green tea leaves which ranges from 10 to 80% as reported by (Armoskaite *et al*.,2011) and normal coffee beans at the same concentrations.

At concentrations of 200µg·ml–[1],these results were also higher than the reported results by (Nayeem *et al*.,2011) for antioxidant levels in leaves of Arabica and Robusta coffee tree.

Literature has revealed that a number of plants posses anti oxidant activity due to the presence of various phyto constituents like phenolic acids, tannins, flavonoids, etc (Singh *et al.,*2010 and Devmurari and NPJivani, 2010).

In this study the level of antioxidant activity for most samples was found to be cohere with levels of phenolic content in the samples. Research have approved that there was a strong relationship between total phenol content and antioxidant activity, as phenols possess strong scavenging ability for free radicals due to their hydroxyl groups. Therefore, the phenolic content of plants may directly contribute to their antioxidant action (Wojdylo *et al.*, 2007, Bendini *et al.*, 2006, Dlugosz *et al.,* 2006).

The significant variation in antioxidant levels of coffee leaves from different sites might be due to variations in locations, climatic conditions, species, cultivation conditions, level of maturity of the coffee leaves, etc. studies reveal that antioxidant levels in plant species vary significantly with respect to growth locations of plants (Zia-ul-haq *et al*.,2008).

In general the results of the anti oxidant activity revealed that the methanolic extracts of the leaves of *Coffee Arabica* had better anti-oxidant activity when compared to the methanolic extracts of normal tea leaves and coffee beans. These leaves may be explored as good source of comparable and promising antioxidant activity.

To avoid the hazards associated with oxidative stress, the external aid of antioxidants in the form of food supplements is required by the human body (Pihlanto *et al*., 2008). For this purpose, synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) have been in use for several years as food additives, but now their use is restricted in dietary items due to reports published on their involvement in number of chronic diseases including cancers and cardiovascular disorders (Anagnostopoulou *et al*., 2006).Therefore, this natural antioxidants are expected to be better options to the synthetic ones.

4.2.3. Tannin analysis

The amount of condensed tannins contents were calculated as catechin equivalent from the calibration curve of standard catechin by plotting the absorbance versus concentration. The trend of tannin composition in current study was observed as Ks>S>Kh>E>Y>M. The results of tannin content in Table 4.4 also showed that coffee leaf samples from Kambata area (Shinshicho Woreda) exhibited the highest value of tannin content as (125.70±20.68) $mg\text{-}^{1}g$. Coffee leaf samples from West Hararge had the least value of tannin content of (63.39±9.79$mg\text{-}^{1}g$).

Statistically there was also significant different in tannin contents of coffee leaf samples from different sites. Samples from Yirgalem area were significantly different from ($p<0.05$) samples from Shambadino and Kambata area (Ks &Kh samples) for their tannin content. However, samples from Kh, Ks, S and E are not significantly different ($p>0.05$) for tannin content.

However, these values are higher when compared with the results of Donald and Gary (1990) for tannin content of tea leaves. The condensed tannins found in present study were also higher than results presented by (Nayeem *et al.,*2011**)** on methnolic extracts of Arabica coffee leaves.

Although tannins have been considered as anti-nutritional factors, their beneficial or anti-nutritional properties depend upon their chemical structure and dosage. If ingested in excessive quantities, tannins inhibit the absorption of minerals, such as iron which may, if prolonged, lead to anemia.

It is possible to reduce the negative effect of tannin on protein and iron absorption by using foods rich in vitamin C. Adding lemon juice to tea will reduce the negative effect of tannins in iron absorption as well. However studies have demonstrated that adding milk to coffee and tea has very little to no influence on the inhibitory effect to tannin (Dommgang *et al*., 1998).

4.2.4. Phytic acid analysis

The amount of phytate concentrations in coffee leaf were calculated as phytic acid equivalents from the calibration curve of standard phytic acid by plotting the absorbance versus concentration.

Accordingly the phytate content of coffee leaves from different sites was found to be in the following order: S>Ks>M>E>Y>Kh>.Coffee leaf samples from Shambadino Woreda contained the highest (36.02±0.50mg-1g) concentration of phytate followed by samples from Kambata area, Shinshicho woredwe, which was found to contain (35.13±0.53mg-g).In contrary to this, samples from Kambata area, Hadero –Tunto Zuriya Woreda were found to contain the least amount of phyteate (29.705±1.77)mg/g.

