ADDIS ABABA UNIVERSITY

COLLEGE OF NATURAL AND COMPUTATIONAL SCIENCES

Department of Chemistry

Determination of selected metals in the stems of *Salix subserrata* (‘Akya’), *Sida cuneifolia* (‘Chifrig’) and *Clausena anisata* (‘Limich’) collected from three different areas of Ethiopia

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Determination of selected metals in the stems of *Salix Subserrata* ('Akya'), *Sida Cuneifolia* ('Chifrig') and *Clausena anisata* ('Limich') collected from three different areas of Ethiopia

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DECLARATIONS

As thesis advisor, I hereby certify that I have read this thesis prepared under my guidance and recommend that it can be accepted as fulfilling the thesis requirement.

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Advisor	Signature	Date

As members of the Examining Board of the Final MSc Open Defense, we certify that we have read and evaluated the thesis prepared by Wubalem Entele Jote entitled: **"Determination of selected metals in the stems of *Salix subserrata* ('Akya'), *Sida cuneifolia* ('Chifrig') and *Clausena anisata* ('Limich') collected from three different areas of Ethiopia"**; and recommend that it can be ace pted as fulfilling the thesis requirement for the degree of Master of Science in Chemistry.

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Name of Chairman	Signature	Date
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Name of Examiner	Signature	Date
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Final approval and acceptance of the thesis is contingent upon the submission of the final copy of the thesis to the Council of Graduate Studies (CGS) through the Departmental Graduate Committee (DGC) of the candidate's major department.

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List of abbreviations and acronyms

ANOVA	Analysis of variance
HCA	Holeta *Clausena anisata*
HSC	Holeta *Sida cunnefolia*
HSS	Holeta *Salix subserrata*
LOD	Limit of detection
LOQ	Limit of quantification
MCA	Muger *Clausena anisata*
MDL	Method detection limit
MSC	Muger *Sida cunnefolia*
MSS	Muger *Salix subserrata*,
MP-AES	Microwave plasma-atomic emission spectrometry
PVC	Poly vinyl chloride
R^2	Correlation coefficient
RSD	Relative standard deviation
SCA	Sendafa *Clausena anisata*
SD	Standard deviation
SSC	Sendafa *Sida cunnefolia*
SSS	Sendafa *Salix subserreta*
WHO	World Health Organization

Abstract

Pencil sized sticks are fashioned from certain plant parts and are chewed on one end until they become frayed into a brush and the brush end is used to clean the teeth in a similar manner to the toothbrush. The plant parts when used in this manner are commonly referred as the "chewing stick". Their use still continues in the modern era of dentistry. It is inexpensive and easily available in rural areas. Further they are used for customary and religious reasons as well. In this study the levels of essential and non-essential metals in the three plants (*Salix subserrata* ('Akya'), *Sida cuneifolia* ('Chifrig') and *Clausena anisata* ('Limich')) were determined in samples collected from three selected areas (Muger, Sendafa and Holleta) of Ethiopia. After proper sample pretreatment, for *Clausena anisata* ('Limich') 3 mL of HNO_3 (69.5%) and 1 mL of $HClO_4$ (70%) for 2:15 h at a temperature of 240 ^oC, for *Sida cuneifolia* ('Chifrig') 4 mL of HNO_3 (69.5%) and 1 mL of $HClO_4$ (70%) for 3 h at a temperature of 210 ^oC and for *Salix subserreta* ('Akeya') 3 mL of HNO_3 (69.5%) and 1 mL of $HClO_4$ (70%) for 2 h at a temperature of 270 ^oC were the optimized digestion conditions. Then using the optimized conditions sample preparation was made and levels of metals were determined by microwave plasma-atomic absorption spectrometry (MP-AES). The mean concentration levels of the metals in the three plant samples collected from the three sampling areas indicated that higher concentrations of Ca and Fe are present in all the samples collected from the three areas.

The overall mean concentrations of the metals (mg/kg) in the *Salix subserrata*, *Sida cunnefolia*, and *Clausena anisata* samples collected from the three sampling areas can be ordered as Ca (14150-25914) > Fe (514-1191) > Al (103-1263) > Zn (152-196) > Mg (46-102) > Ni (4-160) > Mn (25-78) > Cu (13-20) > Cr (7-8). The accuracy of the optimized procedure was evaluated by analyzing the digest of the spiked samples with standard solution and the percentage recoveries varied from 92% to 104%, which is good and is in the allowed range of 90 ± 10%. ANOVA indicated that there is no significance difference between the mean concentrations of Ca among the *Salix subserrata* samples, of Mg, Cu, Zn and Ca among the *Sida cunnefolia* samples and of Al, Cu, Cr, and Ni among the *Clausena anisata* samples, but there is a significant difference for the other studied metals among the corresponding plant samples at 95% confidence level. Since the toxic elements Cd and Pb in the plant samples are not detected, it is possible to conclude that a person who consumes plants from these sampling areas is free from the risks of Cd and Pb toxicity.

1. INTRODUCTION

1.1 Background

1.1.1 The history of traditional tooth brush sticks

There is a long history of the use of plants to improve dental health and promote oral hygiene and is still commonly practiced among Afro-Asian communities. Pencil sized sticks are fashioned from certain plant parts and are chewed on one end until they become frayed into a brush and the brush end is used to clean the teeth in a similar manner to the toothbrush. The plant parts when used in this manner are commonly referred as the "chewing stick". Their use still continues in the modern era of dentistry. It is inexpensive and easily available in rural areas. Further they are used for customary and religious reasons as well.

Dental treatment usually is expensive and not so easily accessible, especially in developing countries, therefore humans have turned to the use of tooth sticks/chewing sticks to prevent dental caries (Akpata and Akinrimisi, 1977; Homer et al., 1990). Typical photo tooth sticks/chewing sticks are shown in Figure 1.1.

Figure 1.1 Traditional tooth brush sticks.

The use of chewing sticks has been documented since ancient times. This kind of tooth brushing has been used by the Babylonians some 7000 years ago. The use of wood stick for brushing teeth continues to be an important tool in many Afro-Asian communities. It has different names in different societies for instance; miswak, siwak or arak is used in the Middle East, miswaki, in

Tanzania, mefaka in Ethiopia and datun in India and Pakistan. The conventional meaning of miswak is stick used to clean teeth and gums. The most commonly used chewing sticks are those having a good flavor, texture and a recognized effect on the teeth and supporting tissue. Freshly cut specimens are always desirable because they are more easily chewed into a brush. The plants used are very carefully selected for such properties as foaminess, hardness or bitterness and certain species are more popular than others.

1.1.2 Common plants used as brushing sticks

Popular plants which are fascinated into chewing and/or tooth brushing sticks include *Salvadora persica* (miswak from arak tree) and *Azadirachta indica* (Neem). Some of the popular species used as natural toothbrush in Southern-Eastern Africa are *Albizia coriaria, Acacia nilotica, Balanites aegyptiaca, Berchemia discolor, Boscia coriacea, Cadaba farinose, Cordia sinensis, Cupressus lusitanica, Dobera glabra, Dodonia angustifolia, Euclea schimperi, Olea europea subsp. africana, Rhus abyssinica, Rhus natalensis, Rhus retinorrohoae, Rhamnus staddo, Sterospermum kunthianum, Salix subserrata, Vernonia amygdalina*, etc. In West Africa the lime tree (*Citrus aurantafolia*) and the orange tree (*Citrus sinesis*) are used.

The roots of Senna (*Cassia vinnea*) were used by black Americans and those of African laburnum (*Cassia sieberianba*) were used in Sierra Leone. arak, a tree used for miswak, is also known as "tooth brush tree". Although the miswak is usually obtained from the roots of the arak tree, some sticks are made from its branches and bark (Almas, 2001).

There are more than 180 plant species that can be used as a natural toothbrush. These species differ from each other on the basis of appearance, scent, texture and taste. Some of the most commonly practiced species are *S. persica* (Peelu), *Azadirachta indica* (Neem), *Olea europaea* (Zaitoon), *Acacia arabica* (Kikar), *Glycosmis pentaphylla* (Ban), *Capparis aphylla* (Khiran). Most of these sticks are easily available in different parts of Pakistan, Middle East and African countries. Arak (*S. persica*) is the most commonly used miswak in Saudi Arabia while litmus and orange tree are common in West Africa *S. persica* obtained from arak tree is the most popular having spongy characteristics and stem that can easily be crushed between teeth. The

stick is widely accepted by people around the world due to its pleasant flavor, texture and its effectiveness in maintaining oral hygiene.

African toothbrush sticks have been used for centuries for the maintenance of oral hygiene. The chewing sticks, termed as such due to the need for the user to chew the stick prior to brushing, have been used throughout the Greek, Roman, Jewish and Islamic Empires (Almas, 2001). Even though many people have abandoned the traditional toothbrush sticks and adapted to the conventional tooth brushing method, some societies still make use of chewing sticks as a daily ritual to maintain oral hygiene. This is especially true in developing countries where economics, customs, religion and the availability of oral hygiene tools play a role in their continued use (Kemoli et al., 2001). Often, chewing sticks are used by the majority of the population, as is the case of Ethiopians (Fadulu, 1975; Olsson, 1978). Although the World Health Organization has promoted the use of toothbrush sticks (Wu et al., 2001) and has encouraged further research of their efficacy, few studies have been undertaken on the potential antimicrobial properties of chewing sticks. Al-hebshi et al. (2006) confirm this in their investigation of crude khat chewing sticks, which have not been investigated previously. Kassu et al. (1999) assessed the most common toothbrush sticks used by rural and urban Ethiopians.

