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DEPARTMENT OF CHEMISTRY

Master's Thesis (Chem-750)

Level of Essential and Non-Essential Metals in Gesho (*Rhamnus prinoides*) Cultivated in Ethiopia

By

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In Partial Fulfillment of the Requirements for Master of Science Degree in Chemistry (Analytical Chemistry)

June 2011

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Abstract

Rhamnus prinoides leaves and stems have been used for bitterness of local alcoholic beverages in Ethiopia for several hundreds of years and as traditional medicine in some African countries like Kenya and South Africa; never the less, level of essential and non-essential elements of this plant has not been conducted so far. Thus, this study has assessed the level of essential (Mg, Ca, Cr, Mn, Fe, Co, Cu and Zn) and non-essential elements (Cd and Pb) of *R. prinoides* leaves and stems cultivated in Ethiopia using flame atomic absorption spectroscopy. The solutions for the analysis of the metals in leaf and stem mentioned above were prepared by the carefully optimized mixture of HNO_3, H_2O_2 and $HClO_4$ reagents using wet digestion with a reflux condenser. The efficiency of the optimized procedure was validated by spiking experiment and the percent of recovery for all the metals was (92-103 for leaf samples and 91-103 for the stem samples). The concentrations of all the metals mentioned above were analyzed and the concentrations were reported in (mg/kg) except the concentration of chromium at Simche zala in stem sample, the concentrations of cadmium in stem samples at two sample sites (Kesatyebirhan ,Gedga Amba and Migra) and the concentrations of lead in stem at all sample sites were below the method detection limit and hence were not reported. The concentrations of the metals found were: Mg (3202–4622), Ca (6304–22236), Cd (0.81–3.10), Cr (5.10–18.7), Mn (8.12–20.6), Fe (47.9–187), Co (22.2–42.1), Ni (12.8–27.3), Cu (6.5–73.0), Zn (12.2–43), Pb (17.7–25.0) in leaf samples and Mg (2635–5528), Ca (3601–5675), Cd (1.00–1.56), Cr (5.42–16.3), Mn (2.16–3.82), Fe (22.0–124), Co (18.7–91.7), Ni (9.68–19.2), Cu (16.6–233) and Zn (17.4–28.2) in stem samples.

Key words: *Rhamnus prinoides*, Gesho, Leaf, Stem, Minerals, Flame atomic absorption spectroscopy, Local alcoholic beverages, Medicinal uses, Wet digestion

1. INTRODUCTION

1.1. Background of the study

1.1.1. Diversity of *Rhamnus prinoides*

*Rhamnus prinoides (*Amharic, Gesho) is in the family *Rhamnaceae*. The common names of this plant are: dark blinkblaar, dog wood and shiny leaf [1]. It is a wide spread plant species in east and south African countries [2]. The only two *Rhamnus* species that occur in Africa are *R. prinoides* and *R. staddo*. *R. prinoides* is common in many parts of eastern and central Africa [3]. This plant is native to Botswana, Eritrea, Lesotho, Namibia, South Africa, Swaziland, Uganda and it is exotic to Kenya [4]. It also occurs outside Ethiopia in Cameroon, Sudan, and Angola [5].

1.1.2. Ecology and distribution of *R. Prinoides*

R. prinoides grows readily in most soils, but thrives in moist, humus-rich soils [4]. *R. prinoides* can easily be grown from seed. It is tough and frost resistant. The natural habitat of *R. prinoides* is widespread and locally common at medium to high altitudes, along water courses, in riverside forest and at the margins of evergreen forests. On grassy hillsides the tree often appears quite black, or at times it glitters in the sun so conspicuously that it can be distinguished at a distance by this aspect alone. It shares this character with two other trees, *Olea africana* and *Bequaertiodendron magalismontanum*. It casts so deep a shade that it often prevents other growth around it. It grows well in light shade under trees and equally well in full sun [1, 4, 6].

Figure 1. *Rhamnus prinoides* plant.

1.1.3. Cultivation of *R. prinoides* in Ethiopia

R. prinoides grows both as a cultivated plant and a natural component of mountain and revering forest, usually on the edges or in clearings, in all parts of the country from 1400 to 3200 m [7]. Ethno-botanical studies in the Keffa-sheka and north Omo zone of the southern region of Ethiopia and the Metekel zone of the Benshangul-Gumuz region indicated that *R. prinoides* was found to occur in the managed ecosystem of the home garden and as a wild habitat [8]. Although it is quite common to find *R. prinoides* cultivations throughout the country, Tigray, North Shoa around Kara Kori and Sebeta, just west of Addis Ababa, are important centers of cultivation of *R. prinoides* [5]. In Tigray, *R. prinoides* is available in the districts of south of Ahferom, Ganta Afeshaum and a small part of Werei Lake. The main crops cultivated in Ahferom district are *R. prinoides* (equivalent to hops) and wheat. *R. prinoides* is a valuable cash crop; leaves/twigs are harvested three times a year and sold year round. And it is taken from the local area to the Enticho market, where it is sold to markets in western Tigray, in Sheraro and Humera, and also to Adigrat and Mekelle [9].

During the sample collection for this study it had been seen a wide cultivation of *R. prinoides* in Amhara region, north Shoa. The important areas are in the districts: Antsekuyana-Gemza particularly Majetye, Efratana-Gidim particularly around Karakori Keblle and Gina Ager, around

72 km to the north east of Debre-Birhan. Huge amount of *R. prinoides* is brought to Addis Ababa form all these areas. Specially, Ginager and Majetye are the main *R. prinoides* cultivation areas by now. Paradoxically, however, cultivation of *R. prinoides* in some areas of north Shoa is dwindling because it is being replaced by other irrigation products like onion in Majetye and by chat in Karakori. The reason for this is currently, chat and onion are more costly than *R. prinoides*. Gedgamba, Migra and Ilamo kebeles in Sebeta district, Oromia region are also major *R. prinoides* cultivation areas.

The samples for this study were collected from different soil types: clay soil, clay-loam, sand-loam, black and white and different climatic zones; temperate and in some areas low land (Kola). This implies that wide cultivation of *R. prinoides* is possible in most of the Ethiopian regions. The most important thing for the effective cultivation of *R. prinoides* as to my observation during sample collection is that it booms in moisture retaining soil. For instance, in purely clay soil unless it gets sufficient rain fall, the tree dries in a short period of time. And I did not get cultivation of *R. prinoides* in purely sand soil, which does not retain water.

1.2. Uses of *R. prinoides*

1.2.1. Alcoholic uses of *R. prinoides*

R. prinoides has tremendous uses. It has been used for bitterness in the traditional brewing process for hundreds of years in East Africa. *R. prinoides* has potential use as a commercial hopping agent in the beer industry and its stem and leaves have been used for bitterness in traditional brewing process for centuries. An extract of this plant (a naphthalene glycoside, Geshoidin) is responsible for this purpose [10, 11].

Geshodin is a non-toxic substance found in bountiful amounts in the stem and leaves of *R. prinoides* [12]. In Ethiopia, the leaves and stems of this plant are used to impart the characteristic bitter flavor to domestically brewed beverages such as *Tella* and *Tej* and it was estimated that well over 5 million people were consuming these beverages everyday [13].

1.2.1.1. *R. prinoides* and some local alcoholic beverages of Ethiopia

Indigenous Ethiopian fermented beverages include *Tej*, *Tella*, *Borde*, and *Arakie*. Of the traditionally fermented beverages in Ethiopia, the most popular alcoholic beverages are *Tej* (honey wine), *Tella* (a malt beverage like beer) and *Arakie* (distilled liquor). According to a census conducted in 1988, about 11,000 persons were engaged in the trade of traditional beverages in Addis Ababa alone. This was about 32% of the population of the city engaged in internal trade [15]. The role of *R. prinoides* and the preparation procedure of these local alcoholic beverages of Ethiopia are summarized below.

Tella

Tella is a malt beverage based on substrates such as barley, wheat, maize, millet, sorghum, teff or other cereals. It is, by far, the most commonly consumed alcoholic beverage in Ethiopia. According to Samuel Sahle and Berhanu Abegaz Gashe [14], over 2 million hectoliters of *Tella* was thought to be produced annually in households and *Tella* vending houses in Addis Ababa. The way of preparing *Tella* differs among the ethnic groups and depends on tradition and the economic situation although the basic processing steps are similar. The clay container *(insera*) is washed with water and fresh leaves of *grawa (Vernonia amygdalina)* several times. The well-cleaned container is then inverted over smoking splinters of *weyra (Olea europae*a) for about 10 minutes. This will eliminate microorganisms sensitive to antimicrobial components of wood smoke. It also contributes to the desirable flavor of the fermented product. *Bikil* (malt) is the source of amylase for the fermenting cereals used in *Tella* preparation. Although *R. prinoides* may have antibacterial effect against some groups of bacteria, its main purpose in the process is to impart the typical bitter taste to *Tella*. It is also thought to be the source of various chemicals. Moreover, it is assumed that it maintains acidic pH during *Tella* fermentation so as to modify the nature of the mash and inhibits the growth of undesirable microorganism [14, 15].

It has been speculated that the role of *R. prinoides* in *Tella* should be similar to that of hops in beer. Hops add bitterness *via* alpha acids being isomerized into more stable and soluble iso-alpha acids assist in the production of tannins that combine with unwanted proteins, add to beer stability due to their antibacterial properties, and impart characteristic aroma to beer through their essential oils. *R. prinoides* is certainly the main agent that imparts the desirable bitter test to *Tella*. However, unlike hops, *R. prinoides* does not contain essential oils [2]. The processes and microbiology of *Tella* fermentation is described as follows: The fermentation is divided into four phases. During the first phase, powdered leaves of *R. prinoides* are mixed with water in a small earthen pot and allowed to ferment for four days. The fermenting material is commonly called *tinsis*. This is transferred to a large earthen pot and the second stage begins by mixing it with barley malt, pounded stems of *R. prinoides*, pieces of *kita* and water. This is left to ferment for two more days. During the third stage, chopped pounded stems of *R. prinoides*, *bikil*, *enkuro* and water are added to the container and the contents are mixed into thick slurry called *difdif*. This is also allowed to ferment for two more days. At the final stage, the container is filled with water to the brim and the contents are again mixed thoroughly. The container is then sealed to create anaerobic conditions and left to ferment for two more days. At the end of the fermentation, most suspended materials settle to the bottom of the container and the clear liquid produced is called *Tella* [14].

Tej

R. prinoides is more or less a staple plant in Ethiopia, where *Tej* is beloved by all and *R. prinoides* is essential to its preparation. No one knows exactly how or when the Ethiopians first decided to mix honey with water and then flavor and ferment it with *R. prinoides*. Excavations at Aksum have found accounts of the consumption of honey wine and its use in rituals. The Encyclopedia of World Environmental History says that *Tej* is thought to be one of the oldest alcoholic beverages ever produced [16].

Tej is a home processed and commercially available honey wine. Often times, widely for commercial purposes, a mixture of honey and sugar may be used as major fermentable substrate [14, 16, 17]. During the preparation of *Tej*, the fermentation pot is seasoned by smoking over glowing splinters of *Olea africana* and smoldering *R. prinoide*s and put back to the fermenting must. Honey, which may contain various impurities including wax, is mixed with water and placed in the smoked pot. The pot is covered with cloth and allowed to ferment in warm place for 2-3 days. At this stage, the wax and top scum is removed. A portion of the fermenting honey is boiled with pieces of washed *R. prinoides* bark and stems and it is put back to the fermenting must. The pot is covered and fermented continuously for five more days, in warmer weather, or for 15-20 days, in cooler environments. The mixture is stirred daily and finally filtered through cloth to remove sediment and *R. prinoides*. Fermentation of *Tej*, like other traditionally fermented alcoholic beverages, relies on the microorganisms present in the substrates, fermentation vats and equipment. The lactic acid bacteria are known to produce a variety of chemical compounds relative to fermentation conditions. Their metabolic products contribute to the acidity and also add distinctive flavor and aroma to the fermenting material. Yeasts of the genus *Saccharomyces* were reported to be responsible generally for the conversion of sugars to ethanol in *Tej*. However, as *Tej* fermentation is a natural fermentation, variability in lactic acid and yeast flora may result in variability in acidity, flavor and alcohol content of the product [14, 16].

Arakie

Arakie is a distilled beverage. It is a colorless, clear, traditional alcoholic beverage which is distilled from fermentation products prepared in almost the same way as *Tella* except that the fermentation mass in this case is more concentrated. *Arakie* is usually brewed in rural and semi-urban areas and is used more commonly by farmers and semi-urban dwellers than by people who live in the cities. In cities, those who drink *Arakie* are predominantly lower class people or those who have economic dependent on alcohol and cannot afford to buy industrially produced alcohol. Ground *R. prinoides* leaves and water are kept for three to four days and after that a *kita* made of *teff* or other cereals and germinated barley or wheat is added. The mixture is allowed to ferment for five to six days and then distilled. In the villages distillation is carried out with primitive equipment made of gourds and wood [15, 17].

Other commonly used Ethiopian alcoholic beverages are *Korefe* and *Katicala*, *Korefe* is the name of the local beer made in Begemder Province among the Koumant ethnic group. Dehusked barley is left in water overnight, and after that toasted and milled. It is mixed with water and dried *R. prinoides* leaves, and fermented in a clay container for two to three months. When the beverage is needed, a small quantity of the mixture is taken, more water is added and after a day's fermentation the beverage is ready for consumption. And *Katicala* is other homemade distilled drink made from maiz or millet [17].

1.2.2. Medicinal uses of *R. prinoides*

R. prinoides has also several traditional medicinal uses in Africa. A decoction of the root is taken as a blood purifier, to treat pneumonia, gonorrhea, rheumatism and stomach-ache and as a gargle, flu/cold, back pains, brucellosis, strength/nutrient supplement, enhancing/facilitating digestion [2, 18, 19].

In Kenya, upper respiratory infections are among the most common infections encountered in outpatient facilities. Some of these infections are becoming difficult to control because some of the causing microorganisms have acquired antibiotic resistance and hence the need to develop new drugs with higher efficacy. Ethno-botanical studies have now been found to be instrumental in improving chances of discovering plants with antimicrobial activity in new drug development. Different parts of *R. prinoides* plant have been used as traditional remedies in central Kenya. The boiling soup of the root is used for the treatment of common cold; the boiled leaves are used for chest pain and the boiled roots or stem are used for the treatment of tonsil [20]. It had been studied that leaf paste mixed with butter as ointment is used for the treatment of Eczema (Amharic, chiffea) by people in Zegie Peninsula, northwestern Ethiopia [21].

