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Original Research

Determination of Selected Essential and Non-essential Metals in the Stems and Leaves of *Rhamnus prinoides* (Gesho)

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Abstract

This study was carried out with the objective of determining the quantity of selected essential and nonessential metals; K, Na, Mg, Ca, Cu, Mn, Cr, Cd, Fe and Zn in the leaf and stem of *Rhamnus prinoides*. Samples were collected from the low-altitude (1500–1670 meters above sea level) and medium-altitude (1670–2000 meters above sea level) areas of Bako Tibe. Wet acid-digestion using a mixture of HNO_3, $HClO_4$ and H_2O_2 for leaf (2.5, 1, 0.5 mL) and for stem (2.5, 1.5, 1 mL) was used. K and Na were analysed using flame photometry, Ca and Mg were determined titrimetrically and the other metals with flame atomic absorption spectrometry (FAAS) after appropriate quality control measures were undertaken to verify and maintain the quality of the data generated. The results of the study showed that the average concentrations determined were ranged from 8855.543 (stem) to 12927.3 (leaf) mg kg^{-1} for K, 226.214 (leaf) to 308.657 (stem) mg kg^{-1} for Na, 6144 (stem) to 11120 (leaf) mg kg^{-1} for Ca, 352.34 (leaf) to 1526.809 (stem) mg kg^{-1} for Mg, 29.0995 (leaf) to 49.913 (stem) mg kg^{-1} for Cu, 3.357 (stem) to 13.107 (stem) mg kg^{-1} for Mn, 1.714 (leaf) to 2.374 (stem) mg kg^{-1} for Cr, 8.58 (leaf) to 10.73 (stem) mg kg^{-1} for Fe, 3.483 (leaf) to 18.36 (stem) mg $kg^{-1}$for Zn and below method detection limit for Cd. The concentrations of the metals were also compared with recommended maximum permissible limits and some international reports; and found to be in a good agreement indicating no exposure risk of using the leaves and stems of *Rhamnus prinoides* under the current situation.

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INTRODUCTION

Rhamnus prinoides L'Herit, common name dogwood, Amharic name Gesho, family Rhamnaceae, is a wide spread plant species in East, Central and South African countries. It is a native plant to Ethiopia, Botswana, Eritrea, Lesotho, Namibia, South Africa, Swaziland, Uganda and exotic to Kenya. It also occurs in Cameroon, Sudan and Angola. The African dogwood, *R. prinoides* (Rhamnaceae) is a dense shrub or a tree that grows up to 6 m high (Berhanu and Teshome, 1995; Hailemichael *et al.,* 2007; Afewerk and Chandravanshi, 2012).

The Gesho plant (*R. prinoides*), which is different from hop (*Humulus lupulus*) is widely cultivated in Ethiopia and is available dried in the local market (Haimanot, 2011); the leaves and stems of this plant are used to impart the characteristic bitter flavour to domestically brewed beverages known as Tella and Tej and it is estimated that well over 5 million people consume these beverages daily (Berhanu and Teshome, 1995). The leaves and steams of Gesho are indispensable ingredients in the making of these traditional fermented beverages. It has been reported that the plant regulates the microflora responsible for the fermentation process. It plays a major role to suppress certain bacteria during the fermentation process (Hailemichael *et al.,* 2007). It is also a valuable cash crop; leaves/twigs or stems are used by most of individuals in the country.

The *Rhamnus prinoides* L'Herit is also used as traditional medicine. In some African countries its leaf is used for the treatment of the diseases/disorders like stomach complications, joint pain, fever, diarrhea, common cold, malaria, body weakness, appetizer and pneumonia, sprains, gonorrhea, colic, rheumatism and ringworm infections (Berhanu and Teshome, 1995; Hailemichael *et al.,* 2007). Specifically, in Southern Africa, the chief use of the tree is for magic; it is widely used as a protective charm toward off lightning and evil influences from homes and crops (Berhanu and Teshome, 1995; Hailemichael *et al.,* 2007).

It is also known that the source of mineral nutrients for human being is mostly plant materials consumed in the form of drinks, food or medicine. So, since *R. prinoides* is one of the main constitutes of traditional drinks and also used as traditional medicines (serve to cure different diseases for human being and domestic animals), the knowledge of their mineral content is very useful. Thus, it is very important to assess the essential, non-essential and toxic mineral nutrients that can be accumulated in the stated plant species so as to address the individual daily intake of mineral nutrients.