Three plant species were identified namely *Salix Subserrata* (‘Akya’), *Sida cuneifolia* (‘Chifrig’) and *Clausena anisata* (‘Limich’) and the level of some selected metals (Cd, Cr, Cu, Mn, Ni, Pb, Al, Mg, Ca, Fe and Zn) were determined in the present study. The samples of the selected plants were collected from three different places near to Addis Ababa; Holleta, Sendafa and Muger.

1.1.3 *Clausena anisata* (‘Limich’)

Clausena anisata (Will). Hook.f. ex. benth., is a tropical shrub or tree growing up to ten meters in height and on the margins of evergreen forests. Different parts (stem bark, roots, and leaves) of this plant are widely used in traditional medicine to treat many diseases. Traditional healers in Tanzania use *Clausena anisata* against oral candidiasis and fungal infections of the skin, whereas in the Temeke district (Daressalam, Tanzania), *Clausena anisata* is used against epilepsy and as an anticonvulsant.

Figure 1.2 *Clausena anisata* ('Limich')

Clausena anisata (Willd.) Hook. is a shrub that belongs to the Rutaceae family; it is characterized by evergreen leaves and is the only species in Africa out of the 15 species of its genus. The various morphological parts of the plant have been identified to be effective remedies against worm infections, respiratory ailments, heart disorders, hypertension, malaria, fever, rheumatism, insanity, convulsion and other inflammatory conditions (Hamza, van den Bout-van den Beukel & Matee 2006; Hutchings et al. 1996). The concoction of the stem bark and the leaves was also identified to be an effective traditional treatment for tuberculosis during an ethno botanical survey among Xhosa people in the Eastern Cape Province (Lawal et al. 2014). The unsustainable harvesting of this shrub, because of its large range of medicinal properties, could perhaps threaten its existence. However, *C. anisata* is classified as a red list plant in South Africa (Raimondo 2009). The essential oil in *C. anisata* has been reported to be responsible for its several pharmacological activities, including antimicrobial, anti-inflammatory and anti-diabetic properties (Ojewole 2002; Senthikumar and Venkatesalu 2009; York et al, 2012).

1.1.4 *Sida cuneifolia* (‘Chifrig’)

Sida cuneifolia which is a shrub that has a tough woody stem and can grow up to a meter tall if left undisturbed. It has yellow flowers with five distinct petals and five sepals. Its leaves are notched at the tip with smooth margins. Traditionally, *Sida cuneifolia* has been used as a herbal

remedy to manage up to 12 diseases. In Busoga, Uganda, it is used to disinfect umbilical cord wounds. Among the Sabaot people around Mt. Elgon in Kenya it is known as *Kupchuwet* and the roots are chewed to treat sore throat.

Figure 1.3 *Sida cunnefolia* (‘Chifrig’)

1.1.5 *Salix subserrata* (‘Akeya’)

Salix subserrata (Synonyms *S. safsaf*) Wild (Salicaceae) is a deciduous bush or small tree 2-10 m high at stream sides in throughout Africa (Gambia, Egypt, Libyan, Zambia and Sudan).

Figure 1.4 *Salix subserrata* (‘Akeya’)

1.1.6 Trace elements found in plants

Many curative effects of medicinal herbs used in the phytotherapy are due to the presence of very minute quantities of trace elements. These elements are iron (Fe), copper (Cu), cobalt,

nickel (Ni), zinc (Zn), magnesium (Mg), manganese (Mn), molybdenum (Mo), chromium (Cr), vanadium (V), lithium (Li), selenium (Se), fluorine (F) and iodine(I) (Shirin et al., 2010). Plants readily assimilate such elements through roots, which are dissolved in water and remains in ionic forms. Other heavy metals like lead (Pb), cadmium (Cd) and mercury (Hg) are toxic at very lower concentration. World Health organization (WHO, 1989) declares the maximum permissible levels in food and drug materials for arsenic (As), Cd and Pb as amount to 1.0, 0.3 and 10 mg/kg, respectively (Basgel and Erdemoglu, 2006). A high supplementation of Cu had been related with liver damage. Zn may produce adverse nutrient interactions with Cu. Zn reduces the immune function and levels of high-density lipoproteins.

Plants may absorb heavy metals from soil, water or air. Medicinal herbs may be easily contaminated during growing and processing. The ability of plants to selectively accumulate essential elements is different for different species and is subjected to certain geochemical characteristics depending on the type of soil (Bin *et al.*, 2001). Usually soil is subjected to contamination through atmospheric deposition of heavy metals from point sources including metalliferous mining, smelting and different industrial activities.

Some other sources of soil contamination involve use of fertilizers, pesticides, sewage sludge and organic manures (Singh *et al*., 1997). Plants readily assimilate such elements through the roots. Metallic ions get dissolved in water and retained. Additional sources of these elements for plants are rainfall, atmospheric dusts and plant protection agents, which could be adsorbed through the leaf blades. An important source of contamination, in vegetable crops, is considered to be the foliar uptake of atmospheric heavy metals emissions by the soil (Salim *et al.*, 1993).

In general, most plants grow by absorbing nutrients from the soil. Their ability to do this depends on the nature of the soil. A soil contains some combination of sand, silt, clay, and organic matter. This combination depends on its location. Soil texture and its pH determine the extent to which nutrients are available to plants. The path taken by metal to transport into the plant is: soil > roots > stems > leaves. The minerals often accompanied by various organic molecules supplied by root cells get transported after being dissolved in the water. This mixture deposits into xylem and from there it moves up in the vessels and tracheids. Minerals enter the root by active transport into the epidermal cells and move towards other parts of plant.

The accumulation of metals in plants is associated with nature of soil and climatic conditions. Bioavailability of metal in plant depends on active and passive transport processes, the response of plant to element, redox state of metal and its solubility and also on plant genotype.

1.1.7 Essential and toxic metals

Essential metals

Calcium

It is one of essential macro-nutrient element. Calcium, the structural element, is found mainly in our bones. Most individuals are aware of the benefits of calcium supplementation. It is important for bone and teeth formation and structure. Calcium also appears to play a role in maintaining normal blood pressure. In addition to this, it is required by the body for blood clotting, muscle contraction and nerve transmission this is because, it is a component of enzymes that contribute to blood clotting, muscle activity and nerve function. It also regulates cell membrane permeability to control nerve impulse transmission and muscle contraction. It regulates hormonal secretion and cell division. Calcium may play a role in triglyceride and cholesterol reduction due to its fat binding properties within the gastrointestinal tract. Good food sources of calcium are dairy products such as cheese and yogurt, fortified bread and flower, yoghurt, tofu, cereals and dark green vegetables, milk, sardines, egg yolks, almonds, sesame seeds, and seaweed. It is also known that over dose consumption of minerals may upset the normal functioning of the body.

Toxicity of calcium occurs with increased intake of calcium. Calcium competes with a number of minerals for absorption therefore supplementation with a multi-mineral may be necessary to prevent decreased levels of other minerals due to calcium. Calcium supplements are usually the cause for the overdose. Bones and teeth are the main storage tissues of calcium in the body.

Chromium

Chromium (Cr), the most abundant environmental form, is an essential element that plays a role in glucose metabolism. Chromium deficiency causes changes in the metabolism of glucose and lipids and may be associated with maturity-onset diabetes, cardiovascular diseases, and nervous system disorders (EMEA, 2007).

Copper

Copper (Cu) is an essential yet potentially toxic element to all organisms. Cu serves as a catalytic and structural cofactor for enzymes that function in energy generation, iron acquisition, oxygen transport, cellular metabolism, peptide hormone maturation, blood clotting, and signal transduction. It is also used as a co-factor in several enzymes and several proteins have evolved to tightly regulate the distribution of copper in the cell. In humans, unbalanced concentration of copper has been implicated in Wilson's disease (excess), Menke's syndrome (deficiency), and neurodegenerative diseases (Alzheimer's disease, amylotrophic lateral sclerosis and prion disease).

Manganese

Manganese (Mn), an essential element, is crucial for a number of biological and physiological processes in the body, including immune function, regulation of cellular energy, reproduction, digestion, bone and connective tissue growth, and blood clotting. Mn also plays an important role as a cofactor for many enzymic reactions including amino acid, lipid, protein, and carbohydrate metabolism (Yoon *et al.,* 2011). Signs of manganese deficiency include impaired growth, skeletal abnormalities, disturbed or depressed reproductive function, ataxia of the newborn, neurotoxic effects, defects in lipid and carbohydrate metabolism (Thompson *et al.*, 2011).

Nickel

The essentiality of nickel in mammals is questionable. It is generally present in the environment and appears to be an essential element for some plant life and bacteria. For the general population, the primary health concern is an allergic response from skin contact. Nickel is one of the few proven human carcinogens. Contact dermatitis is also a common workplace hazard (Gilbert, 2005). Ni is mostly present in the pancreas, where it plays an important role in the production of insulin. Its minute quantity is required and its deficiency results in the disorder of liver, whereas at higher concentration, it shows allergic dermatitis known as Niitch. Arsenic neuropathy is a recognized complication of As toxicity. As is associated with the cancers of skin and internal organs or is associated with vascular diseases. (Gilbert, 2005).

Zinc

Zinc is an essential trace element that plays important role in a wide range of Zn-containing proteins necessary for growth, development, and DNA replication. Zn also has a variety of critical functions including maintaining appetite, wound healing, immune competence, cofactor of the superoxide dismutase enzymes, and stabilization of phosphate groups and coordination with organic bases in DNA. However, it also has the potential to interact with many biological functions to induce adverse effects such as nausea, vomiting, diarrhea, fever and lethargy.

Iron

Fe is an essential element for human beings and animals and is an essential component of hemoglobin. Low Fe content causes gastrointestinal infection, nose bleeding and myocardial infarction. Fe is capable of generating reactive oxygen species, which contributes pathogenesis of diabetes and its complications as diabetic nephropathy.