Statistically there was no siginificant difference ($p>0.05$) for samples from Yirgalem, East Hararge, West Hararge, and Kambata area (Ks) for their phytate concentration. However, there was significant difference ($p<0.05$) between the samples from Shambadino Wereda(S) and Kambata area (kh) for phytate concentration.

When compared with similar leafy and stimulating plants, the phytate concenteration of matured leaves of coffee tree was found to be higher. For instance, the phytate concentration of tea leafs was less than the phytate concentration of coffee leaf .And it was also higher than phatate concentration in normal coffee beans as described by Akande *et al.* (2013)

According to the results of the current study the phytate composition in matured leaves of coffee tree were somewhat high. However, it was less than phytae concentration in cereals and beans and hence might not have significant effect in nutrient and protein absorption of the body.

Phytate has been suggested to serve as a store of cations, of high energy phosphoryl groups, and, by chelating free iron, as a potent natural anti-oxidant (Mueller, 2001).It is ubiquitous among plant seeds and grains, comprising 0.5 to 5 percent (w/w) (Loewus ,2002).

In contrary to its beneficiary effects, because Phytate works in a broad pH-region as a highly negatively charged ion, its high concentration in the diet has a negative impact on the bioavaila bility of divalent, and trivalent mineral ions such as $Zn^{2+,}$ $Fe^{2+/3}$+, $Ca^{2+,}$ Mg^{2+},Mn2+, and $Cu^{2+.}$

4.2.5. High performance liquid chromatographic method of Caffeine analysis

Caffeine concentration was obtained using back calculation method and the results were presented in table 8.

Table 8.caffeine concentration of coffee leaf samples from different sites

Sample Name	Concentration
	Conc.(ppm)
Y	755.825
S	1101.15
KS	691
KH	895.5
E	1443
M	1257.75

In this study data regarding the means of caffeine contents of coffee leaves from different sites was presented in Table (8) .The caffeine contents of the study samples were in the range of (691 to 1257)ppm.

Coffee leaf samples from Eaest Hararge area exhibited the highest caffeine content and samples from Kambata area ,Shinshicho Wored ,exhibited the least caffeine content.

a b c d

Figure 3: Representative chromatograms for standard (a) and samples (b, c and d).

The range of caffeine level in the present samples was slightly higher than the range of (360 to 1074) ppm for different tea brands and (624 to 684) ppm for different coffee brands as this was reported by Wanyika *et al. (*2008).

As it can be understood from the results on table 4.6, Caffeine content among the samples from different sites varied markedly. This was might be due to (Martin *et al*., 1998), environmental, weather (climate), soil conditions and the type of fertilizers applied. For instance, the study conducted by Gonthier *et al.* (2011), described the effect of N fertilization on caffeine synthesis in coffee plants and demonstrated an increase in the caffeine concentration in the phloem exudates as N fertilization increased. As it was described by Palumbo *et al (*2007), the biosynthesis of N-containing secondary metabolites including caffeine is very sensitive to the N levels of leaves.

Recently, (Yun *et al*., 1999) showed that K and Mg fertilizer application increased caffeine in tea leaves (*Camellia sinensis*). Also there is an investigation that report on the effect of mineral nutrition on caffeine contents of leaves and seeds of coffee (Rodriguez, 1961) plant.

Light may also has significant effect on variation of caffeine concentrations in the study samples. As researchers described, on average, exposing the plants to high light (HL) conditions significantly increased the caffeine content and the caffeine content of the high light plants was found to be higher compared to the low light plants (Marcelo *et al*.,2013).

Caffeine contents in coffee were also leveled by a balanced mineral nutrition, considering that the metabolism of caffeine is very similar in fruits and leaves of *C. Arabica (*Suzuki & Waller 1984; Mazzafera *et al*., 1994; Ashihara *et al*., 1996).

The level of maturity of the leaves might be among the factors for caffeine variation between the samples from different sites. As it was described by Marcelo *et al (*2013) caffeine biosynthesis is higher in young leaves and lower in matured once.