Extracts of *R. prinoides* is used for the inhibition of Alzheimer's disease (AD). Alzheimer's disease (AD) is a neurodegenerative disease affecting the brain. It is the most common cause of dementia, leading to deterioration in vital cognitive processes such as memory, understanding and speech Symptoms can also include unpleasant behavioural changes, such as anxiety and dysphoria. Currently there is no cure for AD. To date the most promising target for the symptomatic treatment and slowing of AD progression is cholinesterase inhibitors. In AD the destruction of cholinergic neurones causes a depletion of the neurotransmitter, acetylcholine (ACh). By inhibiting acetylcholinesterase (AChE), the enzyme which catalyses break down of ACh, levels of this neurotransmitter can be elevated and function improved. Sonicated aqueous extracts of *A. nilotica* and *R. prinoides* displayed significant AChE inhibition [19].

Methanolic or hot extract of the leaf and root bark of *R. prinoides* has been also used for malaria treatment either alone or in combination with chloriquine in Kenya. Extracts from eight medicinal plants representing five families, used for malaria treatment in Kenya were screened for their *in vivo* anti malarial activity in mice against a chloroquine (CQ) resistant *Plasmodium berghei* NK65, either alone or in combination with CQ. Extracts of three plants, *Toddalia asiatica* (root bark), *R. prinoides* (leaves and root bark) and *Vernonia lasiopus* (root bark) showed high chemo suppression in the range 51–75% [22, 23].

1.3. Chemical composition of *R. prinoides*

Twenty compounds consisting of seven glycosides of emodin anthrone, five flavonoids and three naphthalenic derivatives have been isolated and characterized from this plant. Organoleptically the most important substance is the naphthalenic glucoside, geshoidin, which is responsible for the characteristic bitter flavor of the beverages derived from this plant [3].

The compounds are: naphthalenic derivatives (musizin, sorigenin and geshoidin), the flavonoids (chrysophanol, physcion, emodin and 10-oxoprinoidin) and the anthraquinone (quercetin, 3-methylquercetin, rhamnocitrin, rhamnazin) glycosides were identified from the leaves of *R. prinoides* [10].

Though this plant has profound of uses, no report was found on the level of essential and non-essential elements in Ethiopia or elsewhere, hence it is worth full investigating the level of essential and non-essential elements and this study will inspire other researchers for collaborate research to be carried out specially, on the role of the leaves and stems of the plant on alcoholic beverages.

1.4. The role of metals in plants to human health

Plants are capable of absorbing a wide range of mineral ions with relevance to human nutrition and health. Hence, plant foods can serve as dietary sources of all essential minerals required by humans. The 14 mineral elements: N, S, P, K, Ca, Mg, Cl, Fe, Zn, Mn, Cu, B, Mo, and Ni defined as essential for plant growth and reproductive success have developed various forms of molecular machinery (membrane transporters) to acquire these mineral nutrients from their soil environment. Na, Cr, I and Se are required by humans, but not by plants. However, plants can acquire these other elements through non-specific influx processes using existing transporters localized to their roots [24]. Plants can manufacture vitamins, essential amino acids, and fatty acids but they cannot manufacture minerals. Instead plants draw in minerals from the soil [25].

1.4.1. Classification and uses of minerals

The elements necessary for living organisms can be classified either based on their importance or amount needed by the body. Based on their importance, they can be essential (like K, Mg, Ca, Mn, Fe, Co, Cu, and Zn) or they may be non-essential (like Cd and Pb). Nutritionally (based on the amount needed), minerals are grouped into macro-minerals and micro-minerals. Elements, such as Mn, Cr, Fe, Co, Cu, Zn, Se, Mo, F and I are essential trace elements, while elements like Ca, Mg and K are grouped under essential macro-elements [26].

The uses of some essential (macro and micro elements) and the role of toxic elements on human health are briefly described below.

1.4.1.1. Macro-essential metals

Macronutrients are found and needed in plants in relatively higher amounts than micronutrients. The elements like C, H, O, N, P, S, K, Ca, Mg, may be defined as macronutrients for plants [27]. The uses of macro elements for human being included in this study are briefly described below.

Calcium

Calcium is used for a variety of structural roles, as a cytoplasmic secondary messenger, linking a range of external stimuli to their physiological responses, and as a counter ion for inorganic anions, forming crystals such as calcium oxalate and calcium carbonate in the vacuole of specialized cells known as idioblasts [28]. Building and maintaining strong bones and teeth, controlling muscle growth, maintaining blood pressure and making blood clot are the structural and physiological roles of calcium. In addition, it enables other molecules to digest food and make energy for the body. Lack of calcium results in several deaths: it is possible to get leg cramps, muscle spasms, our bones may become brittle and we may even have an increased risk of getting colon cancer. Also, when we don't get enough calcium in our diets, our bodies will actually use the calcium that we have stored in our bones. This makes the bones thinner and more brittle [29].

Magnesium

Magnesium is essential element for both plants and animals. It is required by our bodies for numerous different functions. We need it for the proper growth, formation and function of our bones and muscles. It prevents some heart disorders and high blood pressure. Higher intake of magnesium is also associated with improved lung function. Our bodies use it to help convert our food into energy. That is magnesium is essential in allowing our body to control insulin levels in our blood. This means that it is very important in the amount of energy that our body has to operate. As a result, it is suspected that taking extra magnesium might be beneficial for those suffering from fatigue. In addition, it helps our bodies absorb calcium and potassium. It also helps our brains function normally and helps to prevent depression and together with calcium it controls how our muscles contract. It is sometimes injected into patients' veins in emergency situations such as an acute heart attack or acute asthma attack. In non-emergency situations, magnesium is sometimes given to asthma sufferers in a pill form. It relaxes the muscles along the airway to the lungs, which allows asthma patients to breathe easier [29].

Nevertheless, excessive intakes of magnesium are harmful to people with impaired renal function resulting in magnesium retention often associated with hypomagnesaemia. Early symptoms of hypomagnesaemia include nausea, vomiting, and hypotension. And its depletion results gastrointestinal tract abnormalities associated with mal absorption or excessive fluid and electrolyte losses; renal dysfunction with defects in cation reabsorption; and general malnutrition and alcoholism [25].

1.4.1.2. Trace essential metals

Micronutrients are involved in numerous biochemical processes and an adequate intake of certain micronutrients relates to the prevention of deficiency diseases [30]. The elements present only in trace concentrations in the human body but still having a well-defined biochemical function are chromium, cobalt, copper, iodine, iron, manganese, molybdenum, selenium, and zinc [31, 32]. Deficiencies of micronutrients are a major global health problem. More than 2 billion people in the world today are estimated to be deficient in key vitamins and minerals, particularly vitamin A, iodine, iron and zinc. Most of these people live in low income countries are typically deficient in more than one micronutrient [33]. The uses of some trace metals included in this study are briefly described below.

Chromium

Chromium acts as a cofactor in maintaining the normal metabolism of glucose [30]. It has characteristics that range from being an essential trace element, important in glucose metabolism, to a chemically inert and biologically inactive material (the metal itself) or to the extreme case of ultimately being a genotoxic carcinogen, depending on the state of oxidation of the chromium. Generally, trivalent chromium compounds are neutral or essential to organisms, but hexavalent compounds are toxic [34].

Manganese

Manganese (Mn) is regarded as essential for human nutrition because it is an activator and constituent of many enzymes present in humans [32]. It is an essential component of numerous enzymes involved in bone formation and in the metabolism of amino acids, lipids, and carbohydrates. Its deficiency can cause poor reproductive performance, growth retardation, abnormal formation of bone and cartilage, and an impaired glucose tolerance [35]. However, at excessive levels in the brain, produces extra pyramidal symptoms similar to those in patients with Parkinson's disease and decreased learning activity in school-aged children, and increased susceptibility for violence in adults [30].

Iron

The element iron has many functions in the body. This element is used by the body to make tendons and ligaments. Certain chemicals in our brain are controlled by the presence or absence of iron. It is also important for maintaining a healthy immune system and for digesting certain things in the food that we eat. The iron we obtain from our diet is an essential part of hemoglobin the part of our blood that carries oxygen. If we don't get enough iron in our diets, our blood won't carry enough oxygen to our bodies and hence we may feel tired, have decreased alertness and attention span and our muscles may not function properly and we can suffer from anemia [29]. However, great care must be taken not to take too much iron, as excess amounts are stored in the body's tissues and adversely affect the body's immune function, cell growth and heart health [26].

Cobalt

Cobalt (Co) is essential in trace amounts for human life. It is part of vitamin B-12, and plays a key role in the body's synthesis of this essential vitamin. It is a necessary cofactor for making the thyroid hormone thyroxin. It has also been used as a treatment for anemia, because it causes red blood cells to be produced. The toxicity of cobalt is quite low compared to many other metals in soil. Exposure to very high levels of cobalt can cause health effects on the lungs, including asthma, pneumonia, and wheezing [30].

Nickel

Nickel has not been shown to be an essential nutrient for humans, but it may serve as a cofactor or structural component of specific metalloenzymes with a variety of physiologic functions in lower animals. Nickel has been shown to facilitate ferric iron absorption or metabolism [32]. Ni plays some important role in biological systems such as in enzyme activity in hormonal control and also in RNA, DNA, and protein structure or function [30]. However, exposure to nickel by inhalation causes adverse effects, including lung irritation, and pneumonia [36].

Zinc

Zinc is an essential trace element required by all living organisms because of its critical roles both as a structural component of proteins and as a cofactor in enzyme catalysis [31]. More than 300 enzymes require Zn for their activity; it plays an important role in the DNA replication, transcription, and protein synthesis, influencing cell division and differentiation. Zn has a relationship with many enzymes in the body and can prevent cell damage through activation of the antioxidant system. It is an essential component of the oxidant defense system and functions at many levels [41]. Zinc deficiency is the most widespread essential trace element deficiency in the world, perhaps affecting as much as one third of the world´s human population. Large areas of the world have soils that are unable to supply staple crops, such as rice, maize, and wheat, with sufficient zinc. In several countries large proportions of the arable soils are affected by zinc deficiency, such as in India where around 45% of soils are deficient in zinc [31]. Zn deficiency, resulting from poor diet, alcoholism and malabsorption, causes dwarfism, hypogonadism and dermatistis, while toxicity of Zn, due to excessive intake, may lead to electrolyte imbalance, nausea, anemia and lethargy [30]. In addition, excess zinc poses abdominal pain, dizziness and lack of muscular coordination [37].

1.4.1.3. Non- essential metals

The elements such as lead, cadmium, arsenic, beryllium, mercury, and barium have not shown any beneficial function in human beings hence they are classified as non-essential [38].

Cadmium, lead and mercury are common air pollutants, being emitted mainly as a result of various industrial activities [39]. The metals cadmium, mercury, lead, arsenic and aluminum are wide spread toxic elements that are exceptionally toxic to human physiology [40]. Lead and cadmium are the two most abundant toxic metals in the environment [41].

Cadmium

Cadmium (Cd) is one of the most toxic heavy metals and is considered non-essential for living organisms [42]. It is a toxic to virtually every system in the animal body. It is almost absent in the human body at birth, however accumulates with age [43]. Kidney and bone are the critical target organs with regard to environmental exposure to cadmium. The main critical effects include increased excretion of low-molecular weight proteins in the urine as a result of proximal tubular cell, an increased risk of osteoporosis, high blood pressure, destruction of testicular tissues and red blood cells [37, 39]. Cadmium is 10 times more toxic than lead, and is an element to which humans are readily exposed due to its large industrial use. This metal has been also associated with problems in respiratory pathways, including lung cancer and a very small mutagenic effect [38].

Lead

Lead is considered to be among the most dangerous metals for human health because it affects the central nervous system, causes anemia and gastrointestinal damage, and is associated with alterations in genetic expression [38]. Impairment of neurodevelopment in children is the most critical effect of lead. Exposure in uterus, during breastfeeding and in early childhood may all be responsible for the effects. Lead accumulates in the skeleton, and its mobilization from bones during pregnancy and lactation causes exposure to fetuses and breastfed infants. Hence, the lifetime exposure of woman before pregnancy is important [39].

1.4.2. Possible causes of metal exposure

Metals are natural components in soil, however, there are environmental (water, air, soil, dust), occupational, medicinal, and dietary sources of metal exposure [32]. The presence of metals in food can be caused by different sources, such as by direct contamination during production, from metal-rich soil, air, or contaminated water, or from the use of pesticides or fertilizers. Food can also be contaminated during transport, industrial processing, or during storage [38].

1.5. Objectives

1.5.1. General objective

The main objective of this thesis is to investigate the level of essential and non-essential metals in the leaf and stem of *R. prinoides* cultivated in Ethiopia.

1.5.2. Specific objectives

(i) To develop an optimum working procedure for digestion of *R. prinoides* leaf and stem samples for the determination of essential and non-essential metals using FAAS.

(ii) To determine the levels of essential and non-essential metals (Ca, Mg, Fe, Zn, Cu, Co, Cr, Mn, Ni, Cd, and Pb) in *R. prinoides* leaf and stem samples cultivated in Ethiopia.

(iii) To correlate the levels of minerals in the leaf with that of stem of *R. prinoides* taken from the same sample site and to determine the co relational relationship among metals both in leaf and stem.

(iv) To compare the levels of minerals in *R. prinoides* leaf and stem in different sample sites.

2. EXPERIMENTAL PART

2.1. Equipments and reagents

2.1.1. Equipments and glasswares

A drying oven (Digital Heat, J.P. Selecta, Spain) was used to dry *R. prinoides* leaf and stem samples. Electronic blending device (Moulinex, France) was used for grinding and homogenizing the sample. Mortar and pestle was used to grind *R. prinoides* stem sample. Electronic series balance (LA204), with precision of ± 0.0001 g was used for weighing leaf and stem samples. A 100 mL round bottomed flasks fitted with reflux condensers were used in Kjeldahl (Gallankamp, U.K.) apparatus hot plate to digest the dried and powdered leaf and stem samples. Borosilicate volumetric flasks (50 and 100 mL) were used during dilution of sample and preparation of metal standard solutions. 1, 2, 5 and 10 mL Pipettes (Pyrex, USA) were used for measuring oxidizing reagents during dilution of the digestate and for the addition of lanthanum nitrate to the digest ate, micropipettes (Dragonmed, 1-10 L, 100-1000 L, Shangai, China) were used during measuring different quantities of volumes of metal standard solutions for spiking. Flame atomic absorption spectrophotometers (Buck Scientific Model 210VGP AAS, East Norwalk, USA) equipped with deuterium arc back ground correctors and hollow cathode lamps with air-acetylene flame was used for the analysis of the analyte metals (Ca, Mg, Cu, Zn, Mn, Ni, Fe, Co, Cr, Pb and Cd in the leaf and stem samples.