Toxic metals

Cadmium

Cadmium, a universal environmental contaminant, damages several major organs of humans and other mammals (Martin et al., 2007). It can induce various toxic effects such as hepatotoxicity, nephrotoxicity (Tokumoto et al., 2011), osteotoxicity, immunotoxicity, cytotoxicity (Honda et al., 2010; Yang et al., 2007) autophagic cell death (Wang et al., 2009). So far, there are no studies available demonstrating that cadmium is a human teratogen, but is a potent teratogen in laboratory animals, causing exencephaly when administered at early stages of development.

Lead

Lead is one of the most ubiquitous toxic metals known to man. Lead occurs naturally in plants and in soils throughout the world. Chronic lead exposure has been linked with cardiac arrhythmia, renal in sufficiency, hypel1ension, and osteoporosis. Studies indicated that apoptosis may be associated with the lead-induced oxidative stress and DNA damage (Jamieson et al., 2006; Seth et al., 2004). Pb is known to induce renal tumors, reduce cognitive development and increase blood pressure and cardiovascular disease in adults. Cd induces kidney dysfunction, osteomalacia and reproductive deficiencies. Hg causes neurological disorders and has toxic effect on the kidney. Trace amounts of trivalent Cr is required in humans for sugar and lipid metabolism, and its deficiency may cause a disease called Cr deficiency, whereas its hexavalent form is extremely toxic and carcinogenic. It can enter the human cell because of its easy permeation through biological membrane and transferred into more stable form, which can damage DNA. For normal synthesis and secretion of insulin, Mn is required in trace amounts. It acts as a cofactor for a number of enzymatic systems.

1.2 Objectives

1.2.1 General Objective

To determine the levels of some heavy metals (Cd, Cr, Cu, Mn, Ni, Pb, Al, Mg, Ca, Fe and Zn) in three plant species (*Salix subserrata* ('Akya'), *Sida cuneifolia* ('Chifrig') and *Clausena anisata* ('Limich')) used as tooth brush sticks.

1.2.2 Specific Objectives

- To determine the level of selected heavy metals in the selected plant samples.
- To compare the content of heavy metals in the three plant species' from each sampling site and among each other.
- To compare the content of heavy metals in the three plant species' with the metal contents of tooth pastes.

2. EXPERIMENTAL

2.1 Instrument and Apparatus

Stainless steel axe and Teflon (PTFE) knife were used to cut the plant parts in to pieces while air-circulating oven (Digitheat, J.P. Selecta, Spain) was used for drying the samples placed on porcelain. Ceramic pestle and mortal were used for grinding and homogenizing the samples. Digital analytical balance (Mettler Toledo, Model At250, Switzerland) with precision of was used for weighing the samples. Round bottom flasks (250 mL) fitted with reflux condenser were employed in digesting the sample on Kjeldahl heating apparatus (Gallenhamp, England). Borosilicate volumetric flasks (50 mL) were used during dilution of sample and preparation of metal standard. Measuring cylinders (Duran, Germany), pipettes (Pyrex, USA), micropipettes (Dragonmed, 1-10 μL, 100-1000 μL, Shangai, China) were used during measuring different quantities of volumes of sample solution, acid reagents and metal standard solutions. Metals' concentration determination was done by microwave plasma-atomic emission spectroscopy (MP-AES) (Agilent model 4200, USA).

2.2 Chemicals and Reagents

Reagents that were used in the analysis were all analytical grade. 69.5% HNO_3 (Supreme Enterprises Cantt, India) and 70% $HClO_4$ (Aldrich, UK) were used for the digestion of the samples. Stock standard solution of concentration 1000 mg/L in 2% HNO_3 of the metals Ca, Mg, Mn, Fe, Zn, Cu, Zn, Cr, Ni, Co, Cd and Pb (Buck Scientific Puro-Graphictm) from which 10 mg/L of intermediate standard obtained were used for the preparation of the calibration standards of each metal. Working standards were prepared from intermediate standards of each metal. Deionized water was used for sample preparation, dilution, and rinsing apparatus prior to analysis.

2.3 Procedure

2.3.1 Cleaning Apparatus

Apparatus such as volumetric flasks, measuring cylinder and digestion flasks were washed with the tap water using detergent followed by distilled water rinsing. The apparatuses were then soaked with about 50% (v/v) nitric acid for 2 days followed by rinsing with distilled water five times. The apparatus were then dried in hot air oven and kept in dust free place until analysis begins.

2.4 Description of the study area

The study was conducted in selected sites of three regions namely: Muger, Holleta and Sendafa; smaller cities that are found near to Addis Ababa, the capital city of Ethiopia. The selection of these sites was based on some reasons. The study selected the stated areas based on availability of the plant and its popularity in using as tooth brush plants by the local peoples around. Thus having these two reasons, we have selected the stated areas for our study.

Table 2.1 Geographical descriptions of the study areas

Sites	Latitude	Longitude	Altitude (m)	Distance from Addis Ababa (km)
Muger	9°28'57"N	38º21'15.38"W	2450	81.6
Holleta	9°3'N 38°30'E	9.050ºN 38.500ºE	2391	38.8
Sendafa	9°09'N 38°02'E	9.150ºN 39.033ºE	2514	40.5

2.5 Sampling

Sampling plan is composed of three components; (i) sampling, (ii) sample preparation, and (iii) analysis. Nature of the analyte of interest, distribution of the analyte throughout the lot, physical characteristics of the product, accessibility of the product to random representative sampling, sampling procedure, and size of sample are some of the factors affecting the ability of the sampling plan to accomplish the certain objective.

2.5.1 Sample Collection, Preservation and Handling

Salix subserrata (‘Akya’), *Sida cuneifolia* (‘Chifrig’) and *Clausena anisata* (‘Limich’) samples were collected from three different sites which are the most common areas from which the sticks that are used for brushing teeth in Addis Ababa. From each sample types unknown amount of branch stems with leaves samples of *Salix subserrata* (‘Akya’) and *Clausena anisata* (‘Limich’) and roots of *Sida cuneifolia* (‘Chifrig’), since only the roots of this plant are used as chewing sticks. The collected samples were kept in polyethylene bags. The collected plant samples were transported to the laboratory. Some unwanted materials such as leaves and very tiny roots have been removed but the barks were included in the sample because that is how the chewing sticks are prepared.

The stems and roots of the plants were washed well with the running tap water and rinsed first with distilled water and then with deionized water to remove earthly impurities, allowed to dry in air for ten days, chopped and grounded to powder with recommended, acid washed mortar and pestle. Then the powdered samples were screened with recommended sieve size and stored in dry, clean and closely packed polyethylene plastic bag until digestion. Finally, 0.5 g aliquot was taken from each sample for digestion and a solution for final metal determination was prepared.

2.6 Optimization and digestion procedure

To select an optimum procedure for digestion, parameters like digestion time, volume ratio of reagents, and digestion temperature were optimized by varying one parameter at a time and keeping the others constant. Parameters giving clear solution at lower temperature, requiring minimum reagent volume and digestion time were selected as an optimum procedure for digestion of the three plant samples. Finally, the optimum procedure was chosen for each sample.

Table 2.2 Optimization of *Clausena anisata* (‘Limich’)

Trial	Reagents used	Volume Ratio	Temp. (^oC)	Digestion time (h)	Description
1	HNO_3:$HClO_4$	2:2	300	3	Light yellow
2	**HNO_3:$HClO_4$**	**3:1**	**300**	**3**	**Clear and Colorless**
3	HNO_3:$HClO_4$	3:2	300	3	Clear and Colorless
4	HNO_3:$HClO_4$	3:3	300	3	Clear and Colorless
5	HNO_3:$HClO_4$	4:1	300	3	Clear and Colorless
6	HNO_3:$HClO_4$	4:2	300	3	Clear and Colorless
7	HNO_3:$HClO_4$	3:1	150	3	Light yellow
8	HNO_3:$HClO_4$	3:1	180	3	Light yellow
9	HNO_3:$HClO_4$	3:1	210	3	Light yellow
10	**HNO_3:$HClO_4$**	**3:1**	**240**	**3**	**Clear and Colorless**
11	HNO_3:$HClO_4$	3:1	270	3	Clear and Colorless
12	HNO_3:$HClO_4$	3:1	300	3	Clear and Colorless
13	HNO_3:$HClO_4$	3:1	240	1:45	Light yellow
14	HNO_3:$HClO_4$	3:1	240	2:00	Light yellow
15	**HNO_3:$HClO_4$**	**3:1**	**240**	**2:15**	**Clear and Colorless**
16	HNO_3:$HClO_4$	3:1	240	2:30	Clear and Colorless
17	HNO_3:$HClO_4$	3:1	240	2:45	Clear and Colorless
18	HNO_3:$HClO_4$	3:1	240	3:00	Clear and Colorless

*The bold font indicates the optimum reagent volume ratio, temperature and time.