4.3. Sensory attributes analysis

The sensory evaluation of coffee samples for various attributes such as color, flavor, aroma, taste, appearance and overall acceptability was carried out. The product was evaluated by a panel of judges and the results are described below:

Table 9.The mean values of the judge results for sensory attributes of Arabica coffee leaf tea(*kuti)*.

No	Appearance	Taste	Texture	Aroma	Overall acceptability
1	8	8	8	9	8
2	8	4	4	5	5
3	9	8	4	9	5
4	8	9	5	9	9
5	8	8	4	9	9
6	8	9	2	9	9
7	9	9	4	9	9
8	8	6	6	7	7
9	8	7	9	9	9
10	5	9	9	9	8
11	7	6	6	8	6
12	9	8	9	9	9
Mean	**7.91**	**7.58**	**5.83**	**8.41**	**8.5**

4.3.1. Appearance

Color has a profound influence on the acceptance of coffee and other food products. The means of various attributes of coffee leaf brew were presented in the table 9 ppearance of the brew exhibited the mean sensory scores of (7.91).Judges rated the color (appearance) of this brew as best for product development.

4.3.2. Flavor

Flavor is also important for the acceptance of product. It is combined perception of taste; smell and mouth feel. Flavor means in table 9 indicated that the best score for flavor was given for this product with mean value of 7.58 Judges rated the product as best which obtained maximum score (7.58).

4.3.3. Aroma

Aroma also has a profound influence on the acceptance of coffee and other food products. The means of various scales of coffee leaf brew were presented in table 9 which exhibited that the highest scores (8.41) for its aroma. Judges rated the aroma of the product treatment as best which obtained maximum score of (8.41).

4.3.4. Taste

Taste of the product is the vital factor to be considered under organoleptic testing. Table 9 indicated that this product exhibited best score for taste with mean value of 7.58. The judges rated the treatment as best which obtained maximum score (7.58) for its taste.

4.3.5. Over all acceptances

The means for over all acceptance of the product was given in (Table 9) indicated that the product was acceptable for its overall attributes.

4.5. Conclusions and Recommendations

4.5.1. Conclusion

The result of this study showed slightly high accumulation of ash in coffee leafs when compared to ash content n tea and coffee beans. This indicates that there is a significant amount of minerals in the experimental samples and coffee leaves could be able to minimize the macro and micro nutrient shortage of the community.

In present study Coffee leaves were found to boost high amount of protein and essential lipids in that they could be able to play significant role in reducing deficiency of protein and essential fatty acids, especially for women, elders and children who are the first class to consume coffee leaf infusions in some rural areas of the country.

Coffee leaf samples from different sites were found to contain significant amount of trace elements. As compared to other trace elements, calcium was found to be highly concentrated element in coffee leaf samples of all sites.

Coffee leaf was found to contain enormous amounts of secondary plant metabolites such as total polyphenols, tannins, antioxidants and moderate amount of caffeine, which are engrossed in our daily life and should be explored within our daily used commodities with special reference to cost and safety.

Methanolic extracts of coffee leaves were found to contain high level of free radical inhibition activity when compared to antioxidant levels in green tea and normal coffee beans. Amazingly the antioxidant levels in coffee leaf extracts were also found to be higher than the antioxidant levels in ascorbic acid which was used as a control for antioxidant assay.

The sensory attributes of coffee leaf infusions were found to be acceptable by trained panelists and the potential health benefits of coffee-leaf tea, and beverages and any other products made from the leaves of coffee plant are supported by the findings of this study on the bases of high accumulation of phenolic compounds in coffee leaves than coffee beans or normal tea.

The coffee leaf samples from different sites were found to be significantly different for the composition (accumulation of) all the studied parameters and based on this finding it was

possible to conclude that secondary metabolite accumulation in coffee leaves was affected by environmental conditions.

4.5.2. Recommendation

Although coffee leaf infusions have been consumed since the early times in our country, the accumulated influence of these compounds in the human body would require further research. Further research on coffee leaves considering its health benefits and side effects on the consumers is also recommended.

Further research is also recommend in coffee leaves considering its variety for high concentration of secondary metabolites ,specially specific antioxidant levels determination in coffee leaves of different variety was highly recommended.

