ADDIS ABABA UNIVRSITY

SCHOOL OF GRADUATE STUDIES

INVESTIGATION OF THE LEVELS OF SELECTED METALS IN THE LEAVES OF *THYME* (*T. SCHIMPERI AND T. VULGARIS*) GROWN IN ETHIOPIA

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A Graduate Project Submitted to School of Graduate Studies of Addis Ababa University

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Lists of Abbreviations and Acronyms

FAAS: flame atomic absorption spectroscopy

WHO: World Health organization

RSD: relative standard deviation

MDL: method detection limit

DF: freshly prepared thyme samples from near the town of Debresina.

DM: thyme samples bought from town of Debresina

AAM: thyme samples bought from Addis Ababa city

WF: freshly prepared thyme sample from Wondogenet.

ANOVA: analysis of variance

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By Awraris Derbie

Advisor Prof. B. S. Chandravanshi

Abstract

The contents of some selected metals Ca, Mg, Fe, Mn, Zn, Ni, Cu, Co and Cd in different thyme leaf samples readily consumed in Ethiopia were determined by flame atomic absorption spectroscopy (FAAS) after acid digestion with 1:1 HNO_3/$HClO_4$ for 3 h at a temperature of 240 ^{0}C by a Kjeldahl apparatus hot plate digester. The level of the nutrients in the four samples ranged from 1239 – 2517 µg/g, Ca; 1524 – 1786 µg/g, Mg; 728 – 2517 µg/g, Fe; 37.7 – 114 µg/g, Mn; 8.7 – 52 µg/g, Zn; 9.83 – 14.2 µg/g, Ni; 7.69 – 9.3 µg/g, Cu; 2.59 – 4.3 µg/g, Co; and 0.87 – 1.3 µg/g, Cd; respectively. The concentration of Ca was higher than the other metals in the three samples and Cd was the least of all the metals in the analyzed samples. The over all reproducibility of the method obtained from spiking experiment was with in the range ± 10 % except for Fe (± 11.91 %), Cd (± 10.89 %) and Co (± 10.84 %). This result will complement available data on food composition in Ethiopia.

Key words: Thyme leaves, Metals, Debresina, Wondogenet, Ethiopia, FAAS.

1. INTRODUCTION

1.1. Botanical Aspect of Thyme

The genus *Thymus* includes about 350 species worldwide and is widely distributed in temperate zones [1]. The leaves are opposite, grayish-green, entire, linear or elliptic, up to 15 mm long, tomentose beneath. The flowers are small, pale-purple or white, arranged in terminal inflorescences that may be dense or loose. Flowers appear since the beginning of summer until the end of autumn. The fruit is an ovoid smooth achene. The two species, *T. schimperi* Ronniger and *T. serrulatus* Hochst.ex Benth are endemic to the Ethiopian highlands growing on edges of roads, in open grassland, on bare rocks and on slopes, between 2200-4000 m altitudes. Both species are perennial herbs, woody at the base and 5-40 cm high. The inflorescence is commonly crowded into globose and oblong heads with pink corollas [2, 3]. As it is economically important aromatic species as much in the market of products, it presents a challenge to maintain the organoleuptic qualities during the cultivation process. The vegetative propagation is important because it provides the maintenance of the characteristics of the mother plant, standing out via stakes propagation, for the small quantity necessary for the speed obtain the seeding. The potential of the rooting process of stakes of *T. vulgaris* was verified in a study [4].

1.2. Distribution of thyme in the world

Thyme is native to the western Mediterranean area, especially the south of Italy, where from it spread to almost every region. This plant is cultivated in almost every country, as an aromatic for culinary uses (especially in the south of France, Spain, Morocco and North America [5].

1.3. Distribution of thyme in Ethiopia

Thyme is mainly a temperate taxon and is uncommon in the African tropics. There are, however, two species, *T. schimperi* Ronniger and *T. serrulatus* Hochst.ex indigenous to Ethiopia [2, 3] while *T. vulgaris* has been recently introduced. *T. vulgaris* is a species, native to southern Europe, recently introduced into Ethiopia and cultivated in Wondogenet by the Essential Oil Research Center [6]. *Thymus schimperi* is comparatively widespread in central, eastern and northern Ethiopia. It is locally known as *Tosign. T. serrulatus* has obovate to oblanceolate leaf-laminas with weakly crenate margins and is restricted to northern Ethiopia. It is locally known as *Tesni* or *Thasne.* Thymus species in Ethiopia are restricted to afromontane and afroalpine regions and are represented by two species thymus schimperi and thymus serrulatus. Bale, Shewa, Gonder, and Wollo are the major growing areas in Ethiopia [1, 2]. Wild thyme, used to make a tea, is harvested by people living close to the town of Dinsho and near Menz. It is dried, put in plastic bags and sold to travelers on buses [7].

1.4. Uses of Thyme

1.4.1. Medicinal use

According to the World Health Organization the definition of traditional medicine may be summarized as the sum total of all the knowledge and practical, whether explicable or not used in diagnosis, prevention and elimination of physical, mental or social imbalance and relaying exclusively on practical experience and observation handed down from generation to generation, whether verbally or in written. Traditional medicine might also be considered as a solid amalgamation of dynamic know how and ancestral experience [8].

The volatile oil from thyme was found to contain *p*-cymene, γ-terpine, carvacrol, rosmarinic acid, eugenol and thymol [2, 6]. The volatile oil not only has carminative action, but also antiseptic, antimicrobial and antifungal activities [9]. Thyme is prepared as infusion to treat spasmodic cough, laryngitis, bronchitis and urinary infections. It is also used as a decongestant, as a cholagoge, to reduce flatulence and to fight parasites. External uses of thyme include preparations to wash skin wounds or infections [2, 9]. In the Ethiopian traditional medicine the plant has many medicinal applications. Some of the reported applications are for the treatment of gonorrhea, cough, inflammation, spasm, thrombosis, urinary retention, mental illness, eye disease, toothache, stomach problems, leprosy, lung TB, acne and ascaris [8, 10].

1.4.2. Food condiment

The fresh or dried leaves of both species are used locally as condiments in the preparation of chilli powder, stew, bread and tea. Thyme has many uses: in chicken broth or stuffing; in clam chowder and marinades for meats or fish; in sauces; with onions, carrots or peas; in egg dishes with other sweet herbs; even in a baked apple dessert. The flavor can be captured in oils or butter. The caraway-scented form (or chemotype) of *Thymus herb* has a historical association with roast beef (baron of beef). Lemon thyme, *T. xcitriodorus*, is recommended for fish, for tea, and for salad dressings, or anywhere milder thyme is desired [2, 3].

1.4.3. Cosmetic application

Antimicrobial activity

Thyme essence – especially the phenolic components thymol and carvacrol – show antibacterial activity against gram-positive and gram-negative bacteria,

mainly due to their effects on the bacterial membrane. Since thymol and carvacrol are eliminated through the respiratory tract, these compounds have respiratory antiseptic action. Because of its antibacterial activity, thyme is also useful as an antiseptic for the urinary tract, mouth and skin wounds. Furthermore, thymol and carvacrol are antimycotic agents, effective against *Candida albicans*. Thyme water extract showed significant in vitro inhibitory effects on the growth of Helicobacter pylori and its powerful urease activity.

Sebum excess promotes the growth of certain microorganisms on the skin and the scalp. Such microbial flora imbalance is one of the factors causing dandruff and acne. Thyme antimicrobial activity is of great use to formulate cosmetic products aimed at the regulation of sebum hypersecretion and treatment of related disorders. Therefore, thyme extract is recommended to formulate cosmetic products with purifying and antiseptic activity [11, 12].

Anti-inflammatory activity

In vivo tests of thyme ethanolic extract on rats showed anti-inflammatory and analgesic activities. These activities could be related to carvacrol and thymol, which showed inhibitory effects on the enzyme cyclooxygenase in animal models, as well as inhibitory effects on the complement and on nitric oxide synthesis. Carvacrol has inhibitory action on prostaglandin synthesis. This action supports the use of thyme in ointments and other preparations to treat muscle and joint pain. The rosmarinic acid in this extract also has anti-inflammatory activity. Topical applications of thyme essential oil are rubefacient and generate analgesia, beneficial in cases for bruises or sprains. Anti-inflammatory action has been reported for the phenol compounds in thyme extracts. Therefore, thyme extract is recommended to formulate cosmetic products with anti-irritant activity [11, 12].

Antioxidant activity

The Thymol and carvacrol, present in thyme essence, as well as the flavonoids and other polyphenols are considered to be involved in the antioxidant activity. Rosmarinic acid, hydroxycinnamic derivatives and flavonoid compounds showed important in vitro antioxidant activity by inhibiting iron-induced superoxide anion formation and lipid peroxidation in microsomal and mitochondrial systems. Furthermore, the thymol present in the essential oil showed in vitro antioxidant activity by neutralizing the DPPH (diphenyl-picrylhydrazyl) radical. Aged rats, which had been fed on a diet including thyme since young age, showed high proportions of antioxidant enzymes such as superoxide dismutase in liver and heart, as compared with a control group. Therefore, thyme extract is recommended to formulate cosmetic products aimed at the protection of skin and hair integrity against oxidative processes [12 - 15].

1.5. Chemical Composition

Herba thymi contains about 2.5% but not less than 1.0% of volatile oil. The composition of the volatile oil fluctuates depending on the chemotype under consideration. The principal components of *Herba thymi* are thymol and carvacrol (up to 64% of oil), along with linalool, *p*-cymol, cymene, hymene, *α*-pinene, apigenin, luteolin, and 6-hydroxyluteolin glycosides, as well as di-, tri- and tetramethoxylated flavones, all substituted in the 6-position (for example 5,4-dihydroxy-6,7-dimethoxyflavone, 5,4-dihydroxy-6,7,3-trimethoxyflavone and its 8-methoxylated derivative 5,6,4-trihydroxy-7,8,3-trimethoxyflavone. There are several reports in the literature on the chemical composition of *T. vulgaris* oil. Most reports indicate thymol or carvacrol to be the major compounds in the oil [5]. Thymol and γ-terpinene are found to be the major constituents of *T. vulgaris* cultivated in Wondo Genet by the Essential Oil Research Center [6]. Investigation of the chemical compositions of the two

thymus species in Ethiopia (*T. schimperi Ronninger* and *T. serrulatus Hochst.ex Benth*) was limited except for the volatile oil constituents by Asfaw *et al.* [2] and Dagne *et al.* [6] for essential oil constituents.