2.1.2. Reagents and chemicals

HNO_3 (69-72%, Spectrol, BDH, England), $HClO_4$ (70%) (Fine Chem, Mumbai, India) and extra pure hydrogen peroxide 30% H_2O_2 (Scharlau, European Union) were used for digestion of leaf and stem samples. Lanthanum nitrate hydrate (98%, Aldrich, USA) was used to minimize the precipitation of calcium and magnesium ions in the form of phosphates and sulfates. Stock standard solutions containing 1000 mg/L, in 2% HNO_3, of the metals Ca, Mg, Cu, Zn, Mn, Ni, Fe, Co, Cr, Pb and Cd (Buck Scientific Puro-Graphic) were used for preparation of calibration standards and in the spiking experiments. De-ionized water was used for dilution of sample and intermediate metal standard solutions prior to analysis and rinsing glassware. H_2SO_4 (98%) and potassium dichromate were used for washing round bottomed flask.

2.2. Procedures

2.2.1. Sampling of plant material

Sampling, the first and one of the most important steps, has the role to ensure the representative of the sample in the context studied. For this reason it represents the most potential source of errors [44]. If sample taken is not representative of the general population, all the careful and costly work put in to the subsequent analysis will be wasted because the results will be invalid [46]. Careful sampling techniques ensure a representative sample while precise analytical methods ensure reliable data [45].

The elemental content of a plant is not a fixed entity, but varies from month to month, day to day, and even hour, as well as differing between the various parts of the plant itself. In general, tissues that either physiologically young and undergoing rapid change in elemental content or those past full maturity should not be sampled. Frequently, no single time for sampling of a particular plant part is ideal for evaluating every element; therefore, several plant parts at different growth stages may need to be taken. Comparisons of analyses between leaves and petioles, stems and leaves, and upper and lower plant parts may assist in evaluating a plant analysis [45, 46]. In this study, sampling was done in a zigzag manner in area coverage of roughly 2 hectares and the approximate distance between two nearest *R. prinoides* plant from which leaf and stem samples were taken was roughly three meters. Sampling of stem was similar to that of leaf sampling; however, the number of stems sampled was less than the number of leaves sampled. The amount of the samples collected is summarized in Tables 1 and 2.

Table 1. Mass (dry) and approximate number of leaf samples collected.

Name of sample site	Region	Mass of samples collected (g)	Approximate number of leaves
Simchezala	Tigray	136	985
Edaga-Arbi	Tigray	258	1867
Adifeila	Tigray	182	1316
Kesatyebirhan	Tigray	149	1083
Majetye	Amhara	176	1275
Karakori	Amhara	132	954
Tamo	Amhara	289	2096
Gedga Amba	Oromia	120	866
Gedga Amba and Migra	Oromia	250	1813

Table 2. Mass of stem samples collected from different sample areas (dry mass).

Name of sample site	Region	Mass of samples collected (g)
Simchezala	Tigray	69.2
Edaga-Arbi	Tigray	89.2
Adifeila	Tigray	69.0
Kesatyebirhan	Tigray	112
Majetye	Amhara	150
Karakori	Amhara	86.4
Tamo	Amhara	175
Gedgamba	Oromia	50.5
Gedgmba and Migra	Oromia	110

2.2.2. Sample collection and transportation

Nine *R*. *prinoide* leaf and stem samples from three regions of Ethiopia were used in this study. Fresh leaf and stem of *R*. *prinoides* samples were collected from the farmlands of three regions of Ethiopia: Tigray, Amhara and Oromia. In Tigray, the samples were collected from Eastern zone particularly, Gantafeshum district, Kebelle Kesatyebirhan and from Central zone, Ahferom district in particular areas: Adifeila, Edaga Arbi and Smiche zala. In Amhara, the samples were collected from three major cultivation areas of north Shoa. These are Antsekuyanagemza district (particularly, Majetye), Efratanagidim (particularly, Karakori) and Gina Ager district (Particularly, Tamo Kebelle). And in Oromia, the samples were collected from Sebeta district (Gedja Amba and Migra). These sampling sites were selected because they are the major cultivation areas of the plant in the country as it was revealed by the previous researcher Berhanu Abegaz [5].

The samples were collected in a three weeks interval. The leaves sampled were those similar to those that are harvested by the *R. prinoides* cultivators. Very young, very old leaves and yellow leaves were not sampled. The leaves sampled include the bottom, medium and the top part of the plant. The amount of the leaf and stem samples collected from each sample sites was not necessarily equal; the target idea was to take representative sample of the selected areas. The decision for describing as one sample from one block sample site was the soil and climate. Even in a very close area, if either the soil or the climate was believed to be quite different the samples taken from these areas were considered as different samples. This was happened in Ahferom and Sebeta districts.

The samples collected were paced and labeled in polyethylene plastic bags. Both leaf and stem samples were then partially sun dried in the sample collection areas. Finally, the samples from each sample site were brought to Addis Ababa University for further pretreatment and analysis.

2.2.3. Cleaning of apparatus

The apparatus and containers that are used for wet digestion procedures must be carefully cleaned and tested for any possible contamination. Usually, it is sufficient to boil the flasks in concentrated nitric acid followed by rinsing several times with ultra pure water before use. Specially, in trace analysis, not only is the shape and size of the vessel important but the wall material must be chosen to avoid cross contamination as well as losses by adsorption. If the wall surface is large compared to the content of the vessel, adsorption losses easily occur. Those losses in turn may cause contamination of the next sample (memory effects). A material such as PTFE, although seemingly perfectly stable and inert, may gradually increase its surface area (cracks). Losses may occur not only by adsorption but also by volitalization, which occurs especially in open vessels (e.g. dry ashing, fusion) [50, 51]. In this study, volumetric flasks were washed with detergents and tap water and soaked in 10% (v/v) HNO_3 for 24 hours followed by rinsing with deionized water and kept in a refrigerator after drying. The digestion flasks were first washed with tap water and soaked in 1% (w/v) potassium dichromate in 98% (v/v) H_2SO_4 then soaked in 10% (v/v) HNO_3 and finally rinsed with deionized water. The condensers were also washed first with tap water and then soaked in 10% (v/v) HNO_3 for 24 hours at some intervals of time and were cleaned by heating with acidified deionized water for 30 min and then rinsed with deionized water before starting sample digestion.

2.2.4. Sample preparation for elemental analysis

Sample preparation is one of the most critical and time consuming steps involved in elemental analysis of plant tissue [47]. And it involves steps from simple dilution to partial or total digestion [48]. It is also critical in obtaining accurate analytical data and reliable interpretation of plant analysis results. Proven procedures should be followed during decontamination, drying, particle-size reduction, storage, and organic matter destruction. Each of these preparation procedures provides opportunities to enhance the accuracy and reliability of the analytical results [46]. For this purpose, samples are prepared by digestion. Sample digestion is often a necessary step before analysis of metal concentration with highly sensitive spectroscopic techniques. Thus, many methods for the determination of elements require all elements containing species to be converted in one single form which is uniform and well defined; that is all forms of binding present in particular sample are converted in to one or more simplified stages (e.g. ionic) [49]. Sample digestion is a method of converting the components of a matrix in to simple chemical forms. It is carried out by supplying energy, such as heat, by chemical reagent, such as an acid; or by a combination of two methods [48].

The selection of an appropriate treatment for sample dissolution depends on the nature of the sample, and different approaches are required for predominantly inorganic and predominantly organic matrices. There are no absolutes in sample preparation; whichever method produces a sample that lends itself to accurate and reproducible analysis is acceptable. However, there will be occasions when more than one method will be both suitable and available and the selection of either method will depend on other factors [50]. Depending on analysis task, many factors should be considered in the choice of sample preparation method such as levels of contamination introduced during sample preparation, degree of volatilization and/or retention during digestion process, possibilities to handle a representative sample, completeness of digestion and reproducibility in analyte recovery from the matrix, suitability of the analytical technique employed, time needed for sample preparation and economic aspects including labor and reagent consumption, equipment cost, etc. [44, 49, 51].

The digestion techniques can be classified in to: wet digestion, microwave digestion and dry ashing. The most commonly used technique; wet digestion with a reflux condenser was used for this work. Wet digestion involves the destruction of organic matter through the use of both heat and acids. For elemental analysis it involves the chemical degradation of sample matrices in solution, usually with a combination of acids to increase solubility. The various acid and flux treatments are carried out at high temperatures in specially designed vessels that help to minimize contamination of the sample with substances in the air, the local environment, and from the vessel walls. Losses from the sample may occur due to adsorption onto the vessel walls, volatilization, and co extraction. Acid digestions are commonly used for nearly every type of matrix [48, 49].

Most wet oxidation procedures require the use of a combination of acid and oxidant, of which the most commonly used are nitric, sulfuric and perchloric acids and hydrogen peroxide. However, perchloric acid is potentially hazardous during the digestion of biological materials and can cause the loss of certain metals like potassium [52]. The type of acid treatment must be given careful consideration, since particular acids may or may not oxidize the sample, and may be incompatible with certain elements. For example, sulfuric acid cannot be used to dissolve samples containing barium, while hydrochloric acid cannot be used to dissolve silver or samples containing lead and lead compounds. The choice of acid is also restricted by sample volatilization, e.g., hydrochloric acid should be avoided in samples containing arsenic since this is more volatile as a trichloride [50]. However, HCl and HF are also used, depending on the material and on the element under consideration [53].

Generally, the methods of wet oxidation include the digestion of the samples with aqua regia, ternary acid mixture (HNO_3, H_2SO_4 and $HClO_4$), nitric acid-perchloric acid mixture, sulfuric acid-nitric acid mixture, sulfuric acid-perchloric acid mixture, nitric acid-hydrogen peroxide mixture and/or sulfuric acid-hydrogen peroxide mixture [54]. HNO_3 + H_2O_2 + $HClO_4$ combination had been also used for the determination of Cr, Ni and V in biological samples [55].

After the plant sample is digested lanthanum salt is added to the digested solution. It is well known that when the plant samples are digested with a concentrated acid digester, the inorganic elements in plants tissues are available in solution like PO_4^{3-}, K^+, SO_4^{2-}, Ca^{2+}, Mg^{2+}, Fe^{2+}, Mn^{2+}, Zn^{2+} and Cu^{2+}. At this condition the pH of the solution is as below as 2 and there is no precipitation of nutrient elements. Above pH 2 (after dilution), the minerals in the solution may form complex with each other like Ca and Mg with P. The samples containing Ca^{2+}, Mg^{2+}, PO_4^{3-} and SO_4^{2-} may form complexes like $CaSO_4$, $Ca_3(PO_4)_2$, $MgSO_4$ and $Mg_3(PO_4)_2$. After adding 2% $LaCl_3$ with the solution, $LaCl_3$ may react with SO_4^{2-} and PO_4^{3-} of Ca and/or Mg and may form insoluble complex like $La_2(SO_4)_3$ and $LaPO_4$. As a result Ca and or Mg may be free from those anions and the atoms could be measured [54].

A carefully optimized mixture of nitric acid, perchloric acid and hydrogen per oxide was used for this study. Both the leaf and stem sample were further sun dried; leaves for three days and stems for two weeks. Stones and other wastes were removed manually from leaf and stem samples. The dried and cleaned stem samples were crushed with mortar and pestle. Both leaf and stem samples were further dried in an oven at 80 ^{0}C for 20 and 24 hours, respectively, to remove the moisture and so that the concentration of the metals is expressed on a dry mass basis. The dried leaf and stem samples were milled using electronic blender and sieved through 1mm sieve to prepare fine powder of *R. prinoides* stem and leaf samples for digestion.

2.2.5. Optimization of digestion procedure

In this study, sample digestion was carried out using wet digestion with a reflux condenser for both leaf and stem samples. The reagents used were mixture of nitric acid, hydrogen peroxide and perchloric acid. Before the samples were digested for analysis the parameters, temperature, volume of reagents and duration of time for complete digestion were carefully optimized. The optimum condition is one that meets: minimum reagent volume, minimum temperature, minimum digestion time and clear and colorless digested solution. The four parameters, temperature, time, reagent volume and reagent ratio were optimized by varying one parameter and keeping the other parameters constant. Both leaf and stem sample were pre-digested for 30 min to minimize the vigorous reaction that may encounter.

2.2.5.1. Optimization of *R. prinoides* leaf digestion

The volume composition of nitric acid, hydrogen peroxide and perchloric acid, the temperature and duration of time for complete digestion of the leaf sample were carefully optimized. Tables 3 and 4 show the optimization of these parameters.

Table 3. Optimization of volume of reagent for leaf sample.

Total volume (mL)	Mixture of reagents $HNO_3 + H_2O_2 + HClO_4$ (mL)	Temp. (^0C)	Time (h)	Observation
3	2, 1, 0	270	3:00	Deep yellowish green with more residue
3	2, 0, 1	270	3:00	Pale yellowish green and more residue
3	2, 0.5, 0.5	270	3:00	Deep yellowish green and jelly like remain
3	1.5, 0.5, 1	270	3:00	Color less but more residue remained
3	1.5, 1, 0.5	270	3:00	Pale yellowish with small residue
3	1, 1, 1	270	3:00	Very pale yellowish with wore residue
4	2, 1, 1	270	3:00	Colorless with very small residue
4	2, 0.5, 1.5	270	3:00	Pale yellowish green with very small residue
4	2, 0, 2	270	3:00	Deep yellowish green with more residue
4	2, 2, 0	270	3:00	Deep yellowish green with more residue
4	2.5, 0.5, 1	270	3:00	Pale yellowish green with more residue
4	**2.5, 1, 0.5**	**270**	**3:00**	***Clear and colorless**
4	3, 0, 1	270	3:00	Pale yellowish green with more residue
5	3, 1, 1	270	3:00	Deep yellowish green with more residue
5	2, 1, 2	270	3:00	Pale yellowish green with some residue
5	2.5, 1.5, 1	270	3:00	Clear and colorless
5	2.5, 1, 1.5	270	3:00	Clear and colorless
5	3, 1.5, 0.5	270	3:00	Very pale yellowish green with some residue
5	3, 1.5, 0.5	270	3:00	Clear and colorless
5	3, 1, 1	270	3:00	Clear and colorless
5	4, 0.5, 0.5	270	3:00	Deep yellowish green with some residue

* Indicates optimum volume of reagent.

Table 4. Optimization of digestion temperature and time for leaf samples.