The objectives of the study were to: (i) optimize digestion procedure for the stem and leaf of Gesho samples to determine mineral contents by flame photometry and FAAS, (ii) determine the amounts of essential and non-essential metals in stem and leaf of the plant, (iii) compare the amounts of essential and non-essential metals in stem and leaf of the plant within the lowland and medium altitude, as well as between the lowland and medium altitude, and (iv) compare the amounts of essential and non-essential metals in stem and leaf of the plant with the literature values. For which the samples of the leaves and stems of *Rhamnus prinoides* were collected from the sites of lowland and medium altitude of Bako Tibe district of West Showa zone.

MATERIALS AND METHODS

Apparatus and Glassware

A stainless steel cutter, drying oven (DHG-9070A), mortar and pestle, 0.5 mm stainless steel sieve, electronic analytical balance with 0.0001 sensitivity (AA-200DS, Deriver Instrument Company) micropipette (0–50 µL), borosilicate beakers (100, 250 mL), hot plate (Glory dial 1-5), volumetric flasks (50, 100 and 1000 mL), Whatman filter paper (No. 42), funnels, capped glass bottles (50 mL), flame atomic absorption spectrophotometer (SL194, Double beam AAS, Elico) and flame photometer (CL 378, Elico) were used.

Chemicals and Reagents

HNO_3 (65–68%, Uni-Chem® Chemical reagents, India), $HClO_4$ (70–72%, Uni-Chem® Chemical reagents, India), extra pure hydrogen peroxide H_2O_2 (30%, Uni-Chem® Chemical reagents), aqua-regia (1:3, HNO_3: HCl), HCl (35–38%, LR, s.d.fine-cHEM LimTEd, MUMBAI) and sucrose, $C_{12}H_{22}O_{11}$ (HIMEDIA®, A.R.), stock standard solutions (1000 ppm) prepared in 20 mL 1:1HNO_3 for Cu metal diluted to 1 L, 17 mL 1:1 HCl for Zn and Fe diluted to 1 L and distilled water (boiled for 30 minutes) were used.

Stock and Working Intermediate Metal Standard Preparation

The stock standard solutions of each of the metals of interest were prepared at the concentrations of 1000 mg mL^{-1}. The working intermediate metal standard solutions (100 µg mL^{-1} each) were prepared by diluting 10 mL of 1000 µg mL^{-1} individual metal stock standard solutions with distilled water (boiled for 30 minutes) to a 100 mL volume.

Calibration Metal Standard Solutions

A blank and five calibration standard solutions of different concentration levels were prepared for each metal from the respective working intermediate standard solutions (100 µg mL^{-1}). The metal concentrations include 1.5, 2.5, 3.5, 4.5, 5.5 µg mL^{-1} for Cr; 0.25, 0.5, 0.75, 1.0 1.25 µg mL^{-1} for Cd; 1.0, 2.0, 3.0, 4.0, 5.0 µg mL^{-1} for Cu; 1.0, 1.75, 2.5, 3.25, 4.0 µg mL^{-1} for Mn; and 0.5, 1.0, 2.0, 4.0, 8.0 µg mL^{-1} for Fe and Zn. These calibration standard concentrations were within the working linear range of the instrument used for analysis. Beginning with the blank and working toward the highest standard, the solutions were aspirated and the readings were recorded.

Spiking Metal Standard Mixture Solution

A standard mixture solution containing all the metals of interest at varying concentrations (K and Na = 80, Mn = 40, Cu and Cr = 50, Fe and Zn = 60, Cd = 10 µg mL^{-1}) was prepared by taking 4, 2, 2.5, 3 and 0.5 mL stock standard solutions (1000 µg mL^{-1}) respectively in to 50 mL volumetric flask and filling with distilled water (boiled for 30 minute) to the mark.

Sample Collection, Transportation and Pretreatment

The purposive sampling technique was used. The 2 kg each gross samples (a composite sample) of the leaves and stems of *R. prinoides* were collected from the sites of the low-altitude (1500–1670 meters above sea level) and medium-altitude (1670–2000 meters above sea level) of areas Bako Tibe district, latitude of 9°N and longitude of 37.166667°E in January, 2013 G.C.

From the gross samples, sub-samples of 500 g each from each sample sites were placed in the labeled polyethylene plastic bags and brought to Ambo University chemistry laboratory for further pretreatment and analysis. The samples were further powdered by making use of mortar and pestle and sieved with 0.5 mm sieve. Finally, 0.5 g aliquot was taken from each sample for digestion and analysis.