1.6. Risk Associated with the Volatile Oil Extracts of Thyme

The essential oils extracted from thymus vulgaris presents no risk to humans and the environment. The growth inhibiting, reproduction retarding, and repellent effects biological controls have also been demonstrated against storage pests. So far, some attempts have been made to study the effect of medicinal plant powders against *S. zeamais* in stored cone in Chile. The main constituent of essential oil from *Thymus vulgaris* was thymol as reported by various studies [5, 6]. Thymol exhibited stronger insecticidal activity against *A. obtecus* and *S. zeamais* adults. Adult mortality increased along with the dosage of essential oils and the exposure period. The results of the study conducted by Bittner *et al.* show that a dose of 8 µL/L air of *T. vulgaris* is needed to kill close to 55 % of *S. zeamais* adults at 96 h [16].

1.7. Minerals and Their Importance

Life requires a continuous material exchange which, in principle, includes all chemical elements. The occurrence of these elements in organisms depends on external and endogenous conditions; elements can be bioavailable to variable

extents but can also be enriched (bioaccumulated) by organisms using active i.e. energy consuming processes involving a local reduction of entropy. Questions like 'which elements are essential which are beneficial and which are toxic for a certain organism' are continuously being discussed under various aspects, particularly for humans, in popular science. Quantitatively, this is a matter of the physiological state, i.e. of the ability to function properly or even of individual disposition of an organism, depending on the presence, the dose or concentration of an element, often related to its share in the food supply [17]. Some deficiency symptoms of individual inorganic elements are quite familiar, particularly when concerning human beings.

Table 1. Some characteristic symptoms of chemical element deficiency in humans.

Deficient element	**Typical deficiency symptoms**
Ca	-Related Skeletal growth
Mg	-Muscle cramps
Fe	-Anemia disorders of immune system
Zn	-Skin damage, stunted growth, retarded sexual maturation
Cu	-Artery weakness, liver disorders, secondary anemia.
Mn	-Infertility, impaired skeletal growth
Co	-Pernicious anemia
Ni	-Growth depression, dermatitis
Cr	-Diabetes symptoms

The inorganic contents of a nutritionally complete diet are summarized in the following table in the form of RDA (recommended dietary allowances) values of the American food and drug administration. Whether such a composition is really sufficient, how much of it occurs in today's food supply and how far it occurs in today's food supply and how far it can be exceeded via increased

uptake or separate supplementation without detrimental consequences are still open questions in dietetics, particularly from the popular scientific point of view.

Table 2. Essential elements in food for adults and infants [17].

Inorganic constituents	**Recommended daily allowances in mg**	
	Adult	Infant
K	2000 – 5500	530
Na	1100 – 3300	260
Ca	800 –1200	420
Mg	300 - 400	60
Zn	15	5
Fe	10 - 20	7
Mn	2 – 5	1.3
Cu	1.5 - 3	1
Co	0.2	0.001

Trace metals are minerals present in living tissues in small amounts. Some of them are known to be nutritionally essential, others may be essential and the remainders are considered to be nonessential. Trace elements function primarily as catalysts in enzyme systems; some metallic ions, such as iron and copper, participate in oxidation-reduction reactions in energy metabolism. Iron, as a constituent of hemoglobin and myoglobin, also plays a vital role in the transport of oxygen. All trace elements are toxic if consumed at sufficiently high levels for long enough periods. For example high levels of zinc can result in a deficiency of copper, another metal required by the body. The difference between toxic intakes and optimal intakes to meet physiological needs for

essential trace elements is great for some elements but is much smaller for others [18, 19].

Heavy or toxic metals are metals with a density at least five times that of water. As such they are stable elements (meaning they cannot be metabolized by the body) and bio-accumulative (passed up the food chain to humans). These include: mercury, nickel, lead, arsenic, cadmium, aluminum, platinum and copper (the metallic form versus the ionic form required by the body). Most of the heavy metals have no function in the body and can be highly toxic [19]. Nutritionally essential metals may cause adverse health effects at some levels below or beyond the level required for optimum nutrition [20]. Injury to vegetation caused by trace metal has been well recognized because of many botanical and chemical investigations during past 100 years. More than 60 elements in various parts of human body have been detected. Among these at least 25 elements are essential to human health out of which 14 are termed as trace elements [21].

There has been a remarkable expansion in the knowledge of the significance of trace element and the effect of its absence on human health. Recent studies examining the need for various trace and ultratrace elements by animals under some form of nutritional, metabolic, hormonal or physiologic stress have indicated that these are situations in which some of the trace elements may be of nutritional significance [22].

1.8. Physiological Role of Some Metals to Humans

Nutritional elements serve a variety of metabolic functions. As structural components, they are part of the skeletal system, vitamin B_{12}, hemoglobin, and thyroid hormone. As cellular regulators, they are involved in nerve transmission, maintenance of cell membrane permeability, and regulation of osmotic pressure, water balance, and acid-base equilibrium. Additionally,

nutritional elements serve as cofactors in a wide array of enzymatic reactions. Various circumstances may result in inadequate status of nutritional elements: insufficient intake, poor digestion, poor absorption, and competitive inhibition by toxic elements. Maintaining optimal digestive function can therefore be a critically important aspect in elemental nutrition.

Manganese, Mn

Manganese is an antioxidant nutrient and is important in the breakdown of amino acids and the production of energy. It is essentially required for the metabolism of vitamin B-1, C and E and for activation of various enzymes which are important for proper digestion and utilization of foods. Manganese acts as a catalyst in the breakdown of fats and cholesterol and also helps in the nourishment of the nerves and the brain. It is necessary for normal skeletal development and maintains sex hormone production. The best natural sources of manganese are whole grains, cereal products, nuts and green leafy vegetables. Dairy products, meat, fish and poultry are poor sources. A deficiency can cause poor reproductive performance, growth retardation, abnormal formation of bone and cartilage and an impaired glucose tolerance. Lactoferrin saturated with manganese inhibits the replication of polio virus in verocells [23].

Zinc, Zn

Zinc is an essential mineral that is found in almost every cell. It stimulates the activity of approximately 100 enzymes. Zinc supports a healthy immune system and is needed for wound healing, the sense of taste and smell, and for DNA synthesis. Zinc deficiency mostly occurs when zinc intake is inadequate or poorly adsorbed, due to increased losses of zinc from the body, or when the body's requirement for zinc increases. Signs of zinc deficiency include growth

retardation, hair loss, diarrhea, delayed sexual maturation and impotence, eye and skin lesions, and loss of appetite. Zinc deficiency is the most prevalent micronutrient abnormality seen in HIV infection. Low levels of plasma zinc predict a three–fold increase in HIV related mortality. Zinc deficiency characterized by low plasma zinc levels over time enhances HIV associated disease progression, and low dietary zinc intake is an independent predicator of mortality in HIV infected drug users [23].

Copper, Cu

Copper is essential for variety of biochemical processes and needed for certain critical enzymes to function in the body. It is also involved in the functioning of the nervous system, in maintaining the balance of other body functions. Copper is natural element found in the earth's crust and surface water. Copper is an integral part of many important enzymes involved in a number of vital biological processes. Although normally bound to proteins, copper may be released and become free to catalyze the formation of highly hydroxyl radicals that have capacity to initiate oxidative damage and interference with important cellular events [23].

Cobalt, Co

Cobalt is an important constituent of vitamin B_{12}, which is needed to maintain normal bone marrow function for producing erythrocytes. The food sources of cobalt are meat, dairy products, and green leafy vegetables [23].

Nickel, Ni

Nickel is an essential micronutrient. The best sources of nickel include, oatmeal, legumes, cocoa, and vegetable leafs. Nickel is found in blood and

tissues at consistent levels, and is also associated with DNA and RNA in amounts that suggest physiological significance. Nickel is required for normal growth and reproduction in animals, presumably in human beings as well. It appears to have a role in the modulation of the immune system and in development of brain [23].

Cadmium, Cd

Cadmium is a non-essential toxic element which can be regarded as potentially toxic at low concentrations. In its ionic form, Cd^{2+} shows great chemical similarity with the biologically important metals Ca^{2+} and Zn^{2+}. As cadmium is softer and more thiophilic metal it can replace zinc and calcium. Chronic Cd poisoning can cause embrittlement of bones and painful deformations of the skeleton. In the human body cadmium is concentrated in the liver and kidney [16].

Iron, Fe

Iron is an absolute requirement for most forms of life, including humans and most bacterial species, because plants and animals all use iron, it can be found in a wide variety of food sources. Iron is essential to life because of its unique ability to serve as both an electron donor and acceptor. Its ability to donate and accept electron means that if iron is free within the cell, it can catalyze the conversion of hydrogen peroxide to free radicals – damage cellular structures. All life forms that use iron, bind the iron to proteins to prevent this kind of damage. Humans use iron in hemoglobin of red blood cell for oxygen transport. It is also an essential component of myoglobin to store and diffuse oxygen. Humans have no physiologic regulatory mechanism for excreting iron. Most humans prevent iron overload solely by regulating iron absorption. Like most mineral nutrients, iron from digested foods or supplements almost entirely

absorbed in the duodenum. To be absorbed, dietary iron must in its ferrous, Fe^{2+} form [24, 25].

Calcium, Ca

Calcium is needed for strong bones. It is also needed for our heart, muscles and nerves to function properly and for our blood to clot normally. An inadequate calcium intake is thought to play a significant role in contributing to the development of osteoporosis. Each day we lose some calcium in the urine and feces and to a lesser extent through perspiration. These losses must be balanced by consuming adequate amounts of calcium. If the level of calcium in the blood drops below normal, the body takes what it needs from the bone, thereby depleting the calcium reserves [26, 27].