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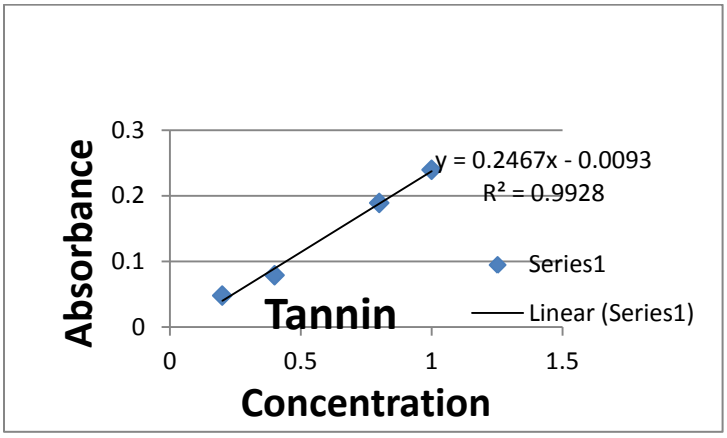
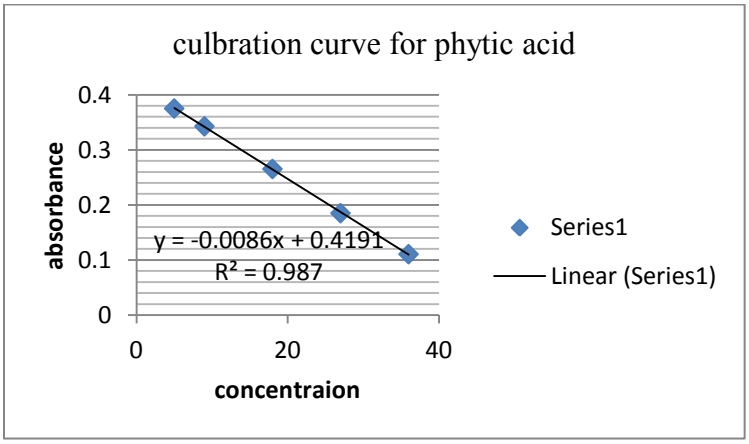
Appendix

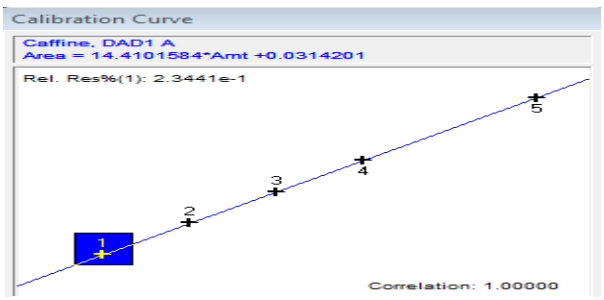
Annex1: Tannin standard calibration curve



Annex 2. Phytic acid standard calibration curve.



Annex 3: Caffeine calibration curve



Annex 4: Percent recovery analysis results

Compound name	CCB ppm 0506	% Recovery	CCVA ppm 050615	% Recovery
Caffeine	2.99	99.67	2.99	99.67

Where: CCVB-Continuous calibration curve verification before sample run

CCVA- Continous calibration curve verification after sample run

Compound	Spike-1	% Recovery	Spike-2	% Recovery	Spike-3	% Recovery
Caffeine	1.2	120	1	100	0.9	90

RSD of spike samples (1ppm), using percent recovery of spiked samples 090615

Compound	Spike-1%	Spike-2% Rec	Spike 3 % Rec	Average	STDV	RSD
Caffeine	120	100	90	103.3	15.3	14.8

Annex 5: Questioner on coffee leaves brew preparation

1. When and how the coffee leave brew preparation was started in your environment?
2. Why people started to prepare the brew and what benefits do they get from it?
3. Which type of the coffee leaves is most likely used to prepare the brew?
4. How do you collect the leaves?
5. How to prepare coffee leave brew?
6. How to dry the ground coffee leaves
7. Is there any difference at the final product due to drying system?
7. Do you use other ingredients when preparing the brew?
8. What types of home equipments are used to prepare the brew?
9. For how long do the infusions are boiled in the water?
10. Do you use salt or sugar when serving the brew?