Determination of Moisture Correction Factor of Stem and Leaf Samples

To determine the moisture content of stem and leaf samples of *R. prinoides* collected from the two sampling areas, the samples were first grounded, sieved through a 0.5 mm stainless sieve and air dried to constant weight for two weeks. Then, the samples were weighed with analytical electronic balance to record the initial weight. The samples were re-weighed and re-dried until they give constant mass after oven drying at 80 °C for two days (24 hrs) and the moisture contents were determined from the differences and the corresponding moisture correction factor (mcf) for analytical results or the multiplication factor for the amount of sample to be weighed for analysis was calculated (Haimanot, 2011).

Optimization of Digestion Procedure for Leaf and Stem Samples of *Rhamnus prinoides*

To prepare a clear colorless leaf and stem sample solutions that were suitable for the analysis using FAAS and flame photometry; the digestion procedures were optimized using different proportions of HNO_3, $HClO_4$ and H_2O_2 mixtures by varying the digestion time and temperature.

Quality Assurance/Control

Calibration and Continuing Calibration Standard

The FAAS was calibrated using a blank and five series of working standard solutions of each metal and the continuing calibration standard (CCS) of metal standard solution of mid-point calibration concentration was run to test the accuracy of the analytical instrument calibration with each batch samples and the deviation from the original value was within ±5% Csuros and Csuros, 2002). The performance of flame photometer for K and Na was checked with the freshly prepared standard solutions of the two respective metals at concentrations of 10, 30, 50, 70, 90 ppm for K and at 1, 5, 10, 20 ppm for Na and the corresponding recovered values 9.567±1.692, 31.033±

1.986, 50.067±0.473, 69.633±0.709 and 89.267±1.274 for K and 0.983±0.104, 5.033±0.153, 10.133±0.306, 15.333± 0.764 and 20.667±0.862 for Na were obtained indicating a good performance of the instrument.

Analysis of Matrix Spike and Matrix Spike Duplicate

Both matrix spike (MS) and matrix spike duplicate (MSD) were prepared for each sample item by spiking aliquots of 0.5 g of each leaf and stem samples with 2.5 mL standards mixture solution giving concentrations of 4.0 µg mL^{-1} for K and Na, 2.5 µg mL^{-1} for Cu and Cr, 0.5 µg mL^{-1} for Cd and 3.0 µg mL^{-1} for Fe and Zn. They were all carried through the same digestion and analysis steps as an un-spiked sample. And the mean recovery values of MS and MSD were used to control overall sample matrix-dependent accuracy of the analytical method and their relative percent differences (RPD) were calculated by dividing the difference of the test results with the average of the test results and expressed as percent to continuously assess the corresponding method precision (FAO, 2008).

Precision and Accuracy

The laboratory precision of the results was assessed by the analysis of laboratory control samples (LCSs, n=3), laboratory control sample duplicates (LCSD), MS (n=3) and MSD. Precision was expressed as the RPD and relative standard deviation (RSD) of replicate results.

The accuracy of the optimized procedure was evaluated by spiking experiments. This included analysis of the MS (n=3), MSD, LCSs (n=3), LCSD and method blanks. Accuracy was expressed as the percent recovery of an analyte that has been spiked to the samples in a known concentration before digestion and subsequent analysis.

Limit of Detection and Quantification

Method detection limit (MDL) and limit of quantification for each metal of interest were estimated by digesting and analyzing seven replicates of the method blanks with the optimized procedure for the leaf and stem samples.

Laboratory Solvent/Reagent Blanks

The instrument was run with reagent blanks/calibration blanks in which no sample present to determine the background signal and establish the baseline of an instrument. The observed readings were below the method detection limit indicating no instrumental variations and/or false positives during the measurement process.

Method Blank

Sucrose was used as matrix since there is no other plant that can serve as the matrix for the leaf and stem samples. The blank which was prepared from the sucrose and any reagents used for the digestion was taken through the entire measurement procedure to detect contamination from reagents, sample handling, and the entire measurement process. The analysis results revealed that there were no readings above the method detection limits of the metals. Hence, it can be concluded that the analytical method was free of overall laboratory contamination.