Total volume (mL)	Mixture of reagent (HNO_3+H_2O_2+$HClO_4$) (mL)	Temp. (^{0}C)	Time (h)	Observation
Optimization of digestion temperature				
4	2.5, 1, 0.5	120	3:00	Yellowish green with some residue
4	2.5, 1, 0.5	150	3:00	Pale yellowish green with some residue
4	**2.5, 1, 0.5**	**180**	**3:00**	***Clear and colorless**
4	2.5, 1, 0.5	210	3:00	Clear and colorless
4	2.5, 1, 0.5	240	3:00	Clear and colorless
4	2.5, 1, 0.5	270	3:00	Clear and colorless
Optimization of digestion time				
4	2.5, 1, 0.5	180	2:00	Pale yellowish green with more residue
4	2.5, 1, 0.5	180	2:10	Very pale yellowish green with some residue
4	2.5, 1, 0.5	180	2:20	Color less with some residue
4	**2.5, 1, 0.5**	**180**	**2:30**	***Clear and colorless**
4	2.5, 1, 0.5	180	2:40	Clear and colorless
4	2.5, 1, 0.5	180	2:50	Clear and colorless
4	2.5, 1, 0.5	180	2:50	Clear and colorless

* Indicates optimum temperature and time of leaf digestion.

2.2.5.2. Optimization of digestion of *R. prinoides* stem samples

The reagents used in stem digestion were the same as the reagents used for leaf digestion.

Table 5. Optimization of volume of reagents for stem samples.

Total volume (mL)	Mixture of reagents ($HNO_3+H_2O_2+HClO_4$) (mL)	Temperature (^0C)	Time (h)	Observation
3	3, 0, 0	270	3:00	Deep yellowish green and some residue
3	2.5, 0, 0.5	270	3:00	Color less with some residue
3	2, 0, 1	270	3:00	Pale yellowish green with some residue
3	2, 0.5, 0.5	270	3:00	Color less with some residue
3	1.5, 1, 0.5	270	3:00	Very pale yellowish green with some residue
3	1.5, 0.5, 1	270	3:00	Very yellowish green with some residue
4	3, 0, 1	270	3:00	Pale yellowish green with more residue
4	2.5, 0.5, 1	270	3:00	Pale yellowish green with some residue
4	2.5, 1, 0.5	270	3:00	Very pale yellowish green
4	3, 0.5, 0.5	270	3:00	Color less with very small residue
4	2, 1, 1	270	3:00	Pale yellowish green with very small residue
5	3, 1, 1	270	3:00	Color less but with very small residue
5	3.5, 0.5, 1	270	3:00	color less but with very small residue
5	**3.5,1, 0.5**	**270**	**3:00**	***Clear and colorless**
5	3,0,2	270	3:00	Yellowish green with more residue
5	2.5, 1, 1.5	270	3:00	Yellowish green with very small remains
5	2.5, 1.5, 1	270	3:00	Color less but with some residue
6	4,1,1	270	3:00	Color less but with very small residue
6	3.5, 1, 1.5	270	3:00	Pale yellowish green but no residue was observed
6	3.5, 1.5, 1	270	3:00	Clear and colorless
6	3, 1, 2	270	3:00	Yellowish green with some residue
6	3, 2, 1	270	3:00	Very pale yellowish green with very small residue
6	3, 1.5, 1.5	270	3:00	Pale yellowish green with some residue

*Indicates optimum volume of reagent.

Table 6. Optimization of temperature and time of *R. prinoides* stem digestion.

Total volume (mL)	Composition of reagent (HNO_3+H_2O_2+$HClO_4$) (mL)	Temperature (C)	Time (h)	Observation
		Optimization of temperature		
5	3.5, 1, 0.5	150	3:00	Pale yellowish green with some residue
5	3.5, 1, 0.5	180	3:00	Colorless but with some residue
5	**3.5, 1, 0.5**	**210**	**3:00**	***Clear and colorless**
5	3.5, 1, 0.5	240	3:00	Clear and colorless
5	3.5, 1, 0.5	270	3:00	Clear and colorless
		Optimization of time		
5	3.5, 1, 0.5	210	1:30	Very pale yellowish green with small residue
5	3.5, 1, 0.5	210	1:40	Very pale yellowish green with very small residue
5	**3.5, 1, 0.5**	**210**	**1:50**	***Clear and colorless**
5	3.5, 1, 0.5	210	2:00	Clear and colorless
5	3.5, 1, 0.5	210	2:10	Clear and colorless
5	3.5, 1, 0.5	210	2:20	Clear and colorless
5	3.5, 1, 0.5	210	2:30	Clear and colorless
5	3.5, 1, 0.5	210	2:40	Clear and colorless
5	3.5, 1, 0.5	210	2:50	Clear and colorless
5	3.5, 1, 0.5	210	3:00	Clear and colorless

* Indicates optimum temperature and time of stem digestion.

2.2.6. Digestion of *R. prinoides* leaf and stem samples

Applying the optimized condition for leaf and stem, 0.5 g of dried and homogenized leaf and stem samples were transferred into a 100 mL round bottomed flask. Then 4 mL mixture of HNO_3 (69-72%), H_2O_2 (30%) and $HClO_4$ (70%) with a volume of 2.5, 1 and 0.5 mL to leaf sample and 5 mL with a volume of 3.5, 1 and 0.5 mL to stem sample were added and the mixtures were made to stay for 30 min at room temperature (pre-digestion) After 30 min the mixtures were digested on a Kjeldahl digestion apparatus fitting the flask to a reflux condenser at the temperature optimized for a duration of the time set for digestion. The digestion was carried out in triplicate for each bulk sample. Digestion of a reagent blank was also performed with the same procedure in parallel with the digestion of the samples keeping all digestion parameters the same. Six blanks were digested for both the leaf and stem samples. The digested solution was allowed to cool to room temperature for 30 min without dismantling the condenser from the flask. To the cooled solution 20 mL of de ionized water was added and the solution was shacked to dissolve precipitates remained on the wall of the flask and to minimize dissolution of filter paper by residue. This solution was then transferred to 50 mL volumetric flask by filtering using Whatman No. 1 (70 mm diameter) filter paper and rinsing the round bottom flask with de-ionized water. Lanthanum nitrate solution (1% w/v) was added and the solution was filled to the mark (50 mL) with de-ionized water. These digested samples were labeled and kept in the refrigerator until the determination of the level of the metals by FAAS.

2.2.7. Analysis of *R. prinoides* leaf and stem samples for metal levels

Metal standard solutions for calibration were prepared from an intermediate standard solution containing 10 mg/L which was prepared from the atomic absorption spectroscopy standard stock solutions that contained 1000 mg/L for each of the metals. Secondary standards for instrument calibration were prepared by diluting with de-ionized water to obtain four working standards for each metal included in this work. Ca, Mg, Cu, Zn, Mn, Ni, Fe, Co, Cr, Pb and Cd were analyzed with FAAS (Buck Scientific Model 210GP, USA) equipped with deuterium arc background corrector and standard air-acetylene flame system using external calibration curve after the parameters: burner and lamp alignment, slit width and wavelength adjustment were optimized for maximum signal intensity of the instrument. Except for lead, hallow cathode lamp for each metal operated at the manufacturer's recommended conditions were used at its respective primary source line. The acetylene and air flow rates were managed to ensure suitable flame conditions. Three replicate determinations were carried out on each sample. The concentration of all the eleven metals were determined by absorption mode and the instrument readout was recorded for each solution manually. The same analytical procedure was employed for the determination of elements in digested blank solutions. The operating conditions for FAAS employed for each analyte are given in Table 7.

Table 7. Instrumental operating conditions for determination of metals in *R. prinoides* leaf and stem samples using FAAS.

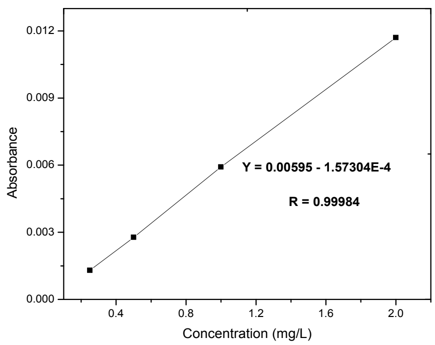
Element	Wavelength (nm)	Detection limit (mg/L)	Slit width (nm)	Lamp current (mA)	Energy (erg)
Mg	285.2	0.001	0.7	1.0	4.091
Ca	422.7	0.01	0.7	2.0	3.723
Cd	228.9	0.005	0.7	2.0	3.221
Cr	357.9	0.005	0.7	2.0	3.576
Mn	279.5	0.01	0.7		3.965
Fe	248.3	0.03	0.2	7.0	2.870
Co	240.7	0.05	0.2	4.5	2.710
Ni	232	0.04	0.2	7.0	2.935
Cu	324.7	0.02	0.7	1.5	3.496
Zn	213.9	0.005	0.7	2.0	3.105
Pb	283.2	0.1	0.7	2.0	3.16

2.2.8. Instrument calibration

The FAAS instrument was calibrated using four series of working standards. The working standard solutions of each metal were prepared freshly by diluting the intermediated standard solutions (10 mg/L). Concentrations of the intermediate standards, working standards and values of correlation coefficient of the calibration curve for each metal are listed in Table 8. The calibration curve of each metals of interest is shown in Figure 2.

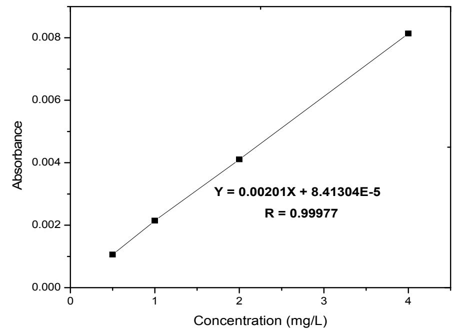
Table 8. Intermediate standards, working standards and correlation coefficients of the calibration curves for determinations of metals using FAAS.

No.	Metal	Wavelength (nm)	Concentration of working standards (mg/L)	Correlation coefficient of calibration curves	Equation for calibration curves
1	Mg	285.2	0.25, 0.5, 1, 2.0	0.99984	Y = 0.00595 X − 1.57304E-4
2	Ca	422.7	0.5, 1.0, 2.0, 4,	0.99977	Y = 0.00201X + 8.41304E-5
3	Cd	228.9	0.25, 0.5, 1.0, 2.0	0.99973	Y = 0.01221X + 3.22179E-4
4	Cr	357.9	0.5, 1.0, 1.5, 3.0	0.99983	Y = 0.00142X − 2.075E-5
5	Mn	279.5	0.25, 0.5, 1.0, 2.0	0.99989	Y = 0.0192X + 4.79696E-4
6	Fe	248.3	0.5, 1.0, 1.5, 3.0	0.99973	Y = 0.00142X − 2.075E-5
7	Co	240.7	0.25, 0.5, 1.0, 2.0	0.99936	Y = 0.00249X + 2.40435E-5
8	Ni	232	0.25, 0.5, 1.0, 2.0	0.99994	Y = 0.005X + 2.65217E-5
9	Cu	324.7	0.5, 1.0, 1.5, 3.0	0.99984	Y = 0.00595X − 1.57304E-4
10	Zn	213.9	0.1, 0.2, 0.4, 0.8	0.99977	Y = 0.06635X − 7.41826E-4
11	Pb	217	1.0, 2.0, 3.0, 4.0	0.9993	Y = 0.00254X + 4.3E-5



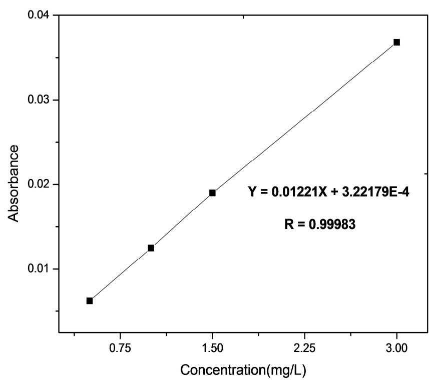
0.012
0.009
0.006
0.003
0.000
Absorbance
0.4
0.8
1.2
1.6
2.0
Concentration (mg/L)
Y = 0.00595 - 1.57304E-4
R = 0.99984

(a)



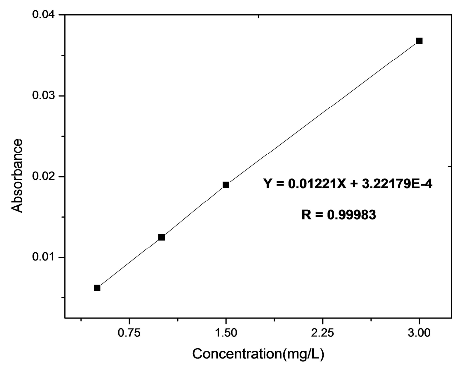
0.008
0.006
0.004
0.002
0.000
Absorbance
0
1
2
3
4
Concentration (mg/L)
Y = 0.00201X + 8.41304E-5
R = 0.99977

(b)



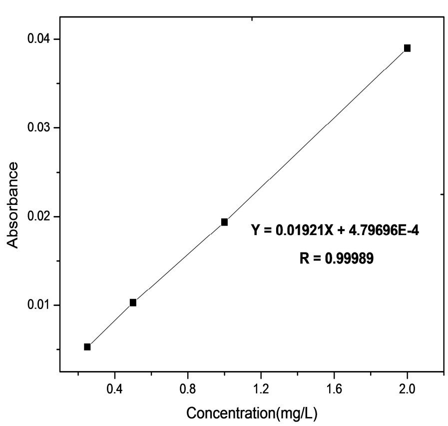
0.04
0.03
0.02
0.01
Absorbance
0.75
1.50
2.25
3.00
Concentration(mg/L)
Y = 0.01221X + 3.22179E-4
R = 0.99983

(c)



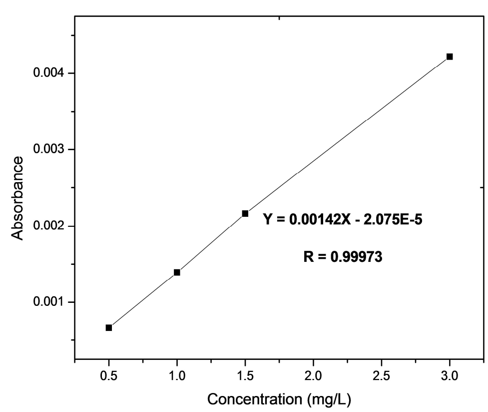
0.04
0.03
0.02
0.01
Absorbance
0.75
1.50
2.25
3.00
Concentration(mg/L)
Y = 0.01221X + 3.22179E-4
R = 0.99983

(d)



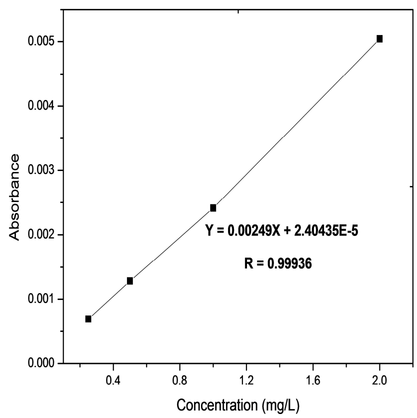
0.04
0.03
0.02
0.01
Absorbance
0.4
0.8
1.2
1.6
2.0
Concentration(mg/L)
Y = 0.01921X + 4.79696E-4
R = 0.99989