Table 2.3 Optimization of *Sida cuneifolia* (‘Chifrig’)

Trial	Reagents used	Volume Ratio	Temp. (^oC)	Digestion time (h)	Description
1	HNO_3:$HClO_4$	2:2	300	3	Light yellow
2	HNO_3:$HClO_4$	3:1	300	3	Light yellow

3	HNO_3:$HClO_4$	3:2	300	3	Yellowish
4	HNO_3:$HClO_4$	3:3	300	3	Yellowish
5	**HNO_3:$HClO_4$**	**4:1**	**300**	**3**	**Clear colorless**
6	HNO_3:$HClO_4$	4:2	300	3	Clear colorless
7	HNO_3:$HClO_4$	4:1	120	3	Deep yellow
8	HNO_3:$HClO_4$	4:1	150	3	Yellowish
9	HNO_3:$HClO_4$	4:1	180	3	Light yellow
10	**HNO_3:$HClO_4$**	**4:1**	**210**	**3**	**Clear and colorless**
11	HNO_3:$HClO_4$	4:1	240	3	Clear and colorless
12	HNO_3:$HClO_4$	4:1	270	3	Clear and colorless
13	HNO_3:$HClO_4$	3:1	210	1:45	deep yellow
14	HNO_3:$HClO_4$	3:1	210	2:00	Yellowish
15	HNO_3:$HClO_4$	3:1	210	2:15	Yellowish
16	HNO_3:$HClO_4$	3:1	210	2:30	Light yellow
17	HNO_3:$HClO_4$	3:1	210	2:45	Light yellow
18	**HNO_3:$HClO_4$**	**3:1**	**210**	**3:00**	**Clear & colorless**

*The bold font indicates the optimum reagent volume ratio, temperature and time.

Table 2.4 Optimization of *Salix subserreta* (‘Akeya’)

Trial	Reagents used	Volume Ratio	Temp. (^oC)	Digestion time (h)	Description
1	HNO_3:$HClO_4$	2:2	300	3	Light yellow
2	**HNO_3:$HClO_4$**	**3:1**	**300**	**3**	**Clear and colorless**
3	HNO_3:$HClO_4$	3:2	300	3	Light yellow
4	HNO_3:$HClO_4$	3:3	300	3	Light yellow
5	HNO_3:$HClO_4$	4:1	300	3	Clear and colorless
6	HNO_3:$HClO_4$	4:2	300	3	Yellowish

7	HNO_3:$HClO_4$	3:1	150	3	Light yellow
8	HNO_3:$HClO_4$	3:1	180	3	Light yellow
9	HNO_3:$HClO_4$	3:1	210	3	Deep yellow
10	HNO_3:$HClO_4$	3:1	240	3	Light yellow
11	**HNO_3:$HClO_4$**	**3:1**	**270**	**3**	**Clear and colorless**
12	HNO_3:$HClO_4$	3:1	300	3	Clear and colorless
13	HNO_3:$HClO_4$	3:1	240	1:45	Light yellow
14	**HNO_3:$HClO_4$**	**3:1**	**240**	**2:00**	**Clear and colorless**
15	HNO_3:$HClO_4$	3:1	240	2:15	Clear and colorless
16	HNO_3:$HClO_4$	3:1	240	2:30	Clear and colorless
17	HNO_3:$HClO_4$	3:1	240	2:45	Clear and colorless
18	HNO_3:$HClO_4$	3:1	240	3:00	Clear and colorless

*The bold font indicates the optimum reagent volume ratio, temperature and time.

Applying the optimized procedure given above, 0.5 g of powdered sample of *Clausena anisata* ('Limich') was weighed on analytical digital balance and placed in a 250 mL round bottom flask. To this, 3 mL of HNO_3 (69.5%) and 1 mL of $HClO_4$ (70%) was added. The round bottom flask was fitted to a reflux condenser and heated on a Kjeldahl apparatus hot plate for 2:15h at a temperature of 240 ^oC. The digest was allowed to cool for 10 min without dismantling the condenser and then further cooled to room temperature for 10 min by dismantling the condenser. The mixture then diluted with 10 mL of distilled-deionized water and filtered with Whatman filter paper No. 42 into a 50 mL volumetric flask. The round bottom flask was further rinsed with 5 mL of distilled-deionized water and added to the filtrate.

Applying the optimized procedure given above, 0.5 g of powdered sample of *Sida cuneifolia* ('Chifrig') was weighed on analytical digital balance and placed in a 250 mL round bottom flask. To this, 4 mL of HNO_3 (69.5%) and 1 mL of $HClO_4$ (70%) was added. The round bottom flask was fitted to a reflux condenser and heated on a Kjeldahl apparatus hot plate for 3h at a temperature of 210 ^oC. The digest was allowed to cool for 10 min without dismantling the condenser and then further cooled to room temperature for 10 min by dismantling the condenser.

The mixture then diluted with 10 mL of distilled-deionized water and filtered with Whatman filter paper No. 42 into a 50 mL volumetric flask. The round bottom flask was further rinsed with 5 mL of distilled-deionized water and added to the filtrate.

Applying the optimized procedure given above, 0.5 g of powdered rue sample of *Salix subserreta* ('Akeya') was weighed on analytical digital balance and placed in a 250 mL round bottom flask. To this, 3 mL of HNO_3 (69.5%) and 1 mL of $HClO_4$ (70%) was added. The round bottom flask was fitted to a reflux condenser and heated on a Kjeldahl apparatus hot plate for 2h at a temperature of 270 ^oC. The digest was allowed to cool for 10 min without dismantling the condenser and then further cooled to room temperature for 10 min by dismantling the condenser. The mixture then diluted with 10 mL of distilled-deionized water and filtered with Whatman filter paper No. 42 into a 50 mL volumetric flask. The round bottom flask was further rinsed with 5 mL of distilled-deionized water and added to the filtrate.

2.8. Determination of the metals by MP-AES

2.8.1 Calibration of the instrument

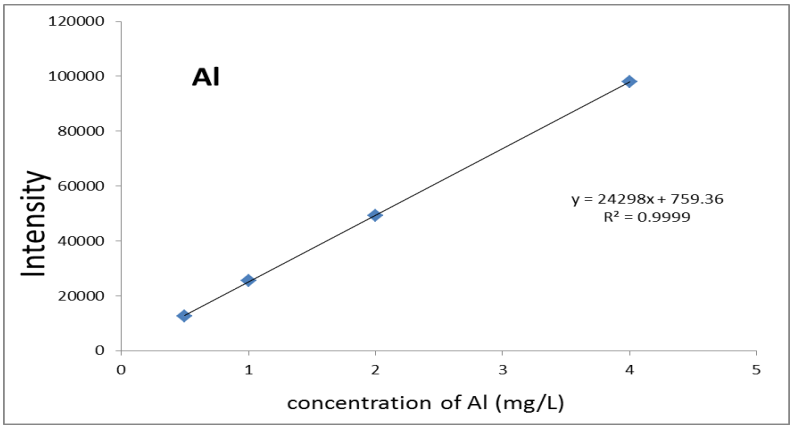
Intermediate standard solutions of metals of interest were prepared from the atomic absorption spectroscopy standard stock solutions that contained 1000 mg/L. These secondary standards were diluted with deionized water to obtain four working standards of each metal, i.e. Al, Ca, Cr, Cu, Zn, Cd, Ni and Pb. The absorbance's' of the working standard solutions were measured and the calibration curves for each of the analyte metal (Ca, Cr, Cu, Zn, Ni and Pb) were constructed. The operating conditions for MP-AES employed for each analytes is given in Table 2.5.

Table 2.5 Instrumental operating conditions for determination of metals in the sample plants

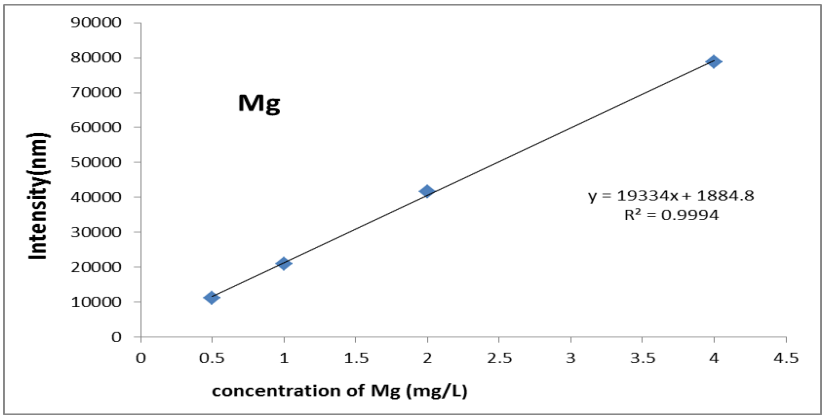
Metals	Parameters											
	Wave length (nm)	Back-ground correction	EGCM setting	Replicates	Pump speed (rpm)	Blank subtraction	Stabilization time (s)	Sample uptake time (s)	Sample uptake fast pump	Rinse time (s)	Read time (s)	Nebulizer flow (L/min)
Cu	327.395	Auto	High	3	15	On	16	28	On	10	3	0.7
Mg	279.553	Auto	High	3	15	On	16	28	On	10	3	0.9
Mn	259.372	Auto	High	3	15	On	16	28	On	10	3	0.9
Ni	305.081	Auto	High	3	15	On	16	28	On	10	3	0.7
Al	396.152	Auto	High	3	15	On	16	28	On	10	3	0.95
Zn	213.857	Auto	High	3	15	On	16	28	On	10	3	0.45
Cr	276.653	Auto	High	3	15	On	16	28	On	10	3	0.9
Ca	422.673	Auto	High	3	15	On	15	27	On	10	3	0.6
Fe	371.993	Auto	High	3	15	On	22	30	On	10	3	0.65

Calibration curves of working standard concentrations of metals versus Intensity

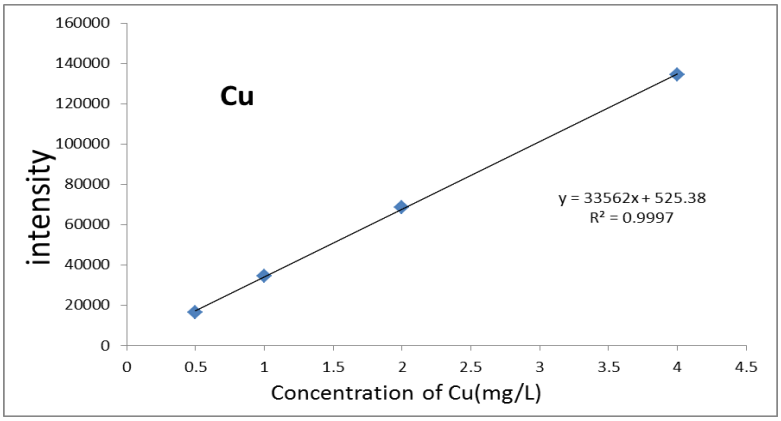
A)