Magnesium, Mg

Magnesium is the fourth most abundant mineral in the body and is essential to good health. Approximately 50 % of the total body Mg is in bone. It is an extremely important and valuable mineral, whose value for good health is just being recognized by conventional physicians. Virtually, all chemical in the body require an enzyme system to help the biochemical reactions takes place. Magnesium is a critical co-factor in more than 300 enzymatic reactions in the human body. Changes in magnesium homeostasis mainly concern the extracellular space, as the intracellular magnesium concentration is well regulated and conserved. The extracellular magnesium concentration is primarily regulated by the kidney [28-32].

1.9. Analytical Techniques for Analysis of Metals in Plant Materials

The determination of metals in plant samples have been carried out by different instrumental techniques depending on the precision and sensitivity required. Flame atomic absorption spectroscopy (FAAS) [33-36], atomic absorption spectroscopy equipped with graphite furnace (GFAAS) [37], electrothermal atomic absorption spectroscopy (ETAAS), inductively coupled plasma atomic emission spectroscopy (ICP- AES) [34], X-ray fluorescence (X-RF) [33, 38, 39], inductively coupled plasma mass spectroscopy (ICP-MS), reversed phase high performance liquid chromatography (RP-HPLC) [40] are the most commonly used instrumental technique for determination of metals in plant samples. In the present study flame atomic absorption spectroscopy (FAAS) was used for the determination of metals in Thyme.

1.10. Scope and Objectives of the Study

Food is the major intake source of toxic trace elements by human beings. Vegetables, fish, meat, grains, soft drinks, condiments, etc. are used as staple part of food. Various studies investigated the concentration of major, minor, and trace elements from the different items of food. Vegetables [21, 41, 42], food condiments [43], water [44], bread [45], fish [46] are some among others. Little information has been reported on the levels of metal concentration of thymus species. Khan *et al.* [47] investigated seven macro-nutrients (Cd, Al, Pb, Ca, K, Na and Mg) and five micro nutrients (Cu, Ni, Mn, Fe, and Cr) from *Thymus vulgaris* by atomic absorption spectrophotometer. No information has been reported yet on the level of the metal concentration on the two thymus species indigenous to Ethiopia and *Thymus vulgaris* cultivated now at Wondo Genet Essential Oil research Center, Ethiopia. A commercially available and freshly prepared *Thymus schimperi* and freshly prepared *Thymus vulgaris* was investigated for their metal content. The research results will be expected to

reveal the level of essential and non essential metals which might be useful to establish the baseline data on the levels and variations of some nutrients in different species of thymus in the country and initiate further studies on nutritional, medicinal and toxicological effects that can be caused due to the use of these species through diet.

1.10.1. General objective

This research project is designed to investigate the levels of essential and non-essential metals (toxic) on the two thymus species *T. schimperi* Ronniger endemic to Ethiopia and *Thymus vulgaris* cultivated at Wondo Genet Essential Oil research Center which is endemic to Western Europe.

1.10.2. Specific objectives

- To determine the concentration of metals (Ca, Mg, Mn, Fe, Cu, Zn, Cd, Co, and Ni) in *Thymus schimperi* and *Thymus vulgaris* by flame atomic absorption spectroscopy.
- To compare the levels of the identified metals in the freshly prepared and market available thyme.
- To compare the levels of the selected metals in Ethiopian *Thymus vulgaris* with the levels of metals in *Thymus vulgaris* from the other part of the world.

2. EXPERIMENTAL

2.1. Instrumentation and Apparatus

Ceramic pestle and mortar were used for grinding and homogenizing of dried thyme leaves; digital analytical balance (Mettler Toledo, Model AT250, Switzerland) with ± 0.0001 g precision and oven (J.P.SELECT, Spain) were used for weighing and drying the samples, respectively. Quick-fit round bottom flasks (150 mL) fitted with reflux condenser were used in Kjeldahl apparatus hot plate to digest the samples. A refrigerator (Hitachi, Tokyo, Japan) was used to keep the samples ready for analysis till determination. Micropipette (1–10 µL, 100–1000 µL, Shanghai, China) was used for spiking of known concentration for recovery test. BUCK SCIENTIFIC MODEL 210VGP (East Norwalk, USA) Flame Atomic Absorption Spectrophotometer equipped with deuterium ark background corrector was used for analysis of the metals (Cu, Cd, Ni, Zn, Mn, Co, Fe, Ca, and Mg). Na and K were determined in the emission mode of the spectrometer.

2.2. Chemicals, Reagents and Standard Solutions

69-72 % HNO_3 and 70 % $HClO_4$ (Research – lab fine chem industries, Mumbai, India) were used for digestion of thyme leaf samples. Stock standard solutions of the metals (Cu, Cd, Ni, Zn, Mn, Co, Fe, Ca, and Mg), 1000 mg/L calibration standards BUCK SCIENTIFIC, prepared as nitrates for each element in 2% HNO_3) were used for the preparation of calibration curves for the determination of metals in the samples. Deionized water for preparation of standard solutions, dilution and for cleaning (rinsing) purpose was used. Lanthanum chloride hydrate (99.9 %, Aldrich, USA) was used to avoid refractory interference for calcium and magnesium analysis from their phosphates.

2.3. Cleaning of apparatus

Apparatus (volumetric flasks, measuring cylinders, digestion flasks, pipettes, funnels, mortar, pestle, porcelain dishes), used for sample preparation and analysis, were washed with tap water, soaked in 10% Nitric acid for two days rinsed with deionized water dried in oven and kept in dust free place prior to usage.

2.4. Sampling

2.4.1. Description of sampling site

Thyme samples were collected from the growing fields of Tarmaber Woreda, Wondogenet essential oil research center and Addis Ababa town. The type of species from Addis Ababa and Tarmaber Woreda was *Thymus schimperi* and from Wondogenet essential oil research center the species was *Thymus vulgaris*. Totally four kinds of samples were taken.

1. Fresh leaves from a highland area which is about 10 km from debresina town (*Thymus Schimperi*).
2. Processed dried thyme leaves from debresina town (*Thymus schimperi*).
3. Processed dried thyme leaves from Addis Ababa city (*Thymus schimperi*).
4. Fresh thyme leaves from the garden of Wondogenet essential oil research center (*Thymus vulgaris*).

The reason for the selection of sampling sites was based on the availability of the sample, proximity to the study area, proximity to the market center, and sampling cost. *Thymus vulgaris*, as stated earlier, was brought from Western Europe and was selected in order to relate and compare the mineral contents with the species growing in Ethiopia and with the same species in other countries.

Debresina is found in the northern part of Ethiopia, 190 km from the capital, Addis Ababa. Wondogenet is in the southern part of Ethiopia and is 305 km of from Addis Ababa.

2.4.2. Collection and preparation of samples

Sampling is obtaining a portion representative of the whole population – the total quantity from which a sample is obtained. Adequate sampling technique helps to ensure that sample quality measurements are accurate and precise estimate of the quality of the population. By sampling only a fraction of the population, a quality estimate can be obtained more quickly and with less expense and personnel time than if the total population were measured. The sample is only an estimate of the true value of the population, but with proper sampling technique it can be very accurate estimate [48].

Sampling solid samples can be effected either manually or continuously [48]. The sampling technique used in the present study was manual random sampling. The leaves of fresh *Thymus schimperi* from Tarmaber woreda was collected from the growing area where 10 bags were taken with in the specific area with a difference of about 100 m between each site. A bulk sample was prepared by taking 50 g from each plastic bag. *Thymus vulgaris* from Wondogenet was sampled by taking randomly from five sites. All the leaves from the five sites were mixed and a bulk sample was prepared. The dried (processed) leaf samples were bought from Debresina and Addis Ababa supermarkets. In both places five supermarkets were randomly selected and from each supermarket three bags were bought. For each sample a bulk sample of 500 g was prepared. The bulk sample was washed with tap water to remove dust material attached to the leaves of thyme. This was followed by washing with distilled and deionized water 10 times in each case for removing trace metal contamination from tap water and the dried with oven at 75^0C for 24 h. The sample was further powdered by making use of mortar and pestle,

sieved, and kept in a desiccator prior to digestion. Finally, 0.5 g aliquot was taken from each sample for digestion and a solution for final metal determination was prepared.

2.4.3. Digestion of samples

Several articles describe the use of different pretreatment for solubilization of thyme involving a wet digestion [43, 47]. In this study the thyme samples were made ready for the analysis after digestion using the Kjeldahl digester heating block. Wet digestion, involving the use of mineral or oxidizing acids and external heat source to decompose the sample matrix, and dry ashing, involving heating of the sample in a muffle furnace in the presence of air at 400–500^{0}C, are the two main methods for metal analysis in food samples [48, 49]. Dry ashing is the simplest of all decomposition systems but it is slow and time consuming [49]. In this study, a wet digestion using a 1:1 mixture of HNO_3 and $HClO_4$ was used.

For preparation of a suitable solution of the analyte, a suitable digestion procedure is crucial. Hence, different digestion procedures were tested by varying the volume of reagent, digestion time, and temperature and reagent composition. The nature of the final digests was examined, clear and colorless solution was selected and the procedure taken as an optimum. The selection considered clearness of the digests, less digestion time, low reagent volume, and low temperature.

0.5 g of powdered thyme sample was weighed on analytical digital balance and added to 250 mL round bottom flask. To this, 3 mL of HNO_3 (69-72 %) and 3 mL of $HClO_4$ (70%) was added. The round bottom flask was fitted to a reflux condenser and made heated on a Kjeldahl apparatus hot plate for 3 h with a temperature of 240^{0}C. The digest was allowed to cool for 10 min without dismantling the condenser and then further cooled to room temperature for 20 min dismantling the condenser. The mixture then diluted with 20 mL of

deionized water and filtered with Whatman filter paper (110 mm, diam) to 50 mL volumetric flask. The round bottom flask further rinsed with 10 mL of deionized water and added to the filtrate. 0.2 % of lanthanum chloride was added to the mixture and the flask containing the filtrate was filled up to the mark. For each sample the digestion was done in triplicate. Blank samples, a mixture of 3 mL of nitric and 3 mL of perchloric acid were digested following the same procedure as the samples. Finally, the digests kept in refrigerator until analysis.