(e)

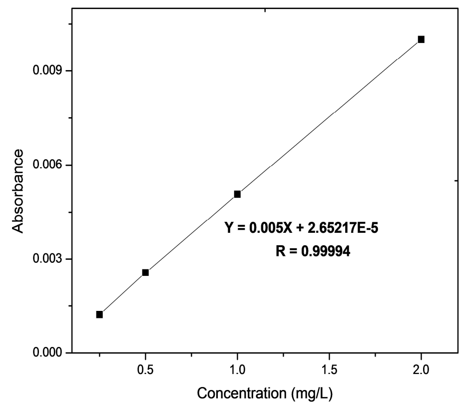


0.004
0.003
0.002
0.001
Absorbance
0.5
1.0
1.5
2.0
2.5
3.0
Concentration (mg/L)
Y = 0.00142X - 2.075E-5
R = 0.99973

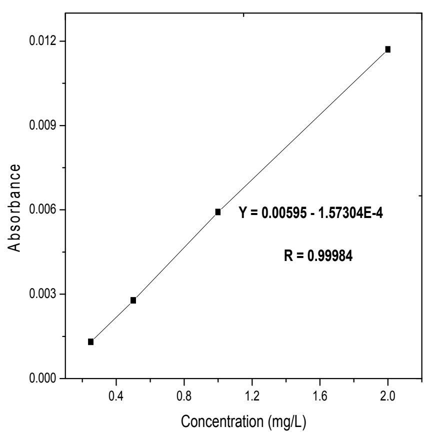
(f)



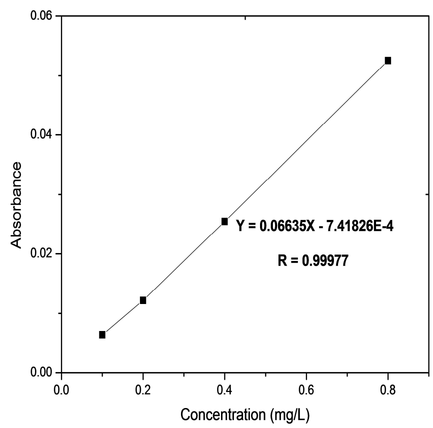
(g)



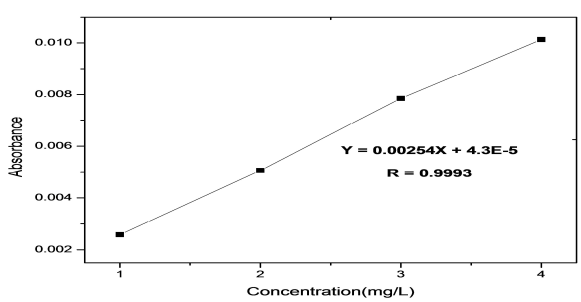
(h)



(i)



(j)



(k)

Figure 2. Calibration curves of (a) magnesium, (b) calcium, (c) cadmium, (d) chromium, (e) manganese, (f) iron, (g) cobalt, (h) nickel, (i) copper, (j) zinc, (k) lead.

2.3. Method performance and method validation

Method validation is the process used to confirm that the analytical procedure employed for a specific test is suitable for its intended use. Results from method validation can be used to judge the quality, reliability and consistency of analytical results; it is an integral part of any good analytical practice. The parameters: accuracy, precision, specificity, limit of detection, limit of quantification etc. are used for method validation [56].

2.3.1. Precision and accuracy

Precision is a measure of the random error associated with a series of repeated measurements of the same parameter with in a sample while Accuracy reflects the closeness of a measured value to a true value. Precision is determined by the absolute standard deviation, relative standard deviation, variance, coefficient of variation, relative percent difference, or the absolute range of a series of measurements [57]. In this work, the data are reported as mean ± SD to show the degree of precision.

2.3.2. Detection limits

There are several different types of detection limits depending on what is being defined. However, the method detection limit (MDL) is the only one designed to be determined. The other detection limits are either determined by the instrument manufacturer or are calculated from the MDL [58]. Method detection limit (MDL) is the minimum concentration of a substance that can be measured and reported with 99% confidence that the analyte concentration is greater than zero. Method detection limits are statistically determined values that define how easily measurements of a substance by a specific analytical protocol can be distinguished from measurements of a blank (background noise). Method detection limits are matrix, instrument and analyst specific. It is worth realizing that instrument detection limit (IDL) is the concentration equivalent to a signal, due to the analyte of interest, which is the smallest signal that can be distinguished from background noise by a particular instrument. The IDL should always be below the method detection limit, and is not used for compliance data reporting. Another related term is lower limit of quantitation (LOQ), it is the level above which quantitative results may be obtained with a specified degree of confidence. The LOQ is mathematically defined as equal to 10 times the standard deviation of the results for a series of replicates used to determine a justifiable limit of detection [57].

In this study, precision of the results was evaluated by standard deviation of the results of the nine measurements, triplicate digestion and triplicate readings. And the method detection limit and limit of quantification for each metal were calculated from the standard deviation of the replicate analysis of six blanks prepared by the optimized procedure by:

MDL = 3×S of blank and LOQ = 10×S of blank and the values are listed in Table 9.

Table 9. Instrument detection limit, method detection limit, and lower limit of quanitification.

Metal	IDL (mg/L)	MDL for leaf sample mg/kg)	MDL for stem sample (mg/kg)	LOQ for leaf sample (mg/kg)	LOQ for stem sample (mg/kg)
Mg	0.001	0.1	0.4	0.4	1.4
Ca	0.01	0.06	0.2	0.2	0.6
Cd	0.005	0.006	0.006	0.2	0.6
Cr	0.05	0.05	0.05	1.8	1.8
Mn	0.01	0.02	0.02	0.05	0.08
Fe	0.03	0.3	0.3	0.9	0.8
Co	0.05	0.06	0.05	0.19	0.18
Ni	0.04	0.06	0.05	0.2	0.18
Cu	0.02	0.06	0.03	0.2	0.1
Zn	0.005	0.03	0.03	0.09	0.11
Pb	0.1	0.12	0.11	0.4	0.37

2.3.3. Validation of optimized procedure

An analytical method can be validated by using an alternative method (against a fully validated method), by using proficiency testing or by using certified reference material [59]. In the present study, the optimized procedures were validated by analyzing metal concentration of a sample which was prepared by the optimized digestion and spiked with a small amount of each metal. Then, the percent recovery of the method was evaluated by calculating percent recovery (% R).

$$(\%\ R) = \frac{C_M \text{ in the spiked sample-} C_M \text{ in the non-spiked}}{C_M \text{ added for spiking}} \times 100$$

Where C_M = concentration of metal.

The recovery of the metals in this study in leaf and stem samples of *R. prinoides* plant are summerized in Tables 10 and 11.

Table 10. Recovery test for the optimized procedure of *R. Prinoides* leaf samples.

Metal	Conc. of metal in the un-spiked sample (mg/L)	Amount added (mg/L)	Conc. of metal in the spiked 0.5 g sample (mg/L)	Percent recovered (%R)
Mg	51.7	12	63.6	99.2
Ca	125	32	156	96.9
Cd	0.044	0.012	0.055	91.7
Cr	0.22	0.058	0.28	103
Mn	0.14	0.033	0.17	103
Fe	2.16	0.53	2.66	94.3
Co	0.6	0.15	0.74	93.3
Ni	0.34	0.075	0.41	93.3
Cu	0.21	0.032	0.24	93.8
Zn	0.41	0.14	0.54	92.9
Pb	0.31	0.095	0.40	94.7

Table 11. Recovery test for the optimized procedure of *R. Prinoides* stem samples.

Metal	Con. of metal in the un-spiked sample (mg/L)	Amount added (mg/L)	Con. of metal in the spiked sample (mg/L)	Percent recovered (%R)
Mg	36.5	11	47.8	103
Ca	57.9	17	74.6	98.2
Cd	0.025	0.06	0.084	98.3
Cr	0.13	0.041	0.17	97.6
Mn	0.056	0.018	0.073	94.4
Fe	2.81	0.81	3.64	103
Co	1.24	0.35	1.57	94.3
Ni	0.19	0.055	0.24	90.9
Cu	2.18	0.50	2.65	94
Zn	0.41	0.12	0.52	91.7
Pb	0.17	0.053	0.22	94.3

3. RESULTS AND DISCUSSION

3.1. Level of metals in *R. prinoides* leaf and stem samples

The concentrations of eleven metals (Ca, Mg, Cr, Mn, Fe, Co, Ni, Cu, Zn, Cd and Pb) in *R. prinoides* leaf and stem sample were determined by FAAS. The concentrations of all metals analyzed in leaf sample from all the sample sites were found to be above the method detection limit whereas, the concentrations of: cadmium in stem samples from two sample sites (Kesatyebirhan, Gedgamba and Migra), lead in the stem samples from all the sample sites and chromium in (Smichezala) stem sample were below the method detection limit. The average concentrations of the metals above the method detection limit were expressed as mean ± standard deviation, SD) in Tables 12 and 13.

Table 12. Average concentration (mean ± SD) of the metals in *R. prinoides* leaf samples.

Sample site	Concentration of metals (mg/kg) ± SD									
	Mg	Ca	Cd	Cr	Mn	Fe	Co	Ni	Cu	Zn	Pb
S/zala	5428 ± 127	22236 ± 293	1.44 ± 0.12	5.08 ± 0.10	17.9 ± 0.3	47.9 ± 2.1	30.0 ± 0.5	16.8 ± 0.7	32.2 ± 1.6	13.5 ± 2.1	17.1± 0.6
Edaga Arbi	3202 ± 18	11546 ± 132	0.81 ± 0.06	9.32 ± 0.06	17.2 ± 0.5	139 ± 3	33.5 ± 0.5	25.3 ± 0.3	73.0 ± 1.1	13.4 ± 1.3	20.2 ± 0.7
Adifeila	5017± 90	6304 ± 195	0.94 ± 0.05	16.1 ± 0.1	11.5 ± 0.3	133 ± 2	22.2 ± 0.3	14.1 ± 0.4	26.8 ± 0.7	27.9 ± 1.1	21.6 ± 0.3
K/birhan	4781 ± 140	7234 ± 175	1.11 ± 0.05	9.42 ± 0.15	13.0 ± 0.2	187 ± 6	42.1 ± 0.7	27.3.± 1.0	30.0 ± 0.7	18.8 ± 0.7	19.5 ± 1.1
Majetye	5706 ± 107	16394 ± 347	2.14 ± 0.12	15.5 ± 0.4	15.5± 0.2	93.4 ± 2.1	35.2 ± 1.0	19.7 ± 0.9	11.7 ± 0.5	17.4 ± 0.7	17.3 ± 1.0
Karakori	4622± 114	12432 ± 398	3.10 ± 0.11	15.8 ± 0.7	10.1 ± 0.3	88.0 ± 4.3	36.2 ± 0.9	25.6 ± 0.6	10.3 ± 0.3	18.3 ± 0.6	20.3 ± 1.2
Tamo	5347± 99.7	7202 ± 80	2.11 ± 0.1	18.7 ± 0.3	8.12 ± 0.22	87.9 ± 2.1	29.6 ± 1.3	18.8 ± 0.7	11.2 ± 0.3	27.9 ± 1.4	25.0 ± 1.40
G/Amba	3361 ± 83	12994 ± 131	1.78 ± 0.09	14.9 ± 0.4	14.9 ± 0.6	88.4 ± 3.2	30.8 ± 1.5	26.4 ± 0.3	19.4 ± 0.5	12.2 ± 0.3	18.4 ± 1.33
G/Amba and Migra	412 1± 93	11358 ± 213	2.18 ± 0.08	20.6 ± 0.3	12.1 ± 0.3	136 ± 4	34.9 ± 0.7	12.8 ± 0.4	6.50 ± 0.4	43.0 ± 1.6	18.8 ± 1.10

Table 13. Average concentration (mean ± SD) of the metals in *R. prinoides* stem samples.

Sample site	Concentration of metals (mg/kg) ± SD										
	Mg	Ca	Cd	Cr	Mn	Fe	Co	Ni	Cu	Zn	Pb
S/zala	3123 ± 85	5102 ± 109	1.26 ± 0.10	4.58 ± 0.11	2.24 ± 0.11	22.0 ± 0.7	18.7 ± 0.4	16.8 ± 0.8	23.4± 1.4	21.5 ± 0.8	ND
E/Arbi	3440 ± 98	4350 ± 116	1.24 ± 0.08	9.51 ± 0.25	3.78 ± 0.15	105 ± 7	20.6 ± 1.5	19.2 ± 0.9	16.8 ± 0.5	17.9 ± 1.0	ND
Adifeila	5102 ± 89	4312 ± 41	1.00 ± 0.04	7.44 ± 0.14	3.82 ± 0.21	115 ± 6	58.1 ± 0.3	14.7 ± 0.4	28.2 ± 1.3	24.3 ± 1.7	ND
K/birhan	5528 ± 128	4272 ± 122	ND	6.58 ± 0.21	3.98 ± 0.10	124 ± 5	50.0 ± 1.5	15.6 ± 0.3	18.6 ± 0.9	19.1 ± 1.0	ND
Majetye	5223 ± 99	4358 ± 63	1.16 ± 0.06	16.3 ± 0.2	2.33 ± 0.13	40.0 ± 2.1	71.3 ± 3.1	9.68 ± 0.50	170 ± 1	28.2 ± 0.9	ND
Karakori	3417 ± 41	5675 ± 133	1.56 ± 0.10	6.83 ± 0.16	2.16 ± 0.13	71.4 ± 5.4	91.7 ± 3.8	11.5 ± 0.4	210 ± 2	19.1 ± 0.7	ND
Tamo	2667 ± 58	3601 ± 85	1.21 ± 0.08	7.65 ± 0.21	2.23 ± 0.06	45.3 ± 1.9	45.4 ± 1.4	11.4 ± 0.6	126 ± 5	18.2 ± 0.6	ND
G/Amba	2642 ± 55	5552 ± 157	1.14 ± 0.09	5.42 ± 0.17	2.28 ± 0.16	47.4 ± 2.7	45.3 ± 2.8	13.6 ± 0.8	233 ± 2	24.4 ± 0.7	ND
G/Amba and Migra	2635 ± 77	4677 ± 115	ND	17.4 ± 0.2	2.36 ± 0.13	120 ± 7	41.0 ± 1.5	13.1 ± 0.8	182 ± 11	17.4 ± 0.8	ND

N.B. ND is less than method detection limit.