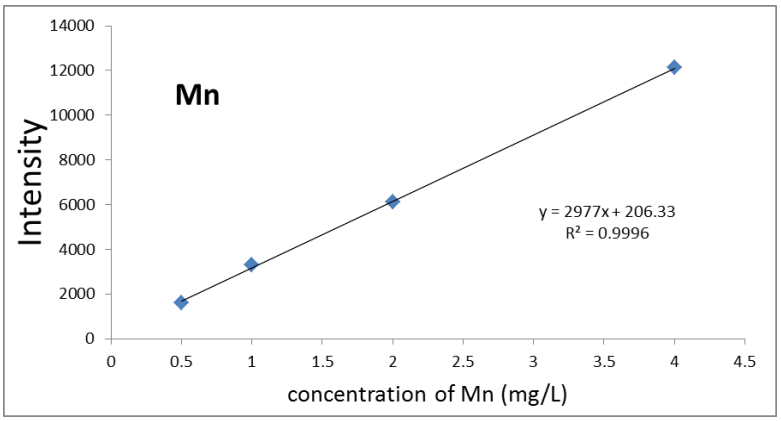
B)



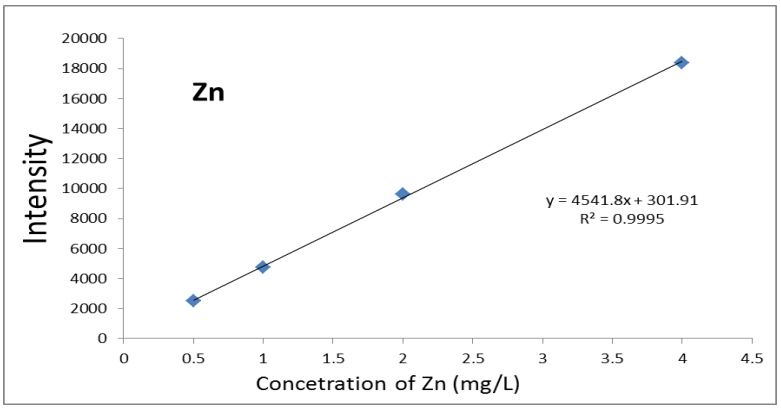
C)


Cu
intensity
y = 33562x + 525.38
R² = 0.9997
Concentration of Cu(mg/L)

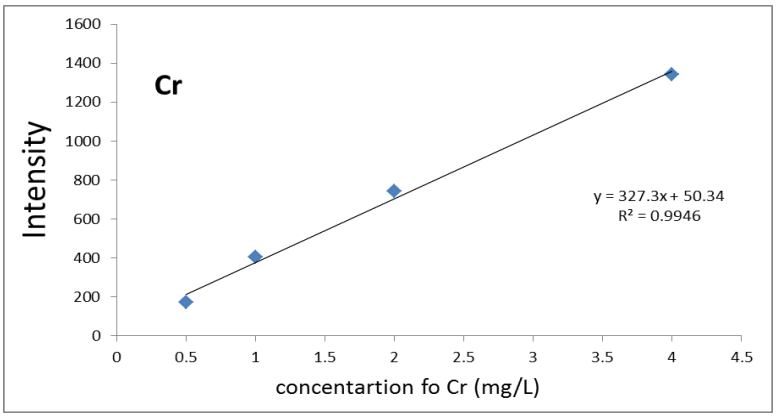
D)


Mn
Intensity
y = 2977x + 206.33
R² = 0.9996
concentration of Mn (mg/L)

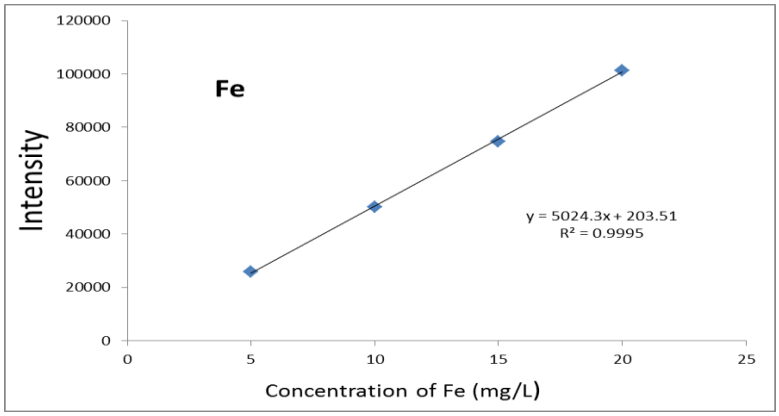
E)


Zn
Intensity
y = 4541.8x + 301.91
R² = 0.9995
Concetration of Zn (mg/L)

F)


Cr
Intensity
y = 327.3x + 50.34
R² = 0.9946
concentartion fo Cr (mg/L)

G)



H)

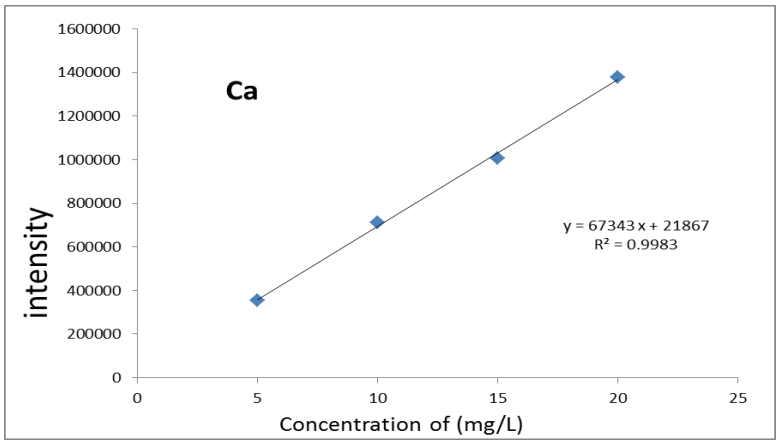


Figure 2.1 Calibration curves of the metals (A-H)

From the correlation coefficients and their corresponding calibration curves in Figure 2.1 (A-H) for each metal, it is possible to say that, the change in intensity with concentration is in good positive correlation and are linearly fit.

2.8.2. Method Detection and Quantification Limit

The limit of detection (LOD) is a measure of how sensitive the analytical method is and is the lowest concentration or weight of analyte that can be measured at a specific confidence level. For the determination of limit of detection of the analytical method (LOD), three blanks were prepared and analyzed for their metal contents. The standard deviation (SD) of the three blanks was calculated and multiplied by three (LOD = 3SD) to determine the method detection limit (Boke et al., 2015). The limit of quantification (LOQ) is the smallest quantity of analyte that can be measured with acceptable accuracy and precision and it is described as ten times of the

standard deviation. In this study the limit of detections for all of the nine elements in the plant samples were obtained and are given in Table 2.6.

Table 2.6 The wavelength, method detection and quantification limit, correlation coefficient and calibration curve equations.

Metals	Wave length (nm)	MDL (mg/kg)	MQL (mg/kg)	Correlation coefficient (R^2)	Calibration curve equation
Al	396.152	0.17	0.57	0.9999	y = 24298x + 759.36
Mg	279.553	0.15	0.50	0.9994	y = 19334x + 1884.8
Cu	327.395	0.40	1.33	0.9997	y = 33562x + 525.38
Mn	259.372	0.02	0.07	0.9996	y = 2977x + 206.33
Ni	305.081	0.27	0.90	0.9997	y = 4453.4x + 270.29
Zn	213.857	0.22	0.73	0.9995	y = 4541.8x + 301.91
Cr	276.653	0.34	1.13	0.9946	y = 327.3x + 50.34
Fe	371.993	0.40	1.33	0.9995	y = 5024.3x + 203.51
Ca	422.673	0.56	1.86	0.9983	y = 67343 x + 21867

Calibration curve for each element was constructed using an appropriate standard at a series of concentrations. Regression equation for each metal was constructed and the best fit of the equation was checked using correlation coefficient (R^2). In all the cases, the regression coefficient (R^2) was found to be the accepted linear range value of 0.999. The calibration curves were with good correlation coefficients.

Spiking experiments were performed to validate the optimized procedure. For this purpose, standard solutions of 1,000 mg/L each metal were used and intermediate standards of each metal (Mg, Cu, Mn, Ni, Zn, and Cr) were prepared from 1,000 mg/L primary standard solution. For

recovery measurement the *Clausena anisata* (‘Limich’) sample from Muger was selected and the spiking was done in triplicate.

From the stock solution of 1000 mg/L, 34.26 µL of Zn, 32.79 µL of Mn, 14.95µL of Cd, 2.63 µL of Cu, 1.92 µL of Cr, and 1.64µL of Ni were added to 0.5 g of *Clausena anisata* sample. The spiked and non-spiked samples were digested and analyzed in similar condition using the optimized procedure. As shown in Table 2.7, six metals were analyzed in triplicate standard metal solutions to evaluate the efficiency of the procedure and the percentage recoveries lies within the range from 83.8 to 104%, and were calculated using the following formula.

$$\% \text{ Recovery} = \frac{\text{spiked sample} - \text{unspiked sample}}{\text{amount added}} x\ 100$$

Table 2.7 Analytical results for recovery test of plant samples.