2.5. Determination of major, minor and trace metals in the samples

10 mg/L (intermediate standard solution) in 100 mL volumetric flask was prepared from by dilution of 1000 mg/L stock solution of FAAS. From the 10 mg/L solution four working standard solutions were prepared at different concentration for each metal (Ca, Mg, Fe, Mn, Zn, Cu, Co, Cd, and Ni). The instrument was calibrated with working standards after the parameters (energy, slit width, lamp current, wavelength and others) were adjusted to give maximum signal intensity. All the thyme and blank samples were analyzed for each metal using absorption mode of FAAS.

Table 3. Instrumental operating conditions for determination of metals using FAAS from thyme leaves.

Element	Wavelength	Detection limit (mg/L)	Slit width (nm)	Lamp current (mA)	Energy
Ca	422.7	0.010	0.7	2.0	**3.690**
Mg	285.2	0.001	0.7	1.0	**3.976**
Cu	324.7	0.020	0.7	1.5	**3.787**
Zn	213.9	0.005	0.7	2.0	**2.986**
Mn	279.5	0.001	0.7	3.0	**3.942**
Ni	341.5	0.040	0.2	3.0	**3.764**
Fe	248.3	0.030	0.2	7.0	**3.635**
Co	240.7	0.050	0.2	4.5	**3.425**
Cd	228.9	0.005	0.7	2.0	**3.043**

2.6. Recovery study

To validate and assess the accuracy of the optimized procedure, spiking experiments were done and the recoveries were examined. This was done by adding a known concentration (1000 mg/L) standard solution of each metal (Buck scientific standard solution) to known weight of the sample before digestion. The spiking was performed in three groups:

1. 400 µL of 1000 mg/L Mg and K respectively were spiked in a flask containing 0.5 g sample.

2. 400 µL and 100 µL of 1000 mg/L Ca, Na, Fe respectively were spiked in other flask with 0.5 g of the same type of sample.
3. 20 µL of 10 mg/L Cd, 20 µL of 100 mg/L Ni, 70 µL of 10 mg/L Co, 20 µL of 100 mg/L Cu, 20 µL of 1000 mg/L Mn and 50 µL of 100 mg/L Zn was spiked in the third group with same amount and type of sample as in the earlier cases. Each group of spike was done in triplicates. All the spiked and un-spiked samples were digested following the procedure described earlier in section 2.4.3 in triplicates. Each sample was analyzed for their mineral contents.

2.7. Method detection limit

Detection limit is defined as the minimum concentration that can be detected by the analytical method with a given certainty. This is often taken as the mean value of the blank plus three times its standard deviation [49]. In the present study, the method detection was estimated from the standard deviation obtained for each metal from the five blank samples.

3. RESULTS AND DISCUSSION

3.1. Optimization of the Digestion Procedure

Degradation and solubilization of the matrix to release all metals for analysis vary with the analytical methods to be used, the concentration range of the analyte, the type of the reagent used, time of digestion, and the type of the matrix in which the analyte exists. A common result of the sample preparation is the dissolution of the entire sample, producing a clear solution. The digestion procedure must be selected to suit the type of sample, the metals being determined, it can give maximum signal of the analyte of interest. [50]

Hence, the choice of the type of reagent, temperature, reagent volume, is a crucial step in developing an optimum digestion procedure. In the present study, a weighed 0.5 g of sample of powdered thyme was digested in a Kjeldahl apparatus hot plate with 3 mL HNO_3 (69 - 72%) and 3 mL $HClO_4$ (70%) at a temperature of 240^0C for 3 h. After venting and cooling, the digest was diluted to 50 mL. Table 4 summarizes different procedures tested for digestion of thyme samples by varying reagent volume, digestion temperature and time.

Table 4. Different procedures tested during procedure optimization for thyme leaf sample digestion.

No	Weight of the sample (g)	Volume of the reagent (mL)			Temperature (^{0}C)	Time (h)	Nature of the digest
		HNO_3	$HClO_4$	Total			
1	0.5	2	1	3	240	3	Yellowish and ppt formed
2	0.5	2	2	4	240	3	Yellowish and ppt formed
3	0.5	3	2	5	240	3	Yellowish
4	0.5	3	1	4	240	3	Yellowish and turbid
5	0.5	4	2	6	240	3	Clear but light yellow
6	**0.5**	**3**	**3**	**6**	**240**	**3**	**Clear and colorless Optimum**
7	0.5	5	1	6	240	3	Clear but yellowish
8	0.5	3	2	5	150	3	Clear but ppt formed
9	0.5	3	3	6	150	3	Clear but light yellow
10	0.5	5	1	6	150	3	Clear but light yellow
11	0.5	2	2	4	240	2. 5	Yellowish and turbid
12	0.5	3	2	5	240	2. 5	Yellowish
13	0.5	3	3	6	240	2. 5	Light yellow
14	0.5	3	3	6	270	3	Clear and colorless
15	0.5 g	3	3	6	270	2 .5	Light yellow
16	0.5 g	3	3	6	240	2.75	Light yellow

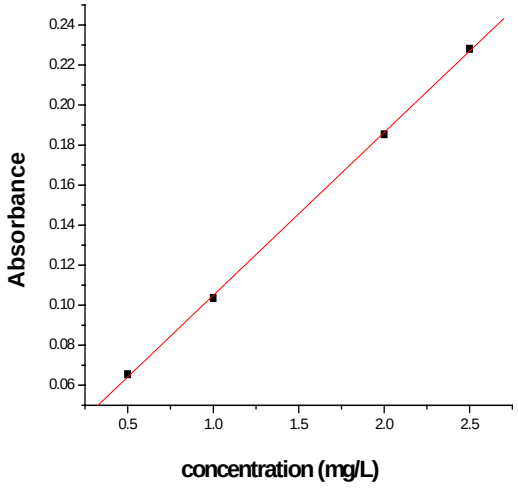
3.2. Instrument Calibration

Calibration requires the establishment of a relationship between signal response and a known set of standards. The standards in atomic spectroscopy refer to the production of a series of aqueous solutions of varying

concentrations (working standard solutions) of the analyte of interest. The choice of the original standard (from which all other dilutions will be made) is important, as any impurity present in this solution will be transferred to all of the working standard solutions [49]. Table 5 shows the wavelengths, the concentrations of the intermediate standards, working standards and correlation coefficients (R) of the calibration curves showed in Fig.1 for each of the analyte metals.

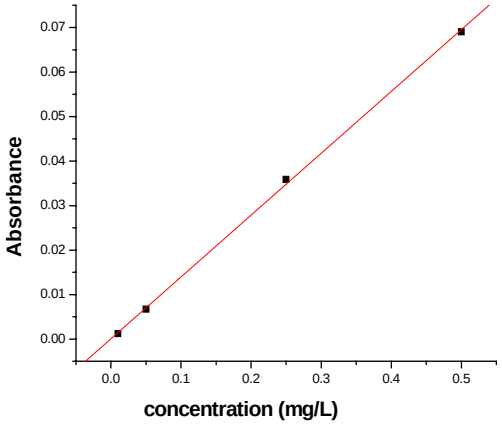
Table 5. Working standards and correlation coefficients of the calibration curves for determinations of metals using flame atomic absorption spectrometer.

No	Metal	Concentration of intermediate standard (mg/L)	Concentration of standards (mg/L)	Correlation coefficient (R) of calibration curves
1	Ca	10	0.5, 1, 2, 2.5	0.9998
2	Mg	10	0.5, 1, 2, 4	0.9994
3	Fe	10	0.5, 1, 1.5, 3	0.9997
4	Mn	10	0.1, 0.4, 1.2, 1.8	0.9997
5	Zn	10	0.1, 0.25, 0.5, 0.75	0.9999
6	Cd	10	0.01, 0.05, 0.25, 0.5	0.9997
7	Ni	10	0.05, 0.3, 1.2, 2	0.9998
8	Co	10	0.1, 0.5, 1, 2	0.9994
9	Cu	10	0.05, 0.1, 0.2, 0.4	0.9990



R =0.9998

(a)



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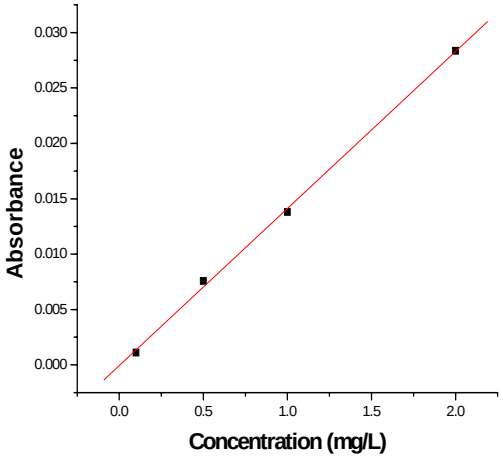
(b)