In both leaf and stem in the entire sample, calcium was found to be the most abundant and magnesium was the second abundant. This is most likely because these elements are among the major elements required by plants. From the micro elements analyzed, iron was the most abundant in leaf samples but there was irregularity in stem samples; copper content was higher in the samples from Gedga Amba, Gedgamba and migra, Gina Ager, Karakori and Majetye and iron content was higher in the samples from Kesatyebirhan, Adifeila, and Edaga Arbi. As it is shown in Tables 11 and 12, concentration of copper in stem samples in the samples from Tigray was by far less than in the samples from other regions. Surprisingly, however, the concentration of copper in leaf samples from Tigray was greater than in stem samples; but it was the reverse in the samples from other regions. Similarly, the concentration of magnesium in leaf samples in the samples from Tigray (Edaga Arbi, Adifeila and K/birhan) was less than in stem samples but it was higher in leaf samples than in stem samples from the rest of the sample sites. Such irregularities cannot be certainly explained, nevertheless, it might be attributed to either effect of age of plant on mineral accumulation; for example, in Tigray, the stem is not harvested unlike in the other regions where both the stem and leaf are harvested, that is stem samples collected from Tigray were older than the stem samples collected from Amhara and Oromia and this in turn may result in a difference in the accumulation of copper in the different sample sites or to a smaller extent it might be due to a difference in time of collection as the concentration of a metal may differ even in hours duration for the samples collected from Tigray were a week before those from Amhara and two weeks before those collected from Oromia.

In case of magnesium, the smaller concentration in leaf samples than stem samples collected from Tigray could be attributed to a difference in season of sampling; these samples were collected early in the morning and hence, they might not get sufficient sun light which in turn results in little rate of photosynthesis and hence less uptake of magnesium by leaf.

Generally, the concentration of the metals analyzed, macro, micro and non-essential was higher in *R. prinoides* leaf than in stem samples. And the difference in concentration of the metals from sample site to sample site was generally larger among leaf samples than among stem samples. Such a difference is probably leaves might be highly sensitive to age difference than stems or the exposure to contamination for instance, dust may differ significantly from sample site to sample site. And it is worth noting that leaves hold more dust and other particles than stems because their wet nature can retain them. Among the micro nutrients the concentration of chromium in leaf was the least in the sample from some sample sites and manganese in the sample from another sample sites but the concentration of manganese in stem was the least from all the sample sites. Among the two toxic elements, concentration of lead was higher in the leaf samples from all the sample sites. Whereas, concentration of lead was below method detection limit in stem samples from in all the sample sites because of its large method detection limit observed. Or such marked differences in concentration of lead in leaf and stem might be attributed to the difference in the degree of exposure to contamination between leaf and stem as it is well illustrated above.

In summary, the relatively low concentration of magnesium in leaf sample at Edaga Arbi, the very small concentration of calcium in leaf sample at Adifeila and Kesatyebirhan, and the extremely high concentration of calcium in leaf samples at Simichezala, the extremely small concentration of iron in both leaf and stem samples at Simichezala and high concentration at Kesatyebirhan, the marked difference of copper concentration at Edaga Arbi, the relatively small concentration of magnesium in stem samples etc. at the sample sites Tamo, Gedga Amba, Gedga Amba and Migra are most likely because of a variation in at least one of the following: bioavailability, physical property of the soil, soil pH, competition of uptake of the metals by the plant, age of the leaf or the stem, season of sample collection and mineral content of the soil [60 - 64]. However, whether there is statistically significant difference in concentration of a metal in both leaf and stem samples at different sample sites will be discussed in statistical analysis section.

3.2. Distribution pattern of metals in different samples

Accumulation of elements in plants depends not only on their absolute content in a soil, but also on the level of soil fertility, acidic–alkaline and reductive–oxidative conditions and content of organic matter [64]. And hence the uptake of elements and their utilization by living cells depends on chemical and physical properties of each element. Transport and utilization of sufficient essential metal could arise from low availability; excessive competition from other metal ions with similar chemical properties, e.g., Co(II) and Ni(II), Ca(II) and Cd(II), PO_4^{3-} and AsO_4^{3-}, Li and Na^+, etc. [60]. Therefore, the differences in concentration of the metals from sample site to sample site are likely to be because of these factors.

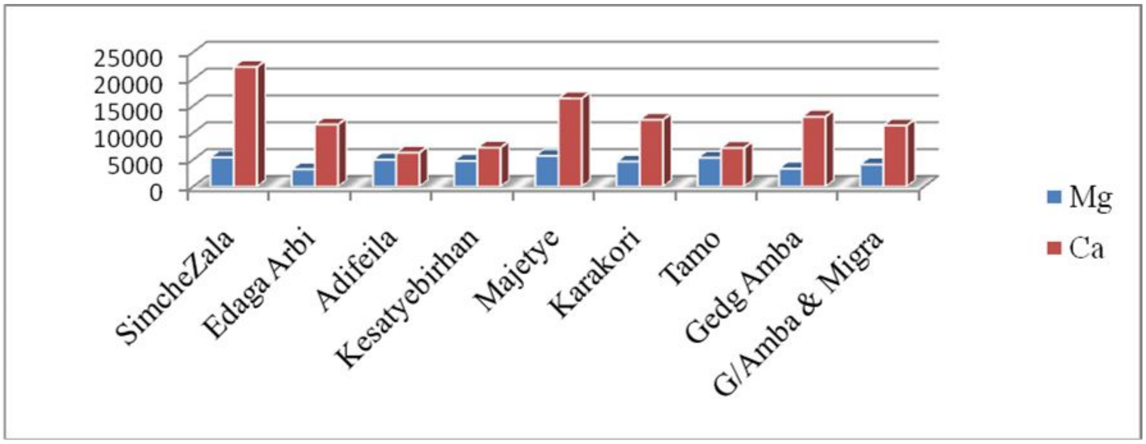
3.2.1. Distribution pattern of the metals in *R. prinoides* leaf samples

As it was discussed above, distribution of calcium in all the samples was the leading one (6304-22236 mg/kg) and magnesium was the second highest (3202-4622 mg/kg). Among the micro nutrients, iron content was the highest (47.9-187 mg/kg) and as it is shown in Table 11 and Figure 3b, copper and zinc which have virtually equal concentration in all the samples were the second highest: 6.5-73, 12.2-43, respectively and this might be attributed to the higher requirement of these metals among the trace elements.

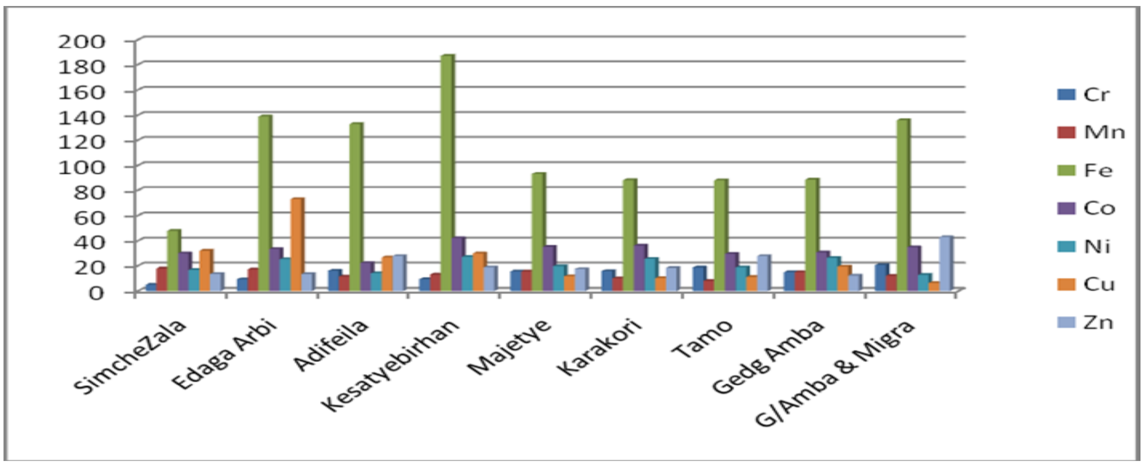
Manganese has the list distribution in the leaf sample at all sample sites. This is probably either the metal is not bioavailable or the intake of this metal by the plant may be small. In general, the concentration of the metals in *R. prinoides* leaf samples is in the order: Ca > Mg > Fe > Cu ≈ Zn ≈ Co > Ni > Pb > (Mn, Cr) > Cd. The range of metal concentration in *R. prinoides* leaf samples is shown in Table 13. And the distribution patterns of metals are shown in Figure 3a, 3b and 3c.

Table 14. Range of concentration of the metals in *R. prinoides* leaf samples.

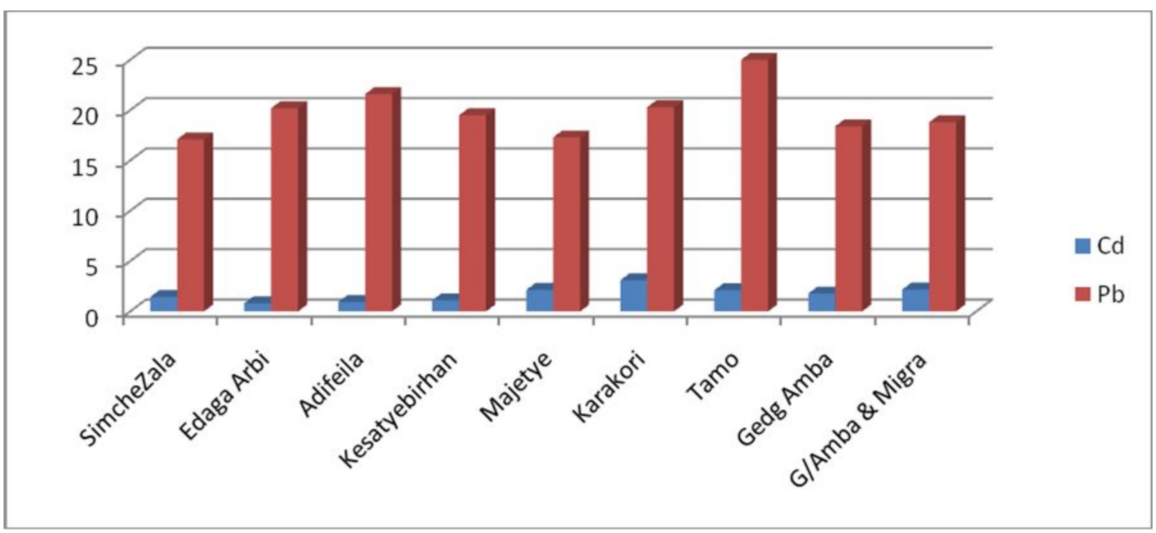
Element	Concentration range (mg/kg)	Element	Concentration range (mg/kg)
Mg	3202-4622	Co	22.2-42.1
Ca	6304-22236	Ni	12.8-27.3
Cd	0.81-3.1	Cu	6.5-73
Cr	5.1-18.7	Zn	12.2-43
Mn	8.1-20.6	Pb	17.7-25
Fe	47.9-187		



(a)



(b)



(c)

Figure 3. Avereage concentration of (a) macro elements, (b) micro elements, and

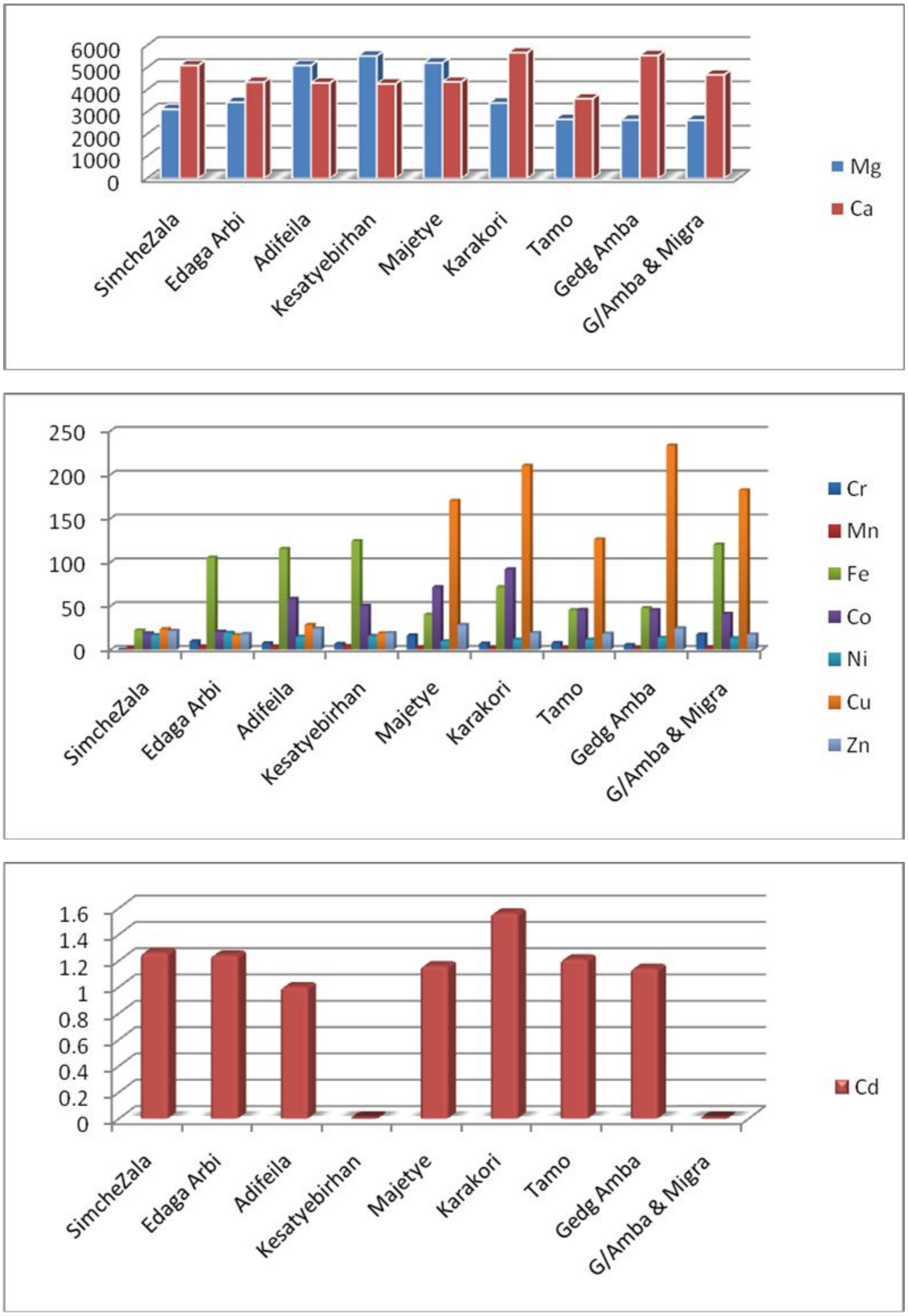
(c) toxic elements in the leaf samples.