Metal	Concentration of metal in un-spiked sample (mg/kg)	% spiked	Amount spiked (mg/L)	Concentration of metal in spiked sample (mg/kg)	% R ($\bar{X}$%R ± SD)
Mg	119	25	29.8	144	83.8±5.60
Cu	13.1	40	5.26	18.6	104 ± 5.50
Mn	327	20	65.5	384	87 ±5.90
Ni	6.55	50	3.28	9.82	99.6±9.70
Zn	342	20	68.51	412	102±2.80
Cr	7.61	50	3.84	11.2	93.4±7.40

3. RESULTS AND DISCUSION

3.1 Calibration curves of standards for each metal

The calibration curves were prepared from standards of known concentration covering the concentration range expected in the sample. Then, the curves were established at five concentration levels corresponding to 0.5, 1, 2 and 4 mg/L for Al, Mg, Cu, Mn, Ni, Zn, and Cr and 5, 10, 15, and 20 mg/L for Fe and Ca. All the working standards of metals solution used for calibration curve exhibited very good linearity with squared regression coefficients (R^2) values ranged from 0.9946 to 0.9999.

3.2 The concentrations of metals in plant samples collected from the sampling areas

As shown in Table 3.1 the concentrations of the metals were determined by using MP-AES and precision of the results was determined by calculating the standard deviation (SD). Mean values were determined from triplicate analysis of each sample and triplicate samples were used for each sample site. As such the mean values determined were triplicate of triplicate analysis for each metal and the results were in terms of mean values ± SD (where n = 3 for each of the metal in this study).

All the results obtained from the MP-AES that are expressed in terms of (mg/L) were converted into (mg/kg) using the following equation.

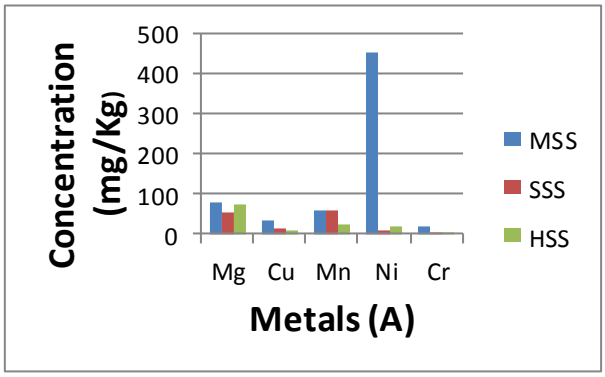
$$\frac{mg}{kg} = \frac{C \times V \times D}{W} \qquad (1)$$

Where; C = concentration in ppm, V = volume in liter, W = weight of sample in grams and D = dilution factor (considered as unity).

The maximum concentration levels in the plant samples for elements Al, Mg, Cu, Mn, Ni, Zn, Cr, Fe and Ca are 1032, 119, 17.1, 101, 452, 342, 19, 3250 and 27990 mg/kg, respectively. Similarly the minimum concentration levels in the plant samples for the elements Al, Cd, Cu, Mn, Ni, Zn, Cr, Fe and Ca are 89.3, 8.08, 11.2, 20.5, 1.95, 108, 2.49, 154 and 10602 mg/kg, respectively.

Table 3.1 Mean concentrations (mean ± SD, n = 3, mg kg^{-1} dry weight) of metals in each sample sites analyzed by MP-AES.

Sites	Type of plant	Al	Mg	Cu	Mn	Ni	Zn	Cr	Fe	Ca	Pb	Cd
Muger	***Salix subserrata***	91.9±0.99	80.0±0.50	36.4±5.48	60.7±6.51	452±8.75	147±6.60	19.0±7.12	1035±8.82	16687±4.98	ND	ND
	Sida cuneifolia	1032±8.05	82.8±6.60	16.0±2.22	61.3±2.32	5.64±0.90	152±1.73	5.98±2.70	193±7.34	26182±6.43	ND	ND
	Clausena Anisata	104±7.10	119±2.86	13.1±0.90	23.1±0.37	6.55±3.27	342±5.42	7.68±1.16	3250±4.91	19285±4.49	ND	ND
Sendafa	***Salix subserrata***	149±7.79	52.7±2.29	12.7±0.74	61.3±2.32	7.65±1.12	281±4.96	2.49±0.33	248±4.55	10602±2.59	ND	ND
	Sida cuneifolia	2291±7.89	104±5.87	20.4±1.96	101±7.68	6.57±0.49	155±7.10	7.71±0.20	2853±3.69	23569±6.14	ND	ND
	Clausena anisata	89.3±3.49	89.3±3.49	13.2±0.85	20.5±0.28	3.51±0.48	122±8.63	7.58±0.36	168±3.37	16288±8.53	ND	ND
Holeta	***Salix subserrata***	122±5.80	76.5±4.98	11.2±0.84	23.1±0.37	19.8±8.52	108±4.34	2.49±0.33	259±7.15	15161±8.64	ND	ND
	Sida cuneifolia	467±3.32	118±8.53	17.1±1.11	71.9±0.57	3.74±0.35	148±5.58	7.71±0.20	507±6.22	27990±8.08	ND	ND
	Clausena Anisata	117±3.84	84.8±3.63	13.0±1.94	31.6±6.07	1.95±0.47	123±6.18	7.58±0.36	154±7.24	18834±6.71	ND	ND



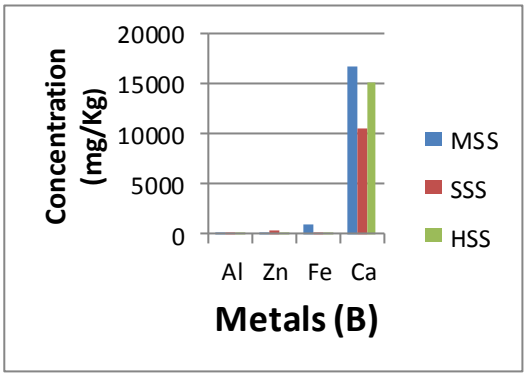
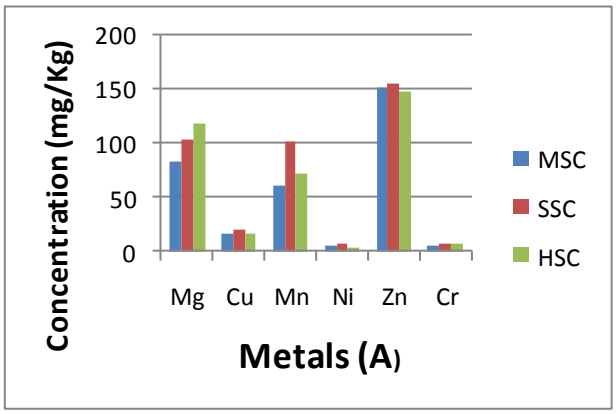


Figure 3.1 Concentrations of metals (mg/kg) in *Salix Subserrata* samples collected from the three sampling areas. (Where: MSS = Muger *Salix subserrata*, SSS = Sendafa *Salix subserreta* and HSS = Holeta *Salix subserrata*).

As shown in Figure 3.1(A), higher concentrations of Ni is observed in the *Salix subserrata* samples collected from the Muger sampling site, and relatively lower amounts of Cr and Cu is observed compared to that of Cd, Mn, and Ni collected from the three sampling sites. Similarly as shown in Figure 3.1(B), relatively higher concentrations of Ca is observed compared to that of Fe, Al and Zn in the *Salix subserrata* samples collected from the three sampling areas. In short as shown in Table 3.1, the mean concentrations of the metals (mg/kg) in the *Salix subserrata* samples collected from the three sampling areas can be ordered as Ca > Fe > Ni > Zn > Al > Mg > Mn > Cu.



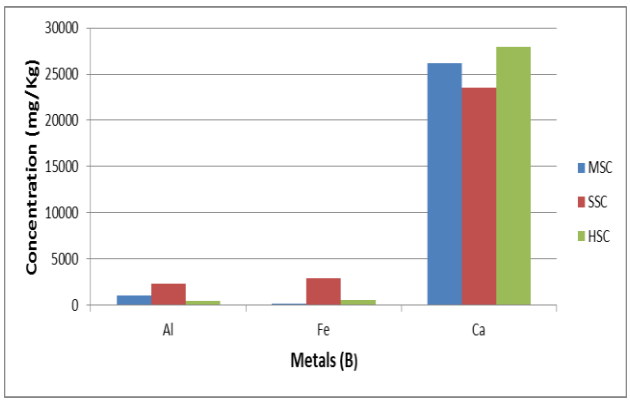


Figure 3.2 The concentrations of metals (mg/kg) in *Sida cunnefolia* samples collected from the three sampling areas. (Where: MSC = Muger *Sida cunnefolia*, SSC = Sendafa *Sida cunnefolia* and HSC = Holeta *Sida cunnefolia*).

As shown in Figure 3.2(A), higher concentrations of Zn, Mn and Cd is observed in the *Sida cunnefolia* samples compared to that of Cu, Cr, and Ni collected from the three sampling sites. Similarly as shown in Figure 3.2(B), relatively higher concentrations of Ca is observed compared to that of Fe and Al in the *Sida cunnefolia* samples collected from the three sampling areas. In short as shown in Table 3.1 the mean concentrations of the metals (mg/kg) in the *Sida cunnefolia* samples collected from the three sampling areas can be ordered as Ca > Fe > Al > Zn > Cd > Mn > Cu > Cr > Ni.