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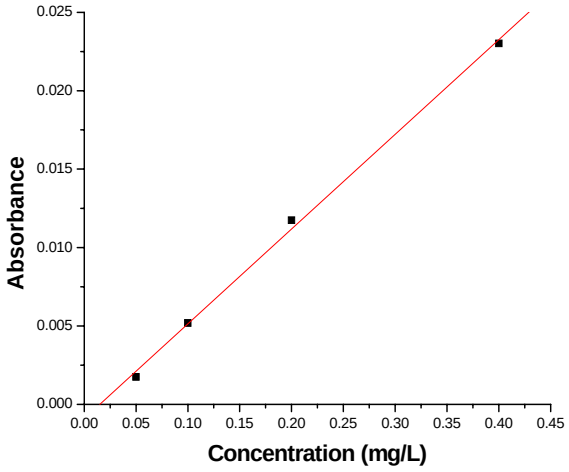
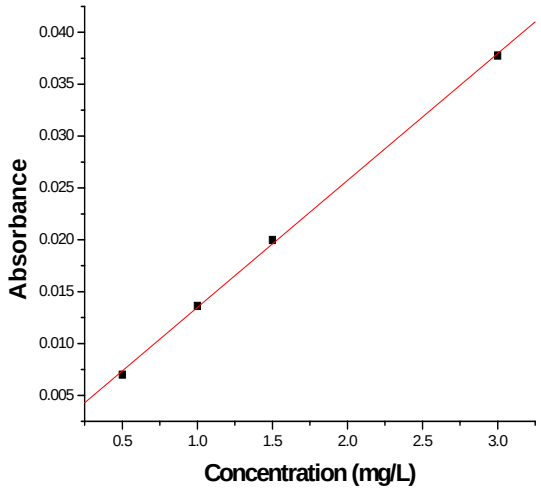
(c)



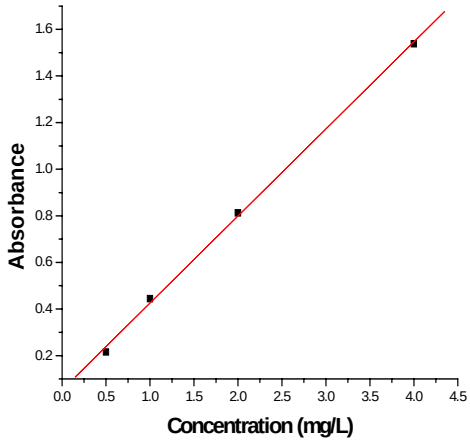
R =0.9990

(d)



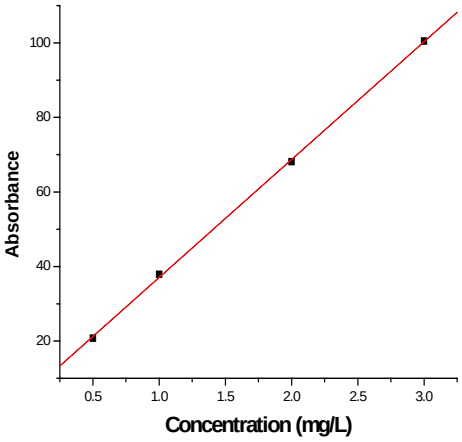
R = 0.9997

(e)



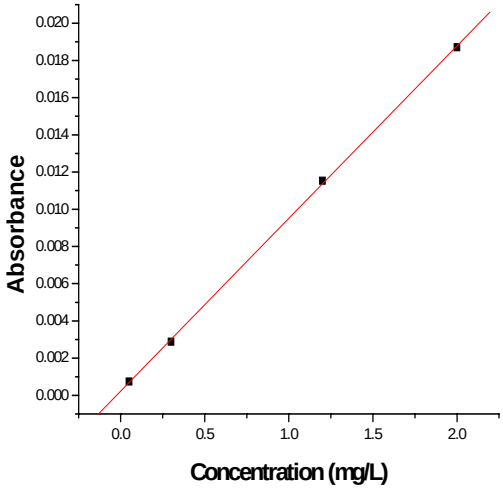
R =0.9994

(f)



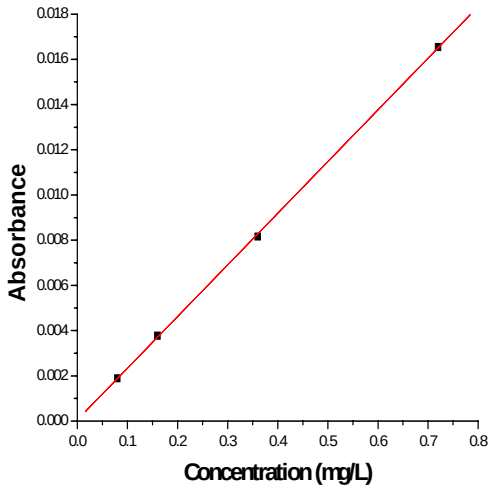
R = 0.9998

(g)



R = 0.9997

(h)



R = 0.9999

(i)

Fig. 1. Calibration curves for analyte metals, (a) Ca; (b) Cd; (c) Co; (d) Cu; (e) Fe; (f) Mg; (g) Ni; (h) Mn; and (i) Zn.

3.3. Evaluation of Analytical Figures of Merit

3.3.1. Precision and accuracy

Precision is defined as the closeness of the grouping of individual results [49]. It can be expressed by repeatability and reproducibility of the individual results. It can be quantitatively expressed by the variance, standard deviation or coefficient of variation of individual measurements [51]. In the present study, the results were evaluated by the pooled standard deviation and relative standard deviation of the results of triplicates (n = 3) for which each sample with triplicate readings. Accuracy is the closeness of the results to the true value [49]. The true value is not known and hence the accuracy and the validity of the analytical method were determined by a spiking experiment.

3.3.2. Method detection limit

The limit of detection of a method may be defined as: the smallest concentration of a determinant for which one can be 95 % confident that the determinant will be detected by the method [51]. The confidence level 95 % can be obliviously altered by the analyst to any level he considers appropriate. This level does however represent a risk of making a wrong decision and should be related to how the determinations are to be used rather than the accuracy of the method [51, 52]. Dean defined the detection limit of a method as the lowest analyte concentration that produces a response detectable above the noise level of the system, plus three times its standard deviation. For example the average blank concentration for calcium in this study was 1.48 ± 0.028 (in mg/L) in 50 mL flask. The method detection limit in 0.5 g of sample diluted to 50 mL where the reagent is included will be 0.084. This implies if the concentration of calcium is less than 8.4 µg/g, the determination was interfered by the noise. In this study; the detection limits for each metal were obtained by multiplying the standard deviation of the five blank solutions by three.

Table 6. Method detection limit for each metal analyte for thyme leaf samples (n = 5).

Metal	Method detection limit (MDL), mg/g
Mg	0.010
Ca	0.0084
Fe	0.034
Mn	0.0069
Zn	0.0018
Cd	0.00006
Cu	0.00047
Co	0.00017
Ni	0.0011

3.3. 3. Method validation

Before a new analytical method or sample preparation technique is to be implemented, it must be validated. The various figures of merit need to be determined during the validation process. Method validation provides a comprehensive picture of the merits of a new method and provides a basis for comparison with the existing methods. A typical validation process may involve one or more of the following steps [50].

- Determination of the single operator figures of merit (accuracy, precision, detection limit).
- Equivalency testing: compared to similar existing methods.
- Collaborative testing with other laboratory.
- Comparing with certified reference material.

Because of the absence of certified reference material for thyme samples in the laboratory for which the analysis made, the validity of the optimized digestion procedure was assured by spiking the samples with a standard of known concentration of the analyte metals. The percentage recovery of each analyte metals as shown in Table 7 is in the range between 92 – 103 % except 89 % for Ni which is in the acceptable range. Hence, the method is of good accuracy.

Table 7. Recovery test for thyme leaf samples.

Metal	Amount Added (µg/0.5 g)	*Amount found (µg/0.5 g)	RSD	%Recovery
Zn	5	4.76 ± 0.323	0.0679	95.1 ± 6.47
Cu	2	2.06 ± 0.132	0.0642	102.76 ± 6.60
Mn	20	19.07 ± 1.483	0.0779	95.35 ± 7.42
Cd	0.2	0.185 ± 0.022	0.1177	92.5 ± 10.89
Co	0.7	0.644 ± 0.076	0.1178	92 ± 10.84
Ni	2	1.78 ± 0.145	0.0812	89.05 ± 7.23
Ca	400	383 ± 15.3	0.0399	95.75 ± 3.83
Mg	400	386 ± 18.45	0.0479	96.5 ± 4.61
Fe	100	96 ± 11.91	0.1240	96 ± 11.91

*Difference between spiked and unspiked samples

RSD = Relative standard deviation

3.3.4. Distribution of macro and micro nutrients in different thyme samples

The concentration of nine metals (Mg, Ca, Fe, Cu, Zn, Co, Ni, Cd, Mn) in some freshly prepared and commercially available Ethiopian thyme samples was determined by FAAS using four point external calibration curve. The results showed that the samples had variable composition of each analyte metals with wide concentration range.

As can be seen from Table 8, Ca (2776 ± 130 μg/g) was observed to be of the highest concentration followed by Mg (1786 ± 13 μg/g). From the studied microelements, Fe (728 ± 58 μg/g) was found in a significant amount compared to Mn, Zn, Ni, Cu, Co and Cd. Cd (1.3 ± 0.08 μg/g) was the least of all the studied metals in DF. The level of metals in this thyme sample as shown in Fig. 2 decreases in the order Ca > Mg > Fe > Mn > Zn > Ni > Cu > Co > Cd.

The study on the thyme sample collected from different supermarkets in Debresina showed incredibly a largest amount of Fe (2517 ± 24 μg/g) followed by Ca (1980 ± 81 μg/g) and Mg (1739 ± 14 μg/g). Cd (0.93 ± 0.09 μg/g) was observed to be the least as in the case of DF. The order of metal concentration follows Fe > Ca > Mg > Mn > Zn > Ni > Cu > Co > Cd.

Ca (2066 ± 11 μg/g) was observed to contain the highest concentration of all the metals studied in AAM followed by Mg (1735 ± 10 μg/g) and Fe (1521 ± 27 μg/g). As in the previous cases Cd (0.87± 0.08 μg/g) was the one with the least concentration. AAM showed a larger amount of Cu (9.30 ± 0.76 μg/g) which was less than Zn and Ni in the case of DF and DM. The level of metals in decreasing order as demonstrated in the graph (Fig.2 and 3) given by Ca > Mg > Fe > Mn > Cu > Ni > Zn > Co > Cd. High amount of Mg (1524 ± 7 μg/g) was obtained from the freshly prepared *Thymus vulgaris* samples, WF. The order, as can be seen in Fig. 2 and 3, of the level of metal is Mg > Ca > Fe > Mn > Zn > Ni > Cu > Co > Cd.