3.2.2. Distribution pattern of the metals in *R. prinoides* stem samples

The average concentrations of the essential and toxic elements are shown in Table 12. Except, the average concentration of lead in stem at all sample sites and cadmium at two sample sites were below the method detection limit. Calcium has the highest concentration in most of the samples except at some sample sites where magnesium was the most abundant. Among the micronutrients, iron content was the highest in some samples and copper in another sample. The concentration of manganese and chromium were the smallest still. Among the concentration of the toxic element, cadmium was the smallest at all the sample sites except lead which was below the method detection limit which implies the stem of this plant takes mainly the essential elements and hence consumption of the stem of this plant for alcoholic purposes may not have noticeable effect on human health. The distribution range of metal concentration is shown in Table 15 and the average concentration of the metals is shown in Figures: 4a, 4b and 4c.

Table 15. Range of concentration of metals in *R. prinoides* stem samples.

Element	Range of concentration	Element	Range of concentration
Mg	2635-5528	Fe	22.0-124
Ca	3601-5675	Co	18.7-91.7
Cd	1.00-1.56	Ni	9.68-19.2
Cr	5.42-16.3	Cu	16.8-233
Mn	2.16-3.82	Zn	17.4-28.2



(a)

(b)

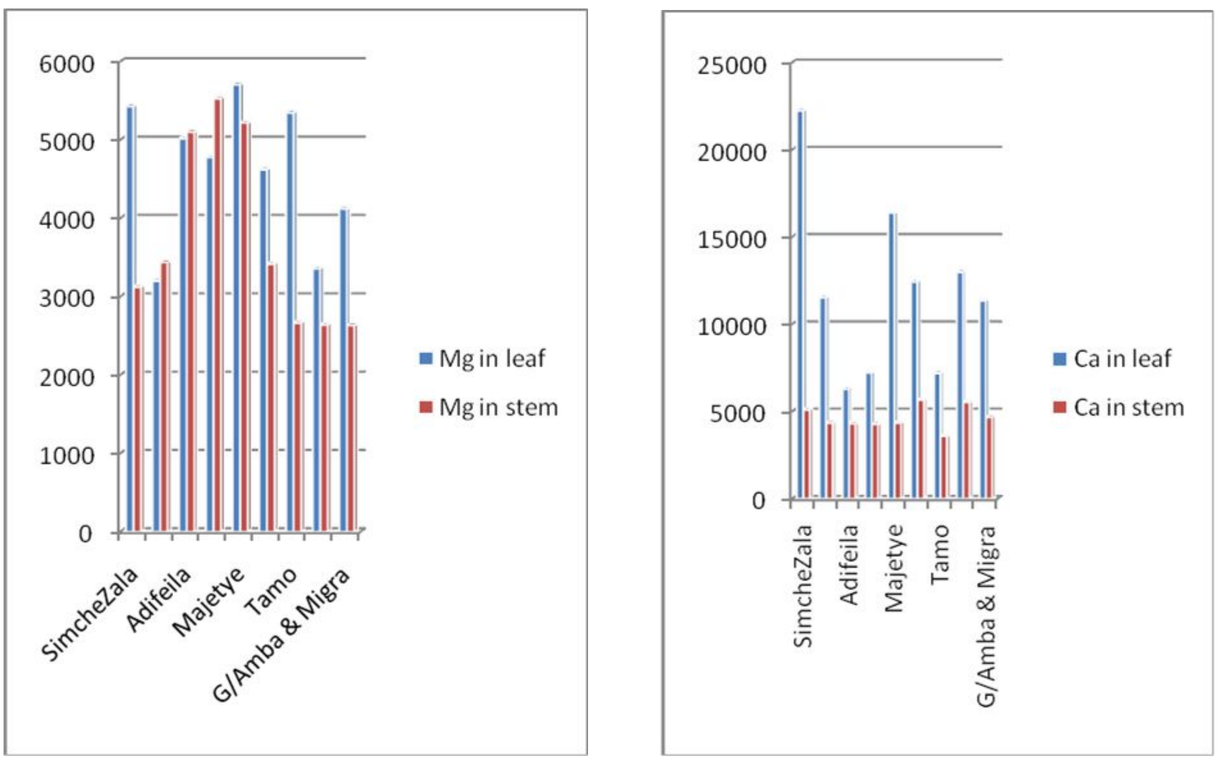
(c)

Figure 4. Average concentration of (a) macro elements, (b) micro elements and (c) cadmium in stem samples.

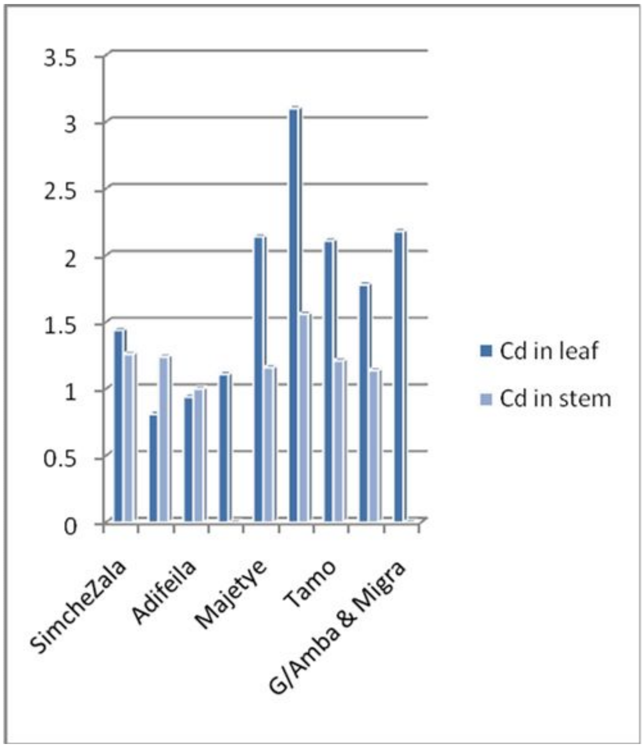
As it is shown in Table 14 and Figures 4a and 4b, there is wide range of magnesium, calcium, iron and cobalt among the sample sites. Such differences may be due to a difference in mineral content of the different soils or other factors like soil pH, soil enrichment factors, a difference in organic matter content of the soil, etc.

3.3. Comparison of metal levels between leaf and stem of *R. prinoides*

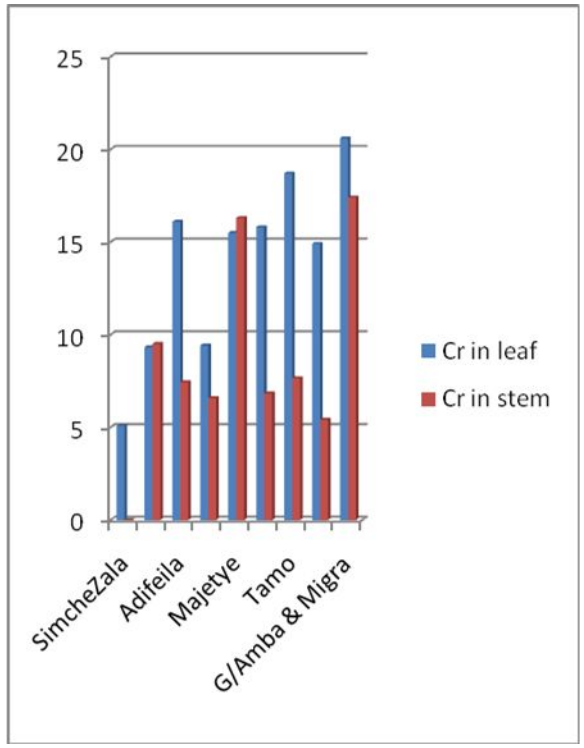
It is reported in many literatures that accumulation of elements in plants is affected by the soil mineral content, the medium of the soil and differences in different parts of the plant. In this study the level of metals in leaf and stem sampled from the same sample site and from the same plants were compared. Figure 5 illustrates the comparison of metal level in leaf with stem of *R. prinoides* plant.



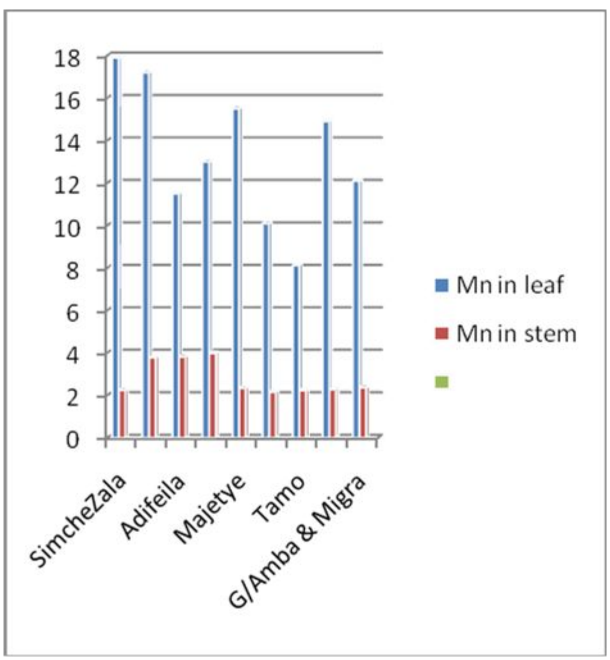
(a) (b)


3.5
3
2.5
2
1.5
1
0.5
0
SimcheZala
Adifeila
Majetye
Tamo
G/Amba & Migra
Cd in leaf
Cd in stem

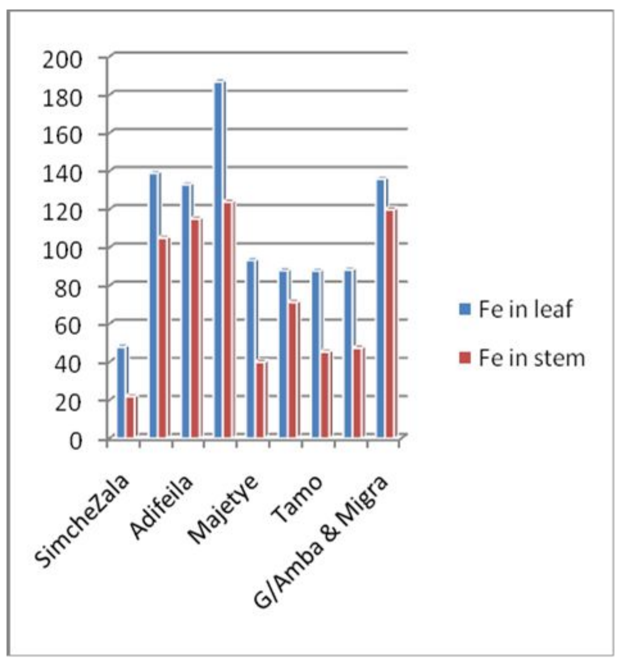
(c)


25
20
15
10
5
0
SimcheZala
Adifeila
Majetye
Tamo
G/Amba & Migra
Cr in leaf
Cr in stem

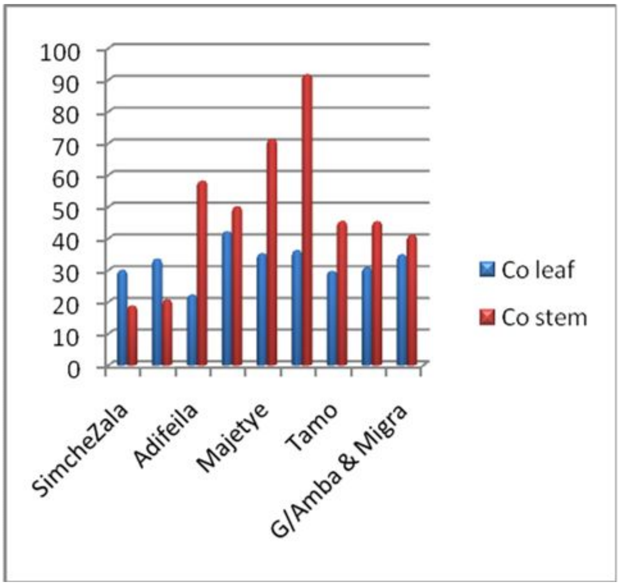
(d)


18
16
14
12
10
8
6
4
2
0
SimcheZala
Adifeila
Majetye
Tamo
G/Amba & Migra
Mn in leaf
Mn in stem

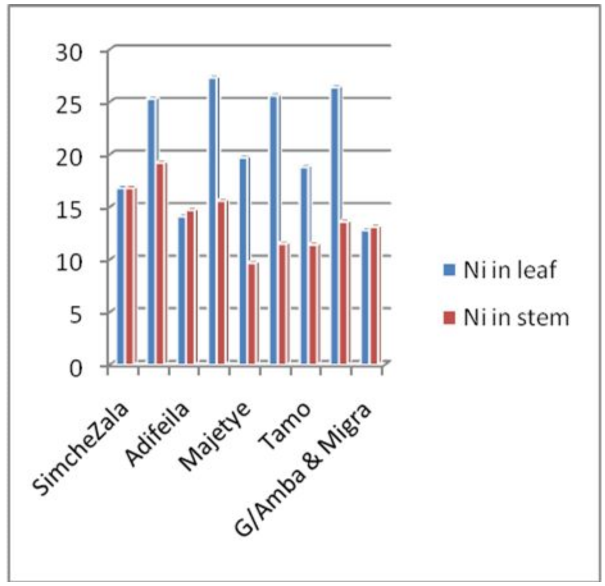
(e)


200
180
160
140
120
100
80
60
40
20
0
SimcheZala
Adifeila
Majetye
Tamo
G/Amba & Migra
Fe in leaf
Fe in stem

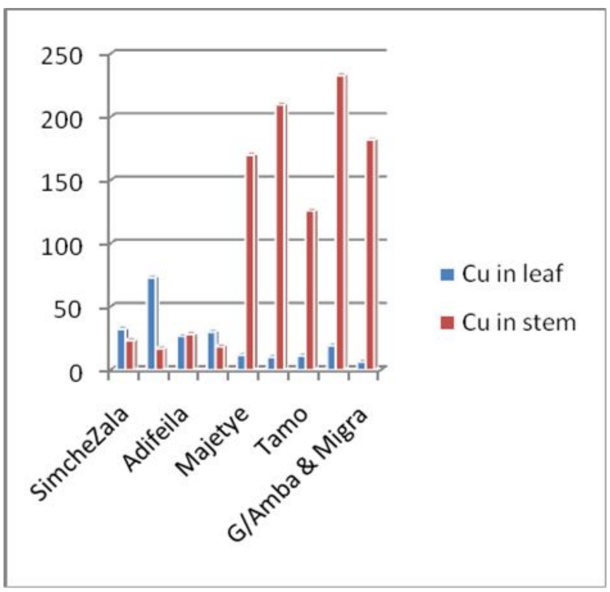
(f)



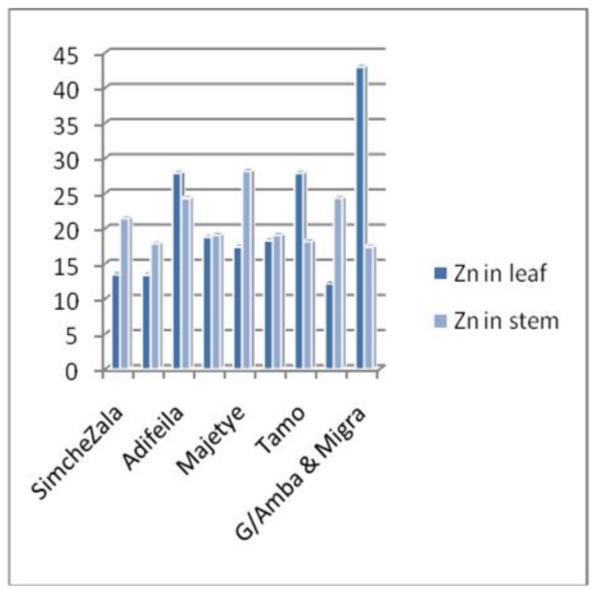
(g)



(h)



(i)



(j)

Figure 5. Comparison of metal level in leaf and stem of *R. prinoides* plant in nine sample sites.