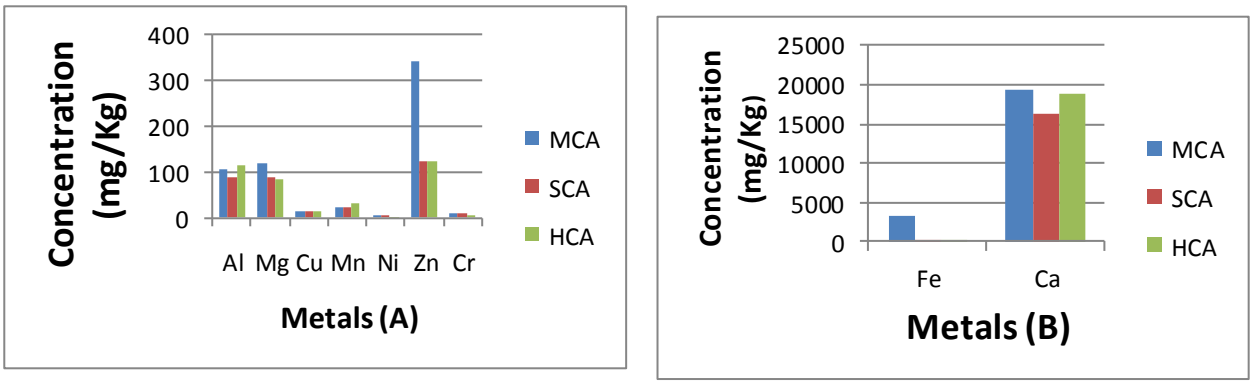


Figure 3.3. The concentrations of metals (mg/kg) in *Clausena anisata* samples collected from the three sampling areas. (Where: MCA = Muger *Clausena anisata*, SCA = *Clausena anisata* and HCA = Holeta *Clausena anisata*).

As shown in Figure (A), higher concentrations of Zn is observed in the *Clausena anisata* samples collected from the Muger sampling site compared to Zn collected from the three sampling sites, and relatively lower amounts of Cr, Ni and Cu is observed compared to that of Al, Cd, and Zn collected from the three sampling sites. Similarly as shown in Figure 2(B), relatively higher concentrations of Ca is observed compared to that of Fe in the *Clausena anisata* samples collected from the three sampling areas.

Table 3.2 Mean concentrations of metals (mg/kg) in the plant samples collected from the three the 3 sampling areas

Metals	*Salix subserrata*	*Sida cunnefolia*	*Clausena anisata*	Over all mean
Al	121	1263	103	496
Mg	46	102	98	82
Cu	20	18	13	17
Mn	48	78	25	50
Ni	160	5	4	56
Zn	179	152	196	176
Cr	8	7	8	8
Fe	514	1184	1191	963
Ca	14150	25914	18136	19400

As shown in Table 3.2 the overall mean concentrations of the metals (mg/L) in the *Salix subserrata*, *Sida cunnefolia* and *Clausena anisata* samples collected from the three sampling areas can be ordered as Ca > Fe > Al > Zn > Mg > Ni > Mn > Cu > Cr. There is significant variation of the mean concentrations of the metals Al, Fe, and Ni between the *Salix subserrata*, *Sida cunnefolia*, and *Clausena anisata* samples. But small variations of the mean concentrations of Cu, Zn, Cr, Ca, Mn and Mg are observed between the *Salix subserrata*, *Sida cunnefolia* and *Clausena aisata* samples collected from the three sampling sites.

3.2.1 Comparison of levels of metals in plant samples with tooth pastes

The plant samples that are studied in this work are used to brush teeth traditionally and hence the study tries to relate the values of some heavy metals determined in a 9 tooth paste samples with the results of this study.

Table 3.3 Comparison of levels of metals in plant samples with tooth paste (mg/kg) (Andrew et al., 2019).

	Cr	Cu	Ni	Zn	Fe	Mn
*Tooth pastes	0.28-7.35	0.73-3.68	0.43-2.54	1842- 2417	1.76- 17.68	0.20-2.07
Plant samples of this study	7-8	13-20	4-160	152-196	514-1191	25-78

*Included conventional, herbal and children's toothpastes.

As can be seen from the Table 3.3 diversified concentration ranges of the studied metals were noticed compared with tooth pastes. The results obtained in this study indicated that Fe, Cu, Mn and Ni are more in the plant samples, Zn is more in pastes than in the plant samples, Cr in the plant samples is comparable with pastes.

3.3 Analysis of Variance (ANOVA)

The concept of ANOVA is to compare different sources of variance and make inferences about their relative sizes. In the study one-way ANOVA (one treatment factor with two or more treatment levels) was used. Microsoft excel 2010 was used for the preparation of calibration curves and the data analysis. One-way ANOVA was used to compare the mean values of the metals between different sample sites. As shown in Tables 3.4–3.6, the statistical analysis of ANOVA indicated that there is significant difference among the mean concentration of Al, Ca, Cd, Cu, Ni, Fe, Zn, Mn and Cr found in the three plant samples collected from the three sampling area at 95% confidence level.

As shown in Table 3.4, the statistical analysis of ANOVA indicated that there is a significant difference among the mean concentrations of Al, Cd, Cu, Mn, Ni, Zn, Cr, and Fe found in the *Salix subserrata* samples collected from the three sampling areas at 95% confidence level. But there is no significant difference on the mean concentrations of Ca between the *Salix subserrata* samples. Similarly, as shown in Table 3.5, the statistical analysis of ANOVA indicated that there is a significant difference among the mean concentrations of Mn, Ni, Cr, and Fe found in the *Sida cunnifolia* samples collected from the three sampling areas at 95% confidence level. But there is no significant difference on the mean concentrations of Al, Cd, Cu, Zn, and Ca between

the *Sida cunnifolia* samples. Finally, as shown in Table 3.6, the statistical analysis of ANOVA indicated that there is a significant difference among the mean concentrations of Cd, Mn, Zn, Fe, and Ca found in the *Clausena anisata* samples collected from the three sampling areas at 95% confidence level. But there is no significant difference on the mean concentrations of Al, Cu, Ni and Cr between the *Clausena anisata* samples. The presence of significance difference may be due to the presence of different geographical distribution, rainfall, soil composition, collecting and storing methods.

Table 3.4 Analysis of variance (ANOVA) between and within *Salix subserrata* samples at 95% confidence level

Metals	F calculated	F critical	Remark
Al	28.6	5.14	Significant difference among the sample means
Mg	363	5.14	Significant difference among the sample means
Cu	790	5.14	Significant difference among the sample means
Mn	591	5.14	Significant difference among the sample means
Ni	96.0	5.14	Significant difference among the sample means
Zn	114	5.14	Significant difference among the sample means
Cr	490	5.14	Significant difference among the sample means
Fe	3572	5.14	Significant difference among the sample means
Ca	3.15	5.14	No Significant difference among the sample means

Table 3.5 Analysis of variance (ANOVA) between and within *Sida cunnefolia* samples at 95% confidence level

Metals	F calculated	F critcal	Remark
Al	0.87	5.14	No Significant difference among the sample means
Mg	3.48	5.14	No Significant difference among the sample means
Cu	4.60	5.14	No Significant difference among the sample means
Mn	19.1	5.14	Significant difference among the sample means
Ni	16.0	5.14	Significant difference among the sample means
Zn	0.79	5.14	No Significant difference among the sample means
Cr	287	5.14	Significant difference among the sample means
Fe	897	5.14	Significant difference among the sample means
Ca	5.84	5.14	No Significant difference among the sample means

Table 3.6 Analysis of variance (ANOVA) between and within *Clausena anisata* samples at 95% confidence level

Metals	F calculated	F critcal	Remark
Al	0.98	5.14	No Significant difference among the sample means
Mg	29.1	5.14	Significant difference among the sample means
Cu	0.03	5.14	No Significant difference among the sample means
Mn	2108	5.14	Significant difference among the sample means
Ni	3.60	5.14	No Significant difference among the sample means
Zn	659	5.14	Significant difference among the sample means
Cr	4.22	5.14	No Significant difference among the sample means
Fe	23.6	5.14	Significant difference among the sample means
Ca	11.9	5.14	Significant difference among the sample means

3.4. Pearson correlation of metals

The Pearson product-moment correlation coefficient is the measure of the strength of a linear association between two variables and attempts to draw a line of best fit through the data of two variables. The Pearson correlation coefficient indicates how far away all these data points are to the line of best fit. A correlation coefficient of +1.0 indicates a perfect positive correlation while a correlation coefficient of −1.0 indicates a perfect negative correlation. The correlation values are categorized as no correlation (R^2 = 0.00-0.19), low correlation (R^2 = 0.20-0.49), medium correlation (R^2 = 0.50-0.75), and higher correlation (R^2 = 0.75-1.00) (Melina et al., 2016). Linear regression correlations tests were performed to investigate the correlations between metal concentrations in the plant samples and are summarized in Table 3.7.

Pearson correlation was evaluated for the overall mean concentration of the individual metals in the three plants. According to the present study highest correlation was observed between Al-Cr (r = -1.0), Mg-Ni (r = -1.0), Mg-Fe (r = 1.0), Mn-Zn (r = -1.0), and Ni-Fe (r = -1.0). Higher correlation was observed between Al-Mn (r = 0.91), Al-Zn (r = -0.93), Al-Ca (r = 0.94), Mg-Ca (r = 0.80), Mn-Cr (r = -0.90), Ni-Ca (r = -0.76), Zn-Cr (r = 0.92) and Cr-Ca (r = -0.94). Medium correlation were observed between Al-Mg (r = 0.54), Mg-Cu (r = -0.67), Mg-Cr (r = -0.55), Cu-Mn (r = 0.64), Cu-Ni (r = 0.72), Cu-Zn (r = 0.59), Cu-Fe (r = 0.73), Mn-Ca (r = 0.71), Ni-Cr (r =

0.50), Ni-Ca (r = -0.76), Zn-Ca (r = -0.74), Fe-Ca (r = 0.75) and low correlation were observed in Al-Cu (r = 0.25), Al-Ni (r = -0.24), Al-Fe (r = 0.48), Cu-Cr (r = 0.24), Cr-Fe (r = -0.49). There is no correlation between Mg-Mn (r = 0.14), Mg-Zn (r = -0.19), Cu-Ca (r = -0.10), Mn-Ni (r = -0.07), Ni-Zn (r = 0.12), Mn-Fe (r = 0.07) and Zn-Fe (r = -0.12).