Table 8. Mean concentration (mean ± SD, n = 9, µg/g dry weight) of major, minor, and trace metals in thyme leaf samples.

Element	Concentration of metals							
	DF[a]	% RSD	WF[b]	% RSD	DM[c]	% RSD	AAM[d]	% RSD
Ca	2776 ± 130	4.7	1239 ± 102	8.2	1980 ± 81	4.1	2066 ± 11	0.5
Mg	1786 ± 13	0.7	1524 ± 7	0.4	1739 ± 14	0.8	1735 ± 10	0.6
Fe	728 ± 58	8.0	1103 ± 71	6.5	2517 ± 24	1	1521± 27	1.8
Mn	114 ± 4	3.1	112 ± 8	6.9	37.7 ± 2.6	6.8	43 ± 2.9	6.8
Zn	42 ± 2.9	6.9	52 ± 3.6	6.9	35.3 ± 2.1	6	8.7 ± 0.75	8.5
Cd	1.3 ± 0.08	6.3	1.08 ± 0.09	8.9	0.93 ± 0.09	9.7	0.87 ± 0.08	9.4
Ni	14.2 ± 1.09	7.7	10.2 ± 0.7	6.7	11.7 ± 1.1	9.6	9.83 ± 0.59	6
Co	4.3 ± 0.25	5.7	3.03 ± 0.14	4.8	4.5 ± 0.29	6.5	2.59 ± 0.16	6.2
Cu	8.9 ± 0.56	5.6	7.69 ± 0.66	8.6	10.1 ± 0.38	3.8	9.30 ± 0.76	8.2

[a]DF = freshly prepared thyme samples near the town of Debresina.

[b]WF = freshly prepared thyme sample from Wondogenet.

[c]DM = thyme samples bought from town of Debresina.

[d]AAM = thyme samples bought from Addis Ababa city.

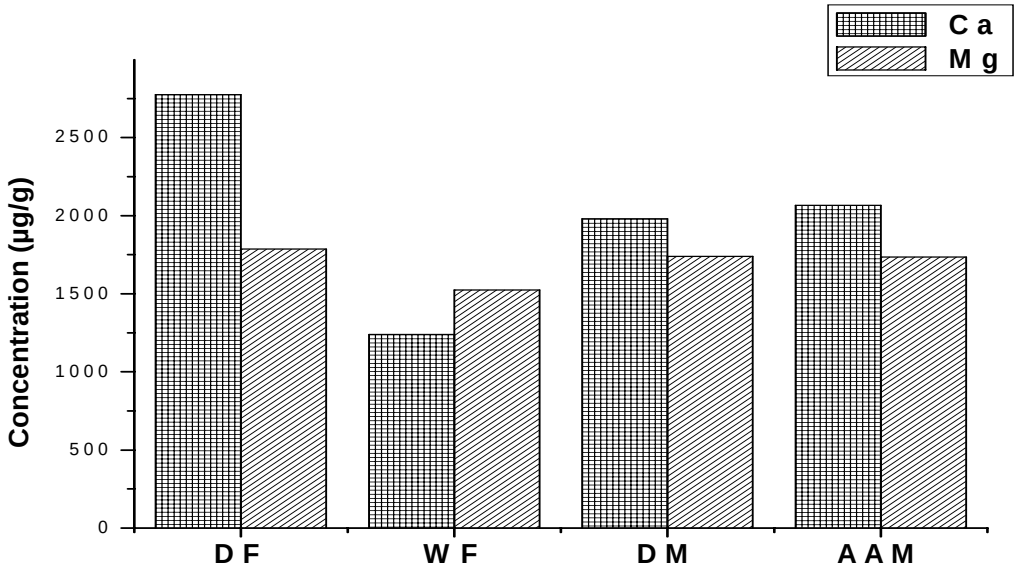
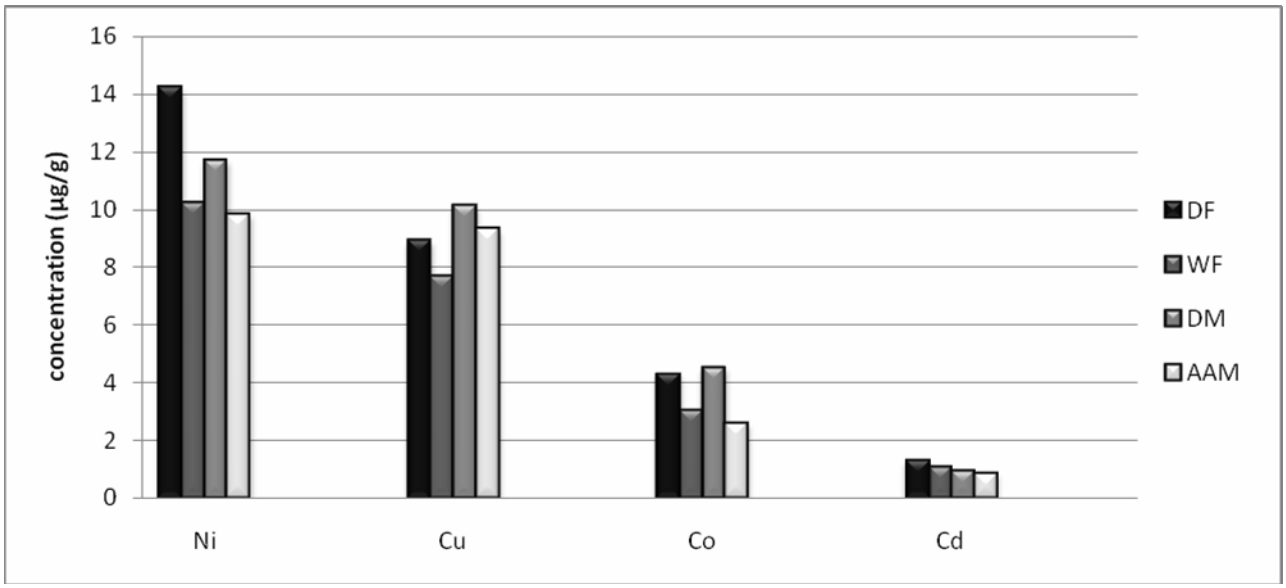
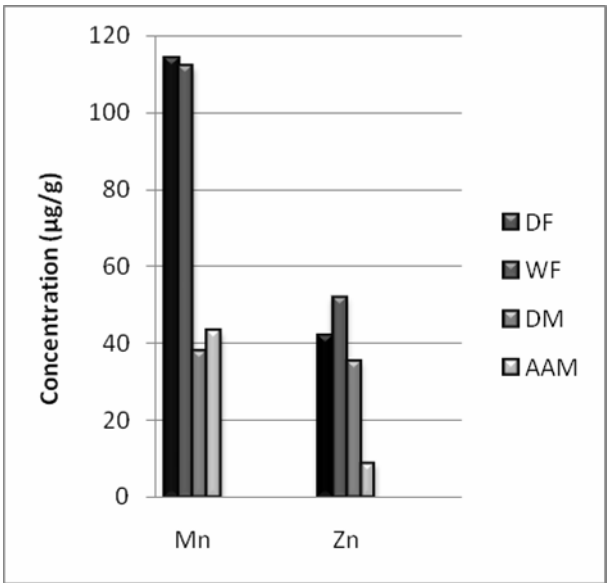
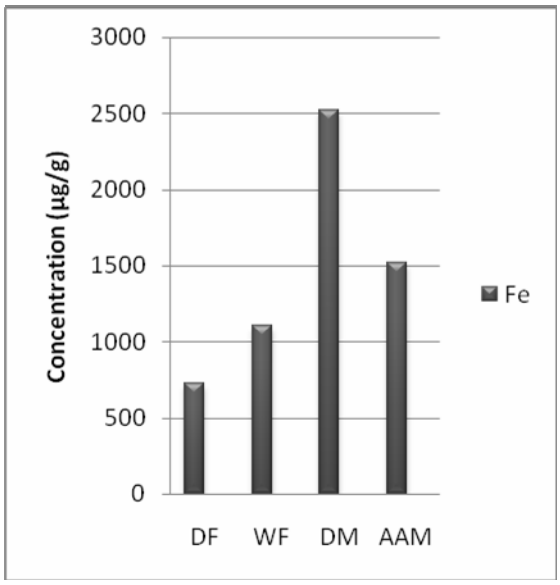


Fig. 2. Concentration of macronutrients in different thyme leaf samples



(a)





(b) (c)

Fig. 3(a, b, c). Concentration of micronutrients in different thyme leaf samples

3.3.5. Comparison of the concentration of macronutrients in different thyme samples

Ca was found to be higher in concentration in the samples analyzed except in WF. It was observed to be 2776 ± 130 µg/g, 1980 ± 81 µg/g, 2066 ± 11 µg/g and 1239 ± 102 µg/g in DF, DM, AAM and WF, respectively. Mg was larger in concentration than Ca (1524 ± 7) in the freshly prepared thyme samples from Wondogenet.

3.3.6. Comparison of the concentration of micronutrients in different thyme samples

All the results from the four samples showed a similar order of magnitude in the level of the concentration of metal except a slight change in AAM where the concentration of Zn is less than Ni and Cu. The order in the other three samples of the level of the microelements is Fe > Mn > Zn > Ni > Cu > Co > Cd.

The results showed a relatively high level of the essential elements Fe, Mn and Zn where Fe exhibited the largest of the three.

The relatively high concentration of Fe reflects the normal composition expected of plant derived products, which most of plant foods and plant derived foods contain Fe in the form of metalloproteins, plant ferritins, Fe present in the sap, and Fe complexed to structural components or storage compounds predominantly as phytates. The incredibly higher concentration of Fe in thyme might be rationalized due to the presence of –OH functional group in the volatile oil constituents which can be readily complexed and hyper accumulated in the plant.

Table 9. Range of concentrations of each analyte metal in all samples.