In general, as it is shown in Tables 11 and 12 and Figure 5, level of the metals was higher in leaf samples than in stem samples. Level of macro elements in leaf samples was greater than in stem except at some sample sites where concentration of magnesium was slightly higher in stem samples. Manganese, iron and nickel and chromium except two sample sites were higher in leaf samples from all the sample sites than in stem samples. The concentration of cobalt was higher in stem samples in most of the sample sites whereas the content of copper and zinc was not regular. And cadmium was higher in leaf samples except two sample sites. These results indicate that the accumulation of the metals has no equal distribution in leaf and stem samples of *R. prinoides* plant. Higher concentration of calcium, chromium, manganese, iron and nickel in leaf samples was most likely; uptake of these elements by leaf of *R. prinoides* was greater than that of stem. On the other hand uptake of cobalt by stem was greater than by leaf. The irregularities of magnesium, copper and zinc may be attributed to either dependence of accumulation of these metals on age or the sampling time might have an effect (example, morning and afternoon or from week to week) on the concentration. Copper was very illustrative; in the samples collected from Tigray where the stem is old, concentration of copper in stem was much more less than those collected from Amhara and Oromia where the stem samples are young. But still, it is not possible to conclude that these differences are only because of these factors as accumulation of minerals by plants depends on several factors.

At last, it is interesting to note that the concentrations of the metals obtained were from complete digestion of the leaf and stem sample but in the actual consumption of the leaf and stem the powdered samples are added to the local alcoholic beverages, and therefore a complete digestion is not expected from the fermentation/metabolism and hence the metal content in the beverage bio-available to human will be less than the amount obtained from the analysis.

3.4. Statistical analysis

3.4.1. Analysis of variance

Whether the difference in the means of more than two means is real or the result of random error is tested by analysis of variance (ANOVA) .The statistical tool F test is applied for the analysis of variance [65]. To detect whether the difference in means of the concentration of the samples is significant at 95% confidence level, the one-way ANOVA, is applied. As it is shown in Tables 16 and 17, there is significance difference between the means of all the metals both in stem and leaf samples collected from nine sample sites. This implies that the variation in metal concentration among sample sites is not due to the random errors introduced from sample preparation to analysis rather it is real. The factors that contribute to such variation are likely to be a difference in the physical and chemical characteristics of the soil, soil enrichment, age and mineral content of the soil.

Table 16. Analysis of variance between and within *R. prinoides* leaf samples at 95% confidence level.

Metal	Comparison	Sum of squares	df	Mean square	F_{cal}	F_{cri}	Remark
Magnesium	Between groups	1964.402	8	245.550	920.245	2.77	Significant difference
	Within groups	4.803	18	0.267			
Calcium	Between groups	44613.324	8	5576.665	8.667	2.77	Significant difference
	Within groups	11581.46	18	643.414			
Cadmium	Between groups	0.001	8	0	633.586	2.77	Significant difference
	Within groups	0	18	0			
Chromium	Between groups	0.045	8	0.06	16530	2.77	Significant difference
	Within groups	0	18	0			
Manganese	Between groups	0.018	8	0.002	1544	2.77	Significant difference
	Within groups		18	0			
Iron	Between groups	4.106	8	0.513	2042	2.77	Significant difference.
	Within groups	0.005	18	0			
Cobalt	Between groups	0.073	8	0.09	136.881	2.77	Significant difference
	Within groups	0.001	18	0			
Nickel	Between groups	0.065	8	0.008	29.407	2.77	Significant difference
	Within groups	0.005	18	0			
Copper	Between groups	0.968	8	0.121	2386	2.77	Significant difference.
	Within groups	0.001	18	0			
Zinc	Between groups	0.219	8	0.027	945.952	2.77	Significant difference
	Within groups	0.001	18	0			
Lead	Between groups	0.015	8	0.002	55.354	2.77	Significant difference
	Within groups	0.001	18	0			

Table 17. Analysis of variance between and within *R. prinoides* stem samples at 95% confidence level.

Metal	Comparison	Sum of squares	df	Mean square	F_{cal}	F_{cri}	Remark.
Magnesium	Between groups	3425.594	8	428.199	2298	2.77	Significant difference
	Within groups	3.354	18	0.186			
Calcium	Between groups	1072.962	8	134.120	267.097	2.77	Significant difference
	Within groups	9.039	18	0.502			
Cadmium	Between groups	0	8	0	787.136	2.77	Significant difference
	Within groups	0	18	0			
Chromium	Between groups	0.052	8	0.006	6700	2.77	Significant difference
	Within groups	0	18	0			
Manganese	Between groups	0.001	8	0	43.174	2.77	Significant difference
	Within groups	0	18	0			
Iron	Between groups	3.884	8	0.486	1249	2.77	Significant difference
	Within groups	0.007	18	0			
Cobalt	Between groups	1.036	8	0.129	12.737	2.77	Significant difference
	Within groups	0.173	17	0.010			
Nickel	Between groups	0.016	8	0.002	6.046	2.77	Significant difference
	Within groups	0.006	17	0			
Copper	Between groups	19.420	8	2.427	1477	2.77	Significant difference
	Within groups	0.030	18	0.002			
Zinc	Between groups	0.036	8	0.005	100.558	2.77	Significant difference
	Within groups	0.001	18	0			

3.4.2. Pearson correlation

Correlation refers to any of a broad class of statistical relationships involving dependence. Correlations are useful because they can indicate a predictive relationship that can be exploited in practice. There are several correlation coefficients, often denoted ρ or r, measuring the degree of correlation. The most common of these is the Pearson correlation coefficient, which is sensitive only to a linear relationship between two variables (which may exist even if one is a nonlinear function of the other) [66]. Pearson's r is a useful descriptor of the degree of linear association between two variables. When it is near zero, there is no correlation, but as it approaches ±1 there is a strong negative or positive relationship between the variables [67]. In this study, Pearson’s correlation of the metals with in leaf, stem and between stem and leaf of *R. prinoides* are shown in Tables 18, 19 and 20.

Table 18. Correlation matrices for metals in *R. prinoides* leaf samples (n = 9).

	Mg	Ca	Cd	Cr	Mn	Fe	Co	Ni	Cu	Zn	Pb
Mg	1										
Ca	0.173	1									
Cd	0.208	0.176	1								
Cr	0.004	-0.486	0.544	1							
Mn	-0.230	0.700	-0.479	-0.728	1						
Fe	-0.305	-0.699	-0.452	0.060	-0.144	1					
Co	-0.107	0.050	0.248	-0.166	0.079	0.398	1				
Ni	-0.426	-0.081	0.010	-0.358	0.141	0.193	0.563	1			
Cu	-0.472	0.031	-0.750	-0.671	0.596	0.277	-0.028	0.326	1		
Zn	0.116	-0.435	0.204	0.721	-0.582	0.293	-0.122	-0.706	-0.479	1	
Pb	0.072	-0.744	0.021	0.424	-0.772	0.147	-0.320	-0.060	-0.045	0.331	1

From Table 18, it can be depicted that there is high positive correlation Ca with (Mn) and Zn with (Cr), a moderate positive correlation Cr with (Cd and Pb), Cu with (Mn), Ni with (Co), a moderate negative correlation Mg with (Ni and Cu), Ca with (Cr and Zn), Cd with (Fe and Mn), Zn with (Mn and Cu) and high negative correlation Ca with (Fe and Pb), Cr with (Mn and Cu), Pb with (Mn) and Ni with (Zn). All the other correlations are poor correlations. The high positive correlations may indicate uptake of the metal by the leaf of this plant facilitates the other, they have the same anthropogenic sources or they have similar function in the leaf of this plant. The high negative correlation on other hand indicates there is a competition in the uptake of these metals. Those that have small positive or negative correlations do not affect one another.

Table 19. Correlation matrices for metals in *R. prinoides* stem samples (n = 9).

	Mg	Ca	Cd	Cr	Mn	Fe	Co	Ni	Cu	Zn
Mg	1									
Ca	-0.310	1								
Cd	-0.387	0.500	1							
Cr	0.085	-0.268	-0.169	1						
Mn	0.618	-0.405	-0.469	-0.155	1					
Fe	0.336	-0.240	-0.184	0.229	0.740	1				
Co	0.353	0.234	0.359	0.146	-0.209	0.011	1			
Ni	-0.030	-0.006	-0.136	-0.395	0.623	0.342	-0.751	1		
Cu	-0.427	0.478	0.345	0.313	0.313	-0.316	0.539	-0.743	1	
Zn	0.455	0.130	-0.534	0.098	-0.123	-0.421	0.301	-0.367	0.186	1

From Table 19, we observe that Mn with (Mg and Ni), Ca with (Cd and Cu), Co with (Cu), Zn with (Mg) have moderate positive correlation and Mn has high positive correlation with (Fe). Mg with (Cu), Ca with (Mn), Cd with (Mn, Zn), Fe with (Zn) have moderate negative correlation and Co with (Ni and Cu) have high negative correlation. The rest combinations have poor correlation. The reasons for the moderate and high positive and moderate and high correlation are associated with the factors mentioned above.

Table 20. Pearson correlation coefficient for metals in *R. prinoides* leaf with stem sample (n = 9).

Metal	Mg	Ca	Cd	Cr	Mn	Fe	Co	Ni	Cu	Zn
R	0.412	0.500	0.710	0.530	0.130	0.913	0.176	0.158	-0.691	-0.346

As it is shown in Table 20, most of the metals have positive correlation within leaves and within stem samples which indicate that similar factors affect the accumulation of these metals. Copper and zinc, however, have a negative correlation which implies that there is a competition in the uptake of these metals between leaf and stem or most likely the difference in the age of the stem may affect the uptake of these metals; as it was discussed in previous sections. And as it can be seen in Tables 18 and 19, the correlation of a given metal with metals in leaf and the correlation of a given metal with metals in stem has not strictly followed the same trend; for example, the correlation of cobalt with nickel with in leaf samples is moderately positive where as it is high negative with in stem samples and magnesium has a moderate positive correlation with manganese with in stem samples but it is weak negative with in leaf samples. It is not possible to explain the reason for such deviation with this study alone never the less a difference in the age of the leaf and stem from sample site to sample site may contribute to such variations.

4. CONCLUSSION AND RECOMMENDATIONS

In this study, the optimized procedure was efficient which gave with good recovery for all the elements (91.7-103 for leaf samples and 90.9-103 for the stem samples). All elements were analyzed in leaf samples but in stem samples the concentrations of: chromium at one sample site, cadmium at two sample sites and lead at all sample sites were below the method detection limit.

The level of calcium was the highest throughout the sample sites in leaf samples and in most of the sample sites in stem samples. This indicates, in addition to its alcoholic beverages, *R. prinoides* can be a good source of calcium. The level of the metals analyzed in general, was in the order: Ca > Mg > Fe > Cu ≈ Zn ≈ Co > Ni > Pb > (Mn, Cr) > Cd in leaf samples but there was no good regular trend in stem samples; calcium was the highest at some sample sites followed by magnesium and the reverse at other sample sites. And from the micro nutrients, iron was the highest at some sample sites followed by copper or cobalt and it was the reverse at other sample sites.

Concentration of most of the elements was larger in leaf samples than stem samples except the concentration of some elements like cobalt, copper and magnesium was higher at some sample sites.

There was large variation in concentration of magnesium in stem samples and copper in both stem and leaf samples. Such variation is probably because these elements are strongly affected in mineral accumulation by sampling season or age of the leaf or the stem.

The concentration difference of all metals among sample sites both for leaf and stem samples were statistically significant which implies that the difference in concentration of the metals was not attributed to the random errors during sample preparation and analysis.

From the micro elements, manganese was the least in concentration in both leaf and stem samples at all the sample sites. This is probably this metal is required in small amount by the plant or the medium of the soil may not be favorable for the bio availability of manganese

Since *R. prinoides* is a cash crop and widely used for the bitterness of local alcoholic beverages in Ethiopia and indeed, it is a potential for commercial alcoholic beverages the assessment of levels of essential and heavy toxic metals is particular interest with respect to human health and the quality of its products. Thus, the present study will give brief information about the mineral contents of it. However, similar studies have not been conducted before and hence the results of the experiments were not compared with any other data or validated by certified reference materials and hence, further investigation is required, particularly the results of lead need to be analyzed by other instruments that have small instrument detection limit like graphite furnace atomic absorption spectroscopy.

In addition, the level of essential and non-essential elements of the local alcoholic beverages and the major contributors have not been studied so far and hence, further study, an extension of this research is required. And government and bear factories should encourage for this work.

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DECLARATION

I, the undersigned, confirm that the results reported in this Master's thesis were my original work under the supervision of my advisor in Faculty of Chemical and Physical Sciences, Department of Chemistry, Addis Ababa University in the academic year 2011 and all sources of materials used for this research have been duly acknowledged.

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This Master's thesis has been submitted for examination with my approval as university advisor.

Advisor Name: __________________________

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Place and date of submission: School of Graduate Studies

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