Table 3.7 Pearson correlation coefficients of the metals in the three plant samples

	Al	Mg	Cu	Mn	Ni	Zn	Cr	Fe	Ca
Al	1.00								
Mg	0.54	1.00							
Cu	0.25	-0.67	1.00						
Mn	0.91	0.14	0.64	1.00					
Ni	-0.48	-1.00	0.72	-0.07	1.00				
Zn	-0.93	-0.19	-0.59	-1.00	0.12	1.00			
Cr	-1.00	-0.55	-0.24	-0.90	0.50	0.92	1.00		
Fe	0.48	1.00	-0.73	0.07	-1.00	-0.12	-0.49	1.00	
Ca	0.94	0.80	-0.10	0.71	-0.76	-0.74	-0.94	0.75	1.00

Pearson correlation was evaluated for the concentration of the individual metals in the *Salix subserrata* samples (Table 3.8). According to the present study highest correlation was observed between Cu-Ni (r = 1.0), Cu-Cr (r = 1.0), Cu-Fe (r = 1.0), Ni-Cr (r = 1.0), Cr-Fe (r = 1.0) and Ni-Fe (r = 1.0). Higher correlation was observed between Al-Ca (r = -0.95), Mg-Cr (r = -0.94), Mg-Fe (r = -0.94), Mg-Ni (r = -0.93), Mg-Cu (r = -0.93), Zn-Ca (r = -0.90), Al-Fe (r = 0.89), Al-Ni (r = -0.89), Al-Cr (r = 0.88) and Al-Cu (r = -0.85). Medium correlation were observed between Al-Mg (r = 0.67), Al-Zn (r = 0.72), Mg-Mn (r = -0.76), Cu-Mn (r = 0.53), Cu-Ca (r = 0.65), Mn-Zn (r = 0.68), Ni-Ca (r = 0.71), Cr-Ca (r = 0.69) and Fe-Ca (r = 0.70) and low correlation were observed between Mg-Ca (r = -0.41), Cu-Zn (r = -0.25), Mn-Ni (r = 0.47), Mn-Cr (r = 0.49), Mn-Fe (r = 0.48), Mn-Ca (r = -0.29), Ni-Zn (r = -0.33), Zn-Cr (r = -0.30) and Zn-Fe (r = -0.31). There is no correlation between Al-Mg (r = -0.02) and Mg-Zn (r = -0.04).

Table 3.8 Pearson correlation coefficients of the metals in *Salix subserrata* samples

	Al	Mg	Cu	Mn	Ni	Zn	Cr	Fe	Ca
Al	1.00								
Mg	0.67	1.00							
Cu	-0.85	-0.96	1.00						
Mn	-0.02	-0.76	0.53	1.00					
Ni	-0.89	-0.93	1.00	0.47	1.00				
Zn	0.72	-0.04	-0.25	0.68	-0.33	1.00			
Cr	-0.88	-0.94	1.00	0.49	1.00	-0.30	1.00		
Fe	-0.89	-0.94	1.00	0.48	1.00	-0.31	1.00	1.00	
Ca	-0.95	-0.41	0.65	-0.29	0.71	-0.90	0.69	0.70	1.00

Pearson correlation was evaluated for the concentration of the individual metals in the *Sida cunnefolia* samples (Table 3.9). According to the present study highest correlation was observed between Cu-Mn (r = 1.0). Higher correlation was observed between Al-Ca (r = -0.99), Mn-Fe (r = 0.99), Cu-Fe (r = 0.99), Ni-Zn (r = 0.99), Zn-Ca (r = -0.98), Al-Zn (r = 0.96), Ni-Ca (r = 0.96), Al-Ni (r = 0.92), Mg-Cr (r = 0.92), Al-Fe (r = 0.91), Fe-Ca (r = -0.86), Al-Mn (r = 0.84), Al-Cu (r = 0.85), Cu-Ca (r = -0.79) and Mn-Ca (r = -0.78). Medium correlation were observed between Mg-Ni (r = -0.57), Cu-Ni (r = 0.57), Cu-Zn (r = 0.66), Cu-Cr (r = 0.69), Mn-Ni (r = 0. 56), Mn-Zn (r = 0.65), Mn-Cr (r = 0.71), Ni-Fe (r = 0.68), Zn-Fe (r = 0.76) and Cr-Fe (r = 0.59) and low correlation were observed in Al-Cr (r = 0.21), Mg-Cu (r = 0.35), Mg-Mn (r = 0.37), Mg-Zn (r = -0.47), Mg-Fe (r = 0.22) and Mg-Ca (r = 0.30). There is no correlation between Ni-Cr (r = -0.19), Zn-Cr (r = -0.08), Al-Mg (r = -0.19) and Cr-Ca (r = -0.10).

Table 3.9 Pearson correlation coefficients of the metals in *Sida cunnefolia* samples

	Al	Mg	Cu	Mn	Ni	Zn	Cr	Fe	Ca
Al	1.00								
Mg	-0.19	1.00							
Cu	0.85	0.35	1.00						
Mn	0.84	0.37	1.00	1.00					
Ni	0.92	-0.57	0.57	0.56	1.00				
Zn	0.96	-0.47	0.66	0.65	0.99	1.00			
Cr	0.21	0.92	0.69	0.71	-0.19	-0.08	1.00		
Fe	0.91	0.22	0.99	0.99	0.68	0.76	0.59	1.00	
Ca	-0.99	0.30	-0.79	-0.78	-0.96	-0.98	-0.10	-0.86	1.00

Pearson correlation was evaluated for the concentration of the individual metals in the *Clausina anisata* samples (Table 3.10). According to the present study highest correlation was observed between Al-Cu (r = -1.0) Zn-Fe (r = 1.0), Cr-Fe (r = 1.0), Zn-Cr (r = 1.0). Higher correlation was observed between Mg-Zn (r = 0.99), Mg-Cu (r = 0.99), Mg-Fe (r = 0.99), Cu-Mn (r = -0.96), Mg-Ni (r = 0.98), Al-Mn (r = 0.95), Ni-Cr (r = 0.94), Ni-Zn (r = 0.94), Ni-Fe (r = 0.94), Al-Ca (r = 0.81) and Cu-Ca (r = -0.79). Medium correlation were observed between Mg-Ca (r = 0.52), Mn-Ni (r = -0.60), Mn-Ca (r = 0.57), Zn-Ca (r = 0.62), Cr-Ca (r = 0.62) and Fe-Ca (r = 0.61) and low correlation were observed in Cu-Ni (r = 0. 33), Mn-Zn (r = -0.29), Mn-Cr (r = -0.29), Mn-Fe (r = -0.30) and Ni-Ca (r = 0.32). There is no correlation between Al-Mg (r = -0.09), Al-Zn (r = 0.04), Al-Cr (r = 0.04), Al-Fe (r = 0.03), Mg-Cu (r = 0.12), Cu-Zn (r = 0.0), Cu-Cr (r = 0.0) and Cu-Fe (r = 0.0).

Table 3.10 Pearson correlation coefficients of the metals in *Clausia anisata* samples

	Al	Mg	Cu	Mn	Ni	Zn	Cr	Fe	Ca
Al	1.00								
Mg	-0.09	1.00							
Cu	-1.00	0.12	1.00						
Mn	0.95	-0.41	-0.96	1.00					
Ni	-0.30	0.98	0.33	-0.60	1.00				
Zn	0.04	0.99	0.00	-0.29	0.94	1.00			
Cr	0.04	0.99	0.00	-0.29	0.94	1.00	1.00		
Fe	0.03	0.99	0.00	-0.30	0.94	1.00	1.00	1.00	
Ca	0.81	0.52	-0.79	0.57	0.32	0.62	0.62	0.61	1.00

4. CONCLUSIONS

An efficient digestion procedure for the determination of metals in the plant samples was optimized and validated through spiking method and a good percentage recovery was obtained for the metals of interest. The overall mean concentrations of the metals (mg/kg) in the *Salix subserrata*, *Sida cunnefolia*, and *Clausena anisata* samples collected from the three sampling areas was in the order of: Ca (14150-25914) > Fe (514-1191) > Al (103-1263) > Zn (152-196) > Mg (46-102) > Ni (4-160) > Mn (25-78) > Cu (13-20) > Cr (7-8). From the results of this work it is possible to conclude that the plant samples collected from the three sites accumulated relatively larger amounts of Ca and Fe among the determined metals and lower amounts of Cu, Cr and Mn. The accuracy of the optimized procedure was evaluated by analyzing the digest of the spiked samples with standard solution and the percentage recoveries varied from 83.8% to 104% which is good and is in the allowed range of 90 ± 10%. ANOVA indicated that there is no significance difference between the mean concentrations of Ca among the *Salix subserrata* samples, of Mg, Cu, Zn and Ca among the *Sida cunnefolia* samples and of Al, Cu, Cr, and Ni among the *Clausena anisata* samples, but there is a significant difference for the other studied metals among the corresponding plant samples at 95% confidence level. Since the toxic elements Cd and Pb in the plant samples were not detected, it is possible to conclude that people who use tooth brush from the three plants from these sampling areas are free from the risks of Cd and Pb toxicity.

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