Metal	Range of concentration (µg/g)
Ca	1239 – 2776
Mg	1524 – 1786
Fe	728 – 2517
Mn	37.7 – 114
Zn	8.7 – 52
Ni	9.83 – 14.2
Cu	7.69 – 9.3
Co	2.59 – 4.3
Cd	0.87 – 1.3

3.3.7. Comparison of the level of metals in *Thymus schimperi* between freshly prepared and market available samples

The concentration of most metals was found higher in the freshly prepared samples than those bought from market except little variation with the micronutrients. The level of metals in DF is significantly (slightly in some cases) higher than DM and AAM. The macro and micronutrients contained with in foods all show varying degrees of stability when foods are stored or processed. In the process of preparation of thyme leaves for usage, there may be a loss of some nutrients and enhancements of certain nutrients through contamination. It is difficult to point out clearly the exact reason for reduction of these nutrients in processed thyme. The accidental (avoidable or unavoidable) losses of the nutrient during storage time and processing hopefully contribute a reasonable effect. Unlike the major nutrients there is a slight increment of some metals in market available thyme. As tried to point out in the above section, as there is nutrient loss during storage and processing, there may be contamination of the nutrients from outside source. The incredible increment of Fe concentration may be attributed to the fact that every processing utilities are made of iron and wood. Moreover there may be a contamination from dust particles during storage and when transported for sale. On the other hand it is difficult to trace the concentration of the metals in the market available with the freshly prepared samples because there is no controlled cultivation in which the leaves are brought to the market place from a specific area. Fig. 4 and 5 summarize the relative concentrations of each of the analyte metals in three samples.

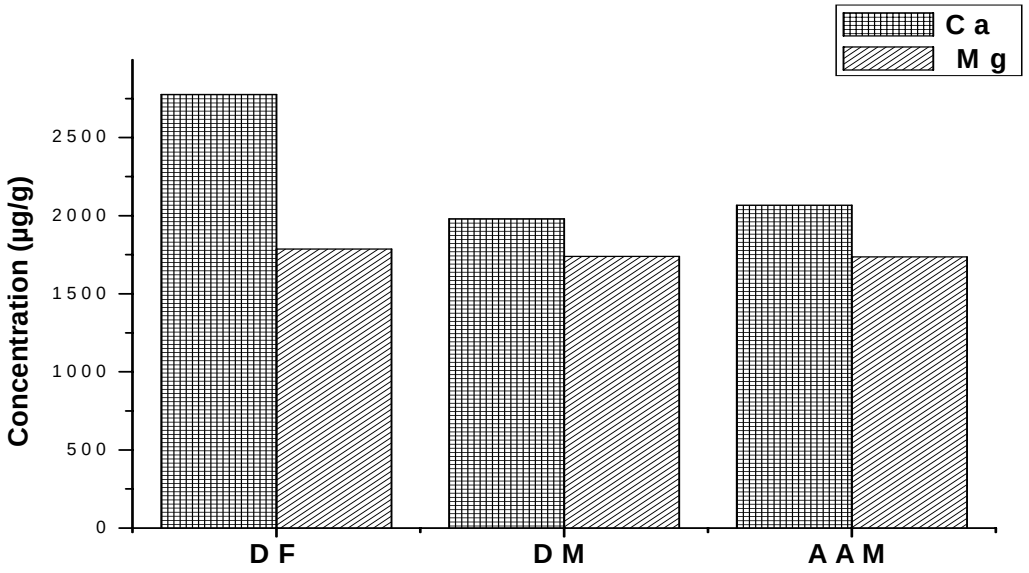
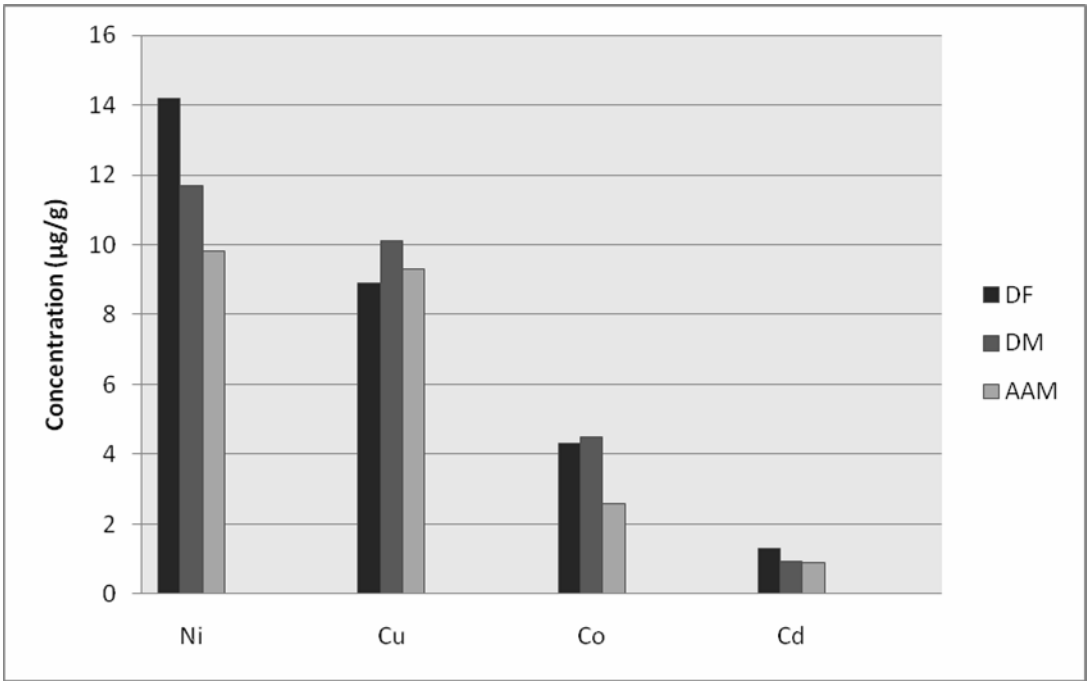


Fig.4

. Distribution of macronutrients in the leaves of *Thymus schimperi*



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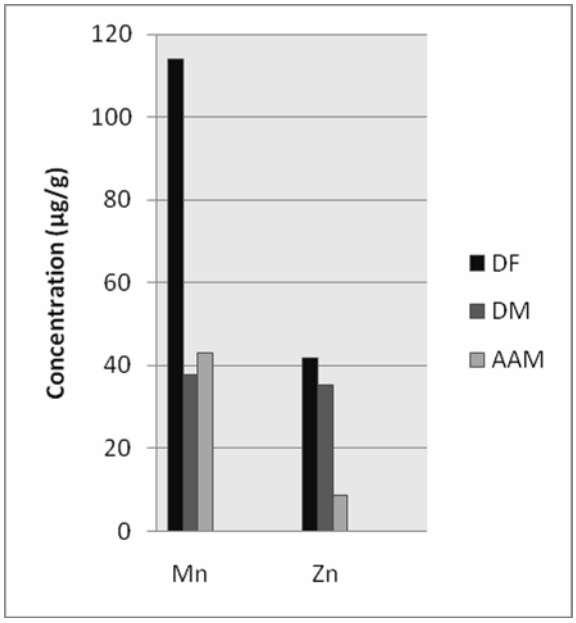

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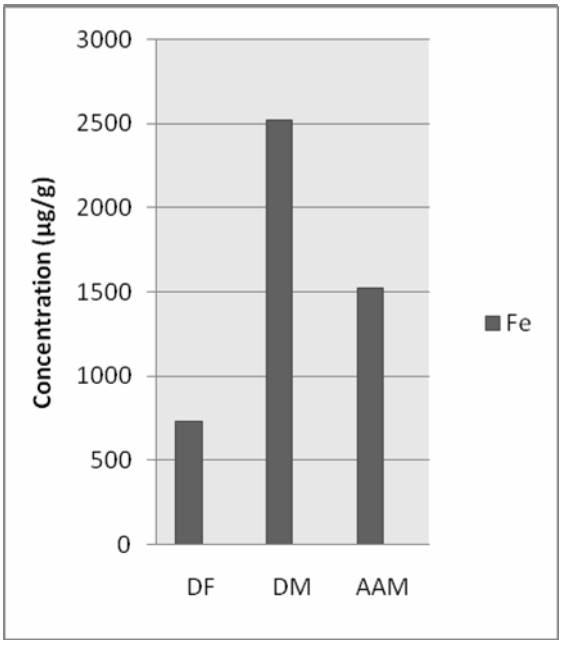

(c)

Fig. 5. Distribution of micronutrients in leaves of *Thymus Schimperi*

3.3.8. Comparison of the level of metal of Ethiopian thyme with other reported values

Earlier studies on different thymus species in different countries, in Ethiopia in particular, focused on the essential oil composition and biological aspects. Studies on the level of major, minor and trace metal composition of the plant was limited except some researchers.

Saeed Khan *et al.* [47] have determined the total metal content using FAAS from the leaves of dried *Thymus vulgaris* samples collected from the growing fields of Zhad near Sharjah. They observed that thyme sample contain; 0.18, 375, 463, 0.09, 25, 96, 1322 in ppm of Cd, Mg, Ca, Cu, Ni, Mn, and Fe respectively. The results revealed that there is a good comparability among the

metals Ni, Cd, and Fe, with the present study even though the concentrations in this study was expressed per dry weight of the powdered sample.

Nnorom *et al.* [43] have also determined the metal content of dried thyme sample collected from a market in southeastern Nigeria using FAAS. They obtained an average concentration of Cd (1.13 ± 0.04), Fe (255.70 ± 231.01) and Zn (37.03 ± 15.66) in µg/g. The results were comparable with the present study except in the case of Fe which is higher in the present case.

Table 10. Comparison of the concentration of macro and microelements in the leaf of thyme sample with earlier reported values.

Metal	**Reported values**			
	United Arab emirates [29] (ppm)	Nigeria [25] (µg/g)	Turkey [47] (mg/kg)	This study (µg/g)
Mg	375	-	-	1524 – 1786
Ca	463	-	-	1239 – 2776
Cu	0.09	-	-	7.69 – 9.3
Ni	25	-	6.93 ± 0.38	9.83 – 14.2
Mn	96	-	-	37.7 – 114
Fe	1322	92.4 – 419.2	-	728 – 2517
Cd	0.18	1.1 – 1.15	-	0.87- 1.3
Zn	-	25.95 – 48.1	-	8.7 – 52

Harum Cifttci *et al.* [40] determined Ni concentration with RP – HPLC from thyme samples collected in Turkey. They used an atomic absorption standard solution of Ni and the chromatographic system was equipped with a Shimadzu LC – 9A pump, SPD – 10AV UV–visible spectrometric detector. A microwave digestion system employing a mixture of HNO_3 and $HClO_4$ was used for sample

decomposition. The result showed that the concentration of nickel in thyme sample to be 6.93 ± 0.38 mg/kg dry matter which was in a good comparability with the result obtained in this study.

3.3.9. Comparison of the concentration level of some selected metals in other plants and thyme

Many authors reported the concentration of metals in different kinds of food items, drinks, and other materials in which human being used for their day-to-day consumptions. Vegetables, medicinal plants spices and condiments are some of the concern areas in different parts of the world. Nnorom *et al*. [43], khan *et al*. [47], Ozkutulu [33], Aiwonegbe [42], and Hashmi [21] are some of them who reported certain selected mineral nutrients.

The concentration of some micronutrients studied by different researchers from different countries showed a varied concentration as can be seen from Table 11. The concentrations of Fe, Mn, Zn, Ni, Cu, Cd ranges from 2.5 – 32.3, 1.7 - 2.6, < 0.01 – 5.93, 0.19 – 0.37, 1.1 – 3.3, and ND – 0.12 in μg/g respectively in different vegetables. The results obtained from thyme in this study are larger than those reported values for different kind of vegetables. Some of the spices and condiments studied and cited in this paper contain lesser amount of Cd (black paper, ginger and clove) and some others contain larger amount except curry powder (ND – 1.8 μg/g) which is in comparable range. The Mn content of all the medicinal plants, spices and condiments are in a comparable range with thyme except a slight increase in the case of clove. On the other hand, Cu, Ni, Zn, and Fe are found in a larger amount in thyme than the other kinds of food materials reported and cited as indicated on Table 11.

Table 11. Comparison some of the observed metal concentration in thyme leaves with some reported values in vegetables, medicinal plants, spices and condiments.

Food types		Metal concentration(µg/g)										Reference
		Cd		Fe		Zn		Ni				
Vegetables (leaf)	➢ Spinach ➢ Cabbage ➢ Carrot	0.12±0.04 ND <0.01±0.00		13.97±0.04 2.5±0.05 4.04±0.03		5.93±0.03 <0.01±0.00 4.03±0.02		0.19±0.05 0.37±005 0.25±0.01				42
		Fe		Cu		Mn		Zn				21
	➢ Mustard ➢ Cabbage ➢ Spinach	16.3±0.03 17.7±0.03 32.3±0.05		3.3±0.01 1.1±0.00 2.9±0.01		2.6±0.01 1.7±0.01 1.8±0.01		5.4±0.03 3.8±0.02 6.1±0.03				
Medicinal plants		Fe (ppm)	Cu (ppm)	Mn (ppm)	Cd (ppm)	Ni (ppm)						47
	➢ Onion ➢ Mint ➢ Coriander	256 568 348	0.1 0.08 0.08	76 72 77	0.06 0.34 0.28	12.8 7 3.44						
Spices and condiments		Cd		Fe		Zn						43
	➢ Bouillon cubes ➢ Chicken seasoning ➢ Curry powder ➢ Beef seasoning ➢ Mixed spices	3.6 – 3.65 3.9 – 5.05 ND – 1.8 0.85 – 4.8 0.80 – 4.9		3.65 – 8.95 11.05 – 32.7 32.35 – 320.85 32.70 – 73. 20 ND – 50.60		1.6 – 4.4 3 – 3.7 13.65 – 29.90 3.70 – 21.25 3.40 – 22.55						
		Cd	Cu	Fe	Mn	Zn						33
	➢ Black paper ➢ Ginger ➢ Clove	0.206 ± 0.0067 0.072 ± 0.0041 0.013 ± 0.0017	11 ±0.05 3 ±0.8 4 ±1.0	374 ±7 34 ±1.1 52 ±4.6	191 ±5 73 ±3.4 355 ±0.7	11 ±0.6 5 ±0.9 6 ±0.5						
Thyme	Fe	Mn	Zn	Ni	Cu	Cd						This study
	728 – 2517	37.7 – 114	8.7 – 52	9.83 – 14.2	7.69 – 9.3	0.87 – 1.3						

ND = Not detected.

Generally, the concentration of metals as compared to other kind of food items studied earlier was quite larger in thyme species reported in this paper and by different authors [43, 47]. This may be attributed to the presence of polyphenols in the leaves of thyme. The polyphenols can be readily complexed with the metals so that they are accumulated in better amount than in the other kind of food condiments, spices, medicinal plants and vegetables.

3.4. Statistical Analysis

In analytical work there may be two or more than two means to be compared to check whether there is a significant difference between them or not. For example, a variation between the means of different sample can be resulted from random and controlled sources of error. This kind of variation and the variation due to the difference in the original sample type can be separated and estimated by a powerful statistical technique known as Analysis of Variance (often abbreviated as ANOVA) [53]. In this study, a one way ANOVA and SPSS (SPSS 15.0 for windows, The Apache software foundation, 2000) software was used to know the variation between samples analyzed was significant or not for the mean concentrations of each metal in triplicate analysis.

For Cd, there is no significance difference ($p \geq 0.05$) at 95 % confidence interval observed between the mean concentrations of the four thyme samples (DF, WF, DM, and AAM). The mean concentration of Ca, Mg, K, Na, Mn, and Cu do not differ significantly ($p \geq 0.05$) for DM compared to AAM. As can be seen from Table 12, the mean concentration of the four samples shows a significant difference ($p \leq 0.05$) for Fe at 95 % confidence level. The samples taken from Debresina and Wondogenet (both freshly prepared) show a significant difference ($p \leq 0.05$) for Ca, Mg, Na, Fe, Zn, Ni and Co at 95 % confidence interval.

Table 12. Observed significance difference in the mean concentrations between the four samples of thyme at 95 % confidence interval.

Metals	Sample type with no significant difference ($p \geq 0.05$)	Sample type with significant difference ($p \leq 0.05$)
Ca	DM and AAM	DF and WF, DM, AAM WF and DM, AAM
Mg	DM and AAM	DF and WF, DM, AAM WF and DM, AAM
Fe	-	DF and WF, DM, AAM WF and DM, AAM DM and AAM
Mn	DM and AAM, DF and WF	DF and DM, AAM WF and DM, AAM
Zn	DF and DM	DF and WF, AAM WF and DM, AAM DM and AAM
Ni	WF and AAM, WF and DM	DF and WF, DM, AAM DM and AAM
Cu	DF and WF, DM, AAM DM and AAM	WF and DM, AAM
Co	WF and AAM, DF and DM	DF and WF, AAM WF and DM DM and AAM
Cd	DF and WF, DM, AAM WF and DM, AAM DM and AAM	-

DF = freshly prepared thyme samples near the town of Debresina.

DM = thyme samples bought from town of Debresina

AAM = thyme samples bought from Addis Ababa city

WF = freshly prepared thyme sample from Wondogenet.

The one way ANOVA shows a difference in concentration between different samples for some metals. This difference could be attributed to the soil type for which the plant grow, the geographical location, difference in processing of thyme and difference that can be resulted from way of transportation to the market place.

4. CONCLUSIONS

In this study the metal content of some freshly prepared and commercially available Ethiopian thyme has been investigated. The concentrations of macronutrients (Ca and Mg) and micronutrients (Fe, Mn, Zn, Ni, Cu, Co and Cd) have been analyzed by flame atomic absorption spectroscopy. The powdered samples were ready for analysis after a digestion of 3 h by a mixture of 3 mL HNO_3 and 3 mL $HClO_4$ with a Kjeldahl apparatus hot plate digester. The recoveries for the eleven analyzed metals were between 92 and 102. 76 % except for Ni (89.05 %) revealing an acceptable digestion method for the analysis of thyme leaf samples.

The level of metals obtained shows a comparable result with other reported values in some cases. The concentration of Fe investigated in this study is higher than the values reported by different authors cited in this paper. The concentration of the non-essential metal Cd, potentially toxic if it is above the recommended level, ranges from 0.87 ± 0.08 to 1.3 ± 0.08 μg/g. This concentration is above the WHO acceptable level (maximum 0. 3 mg/kg) [6]. The Cd pollution may result from household materials like cosmetic containers, fossil fuels, car batteries, etc. Therefore, to use thyme for a day-to-day consumption, it has to be taken care in cultivation, processing, transporting and selling of thyme leaves to the consumer. Statistical analysis shows that there is a variation in the level of some metals between the different samples studied.

As thyme leaves are used for different uses in different areas (added to staple foods for flavoring, used for making tea, used for medication, etc.), the composition with regard the level of minerals is crucial. This study will give brief information about the mineral content of thymus leaves. The study could also be extended to other parts of thyme growing areas so that complete and general information can be obtained about the mineral composition of different thyme species in different thyme species of the country. The study can be

further expanded on the other minerals which were not dealt in this research. Moreover, the mineral content of the soil, where thyme is growing, should be studied so that the level of toxic metals can be monitored and fertilizers can be supplemented for deficient minerals.

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Declaration

I the undersigned confirm that the results reported in this work were obtained by research carried by me under the provision of my advisor in the Faculty of Science, Department of Chemistry, Addis Ababa University in the Academic year 2008/2009.

Name: Awraris Derbie Signature _____

This project has been submitted for examination with my approval as a university advisor.

Advisor_____

Signature _____

Place and date of submission: School of Graduate Studies

Addis Ababa University

June, 2009