Analysis of Laboratory Control Samples

Triplicates of LCSs prepared from sucrose spiked with the target analyte of known concentration the same to sample spike were analyzed along with each batch of the leaf and stem samples. LCS recoveries and the RSD values of the LCS recoveries values for each metal of interest were calculated and used to control overall analytical method accuracy and precision, respectively which were independent of sample matrix effects (Csuros and Csuros, 2002; Miller and Miller, 2010).

Analysis of Stem and Leaf of *R. prinoides* Samples for Metal Levels

Five replicate analyses were carried out on each sample for the determinations of Na and K with flame photometry and Cu, Mn, Cr, Cd, Fe and Zn with FAAS; and the concentrations of Ca and Mg were determined by complexometric EDTA titration with the procedure given in FAO (Haimanot, 2011).

Statistical Analysis

The metal level of each *R. prinoides* samples were analysed by FAAS, FAES and titration. During the processes of sample preparation and analysis a number of random errors may be introduced in each aliquot and in each replicate measurement. The variation in means of the analytes of samples collected from the two sites was tested by using T-test of SPSS Version20 Software to test whether the source for variation was from experimental procedure or heterogeneity among the samples.

RESULTS AND DISCUSSIONS

Moisture Correction Factor of Stem and Leaf Samples

The moisture content of the four samples of air dried *R. prinoides* was 2.582–3.409%. Therefore, the air dried gesho cultivated in lowland and medium altitude areas of Bako has comparable moisture content with the value 2.5-14.4% reported for typical analysis of lumber (Simpson, 1999).

Optimization of Digestion Procedures for Leaf and Stem Samples

From the optimized procedures, the mixture of 2.5 mL HNO_3, 1 mL HClO4 and 0.5 mL H_2O_2, digestion time of 120 min and digestion temperature of 170±4.397 ^oC (at dial 2) were found to be the optimal condition for 0.5 g *R. prinoides* leaf sample and applied for complete digestion of samples. After digestion, the samples were cooled and diluted to 50 mL.

In the same manner, the optimum conditions 2.5 mL HNO_3, 1.5 mL $HClO_4$ and 1 mL H_2O_2 mixtures, digestion time of 110 min and digestion temperature of 180±2.582 ^oC (at dial 2.5) were obtained for 0.5 g *R. prinoides* stem samples.

Quality Assurance/Control

Calibration

Calibration curves for the various concentration ranges showed good correlation coefficients ranged between 0.996 and 0.999 (Table 3), which were all greater than the required limit (0.995) for trace element analysis (US EPA, 2007). This showed that there was good correlation (or relationship) between concentration and absorbance indicating good calibration of the instrument.

Matrix Spike and Matrix Spike Duplicate Results

The highest average recovery obtained was 103.409% (Cu in stem) and the lowest 91.875% (Na in leaf) (Table 2) where all were in the range 80–120%, which is recommended for matrix spike recovery of major element analyses by FAAS (US EPA, 2008). This indicated that the analytical process has provided the required matrix-dependant accuracy. Therefore, the method was well applicable for all the matrices and all the results obtained were trustable.

RPD values of MS and MSD obtained ranged between 1.556 (K in stem) and 13.451 (Fe in stem) (Table 2) which were in the acceptance limit (≤15%) (FAO, 2008). This showed that the overall analytical process was less affected by matrix-specific variability and the data generated was in the acceptable quality range.

Method Accuracy

The percent recoveries of each sample spike (n=3) were determined for each metal as shown in Table 1 and ranged between the highest 99.94 (Cu in stem) and lowest 92.583 (Na in leaf), all lied in the acceptable range (80–120%) for metal analysis (US EPA, 2008). This showed that the analytical method provided results in the required level of accuracy.

Method Precision

Triplicates of each sample spike were digested and analyzed following the same analytical procedure as samples and, then the RSD of the recovery values were calculated as shown in Table 1 and all were under the required limit (≤15%) (Csuros and Csuros, 2002). This indicated that the analytical method, which covers digestion and instrumental measurement steps, has provided acceptable repeatability or precision.

Instrument Detection, Method Detection and Quantification Limits

The instrument detection limits ranged between 0.21 and 0.72 mg kg^{-1}, indicating good sensitivity of the measuring instrument for analyses. The MDL values lied in the range 0.797 (Cr in leaf)–4.73 (Fe in stem) mg kg^{-1} and method quantification limit values 1.76 (Cd in leaf)–11 (Fe in stem) mg kg^{-1} (Table 3). All the values were found to be in the required limit (Temminghoff and Houba, 2004). This indicated that the method was well applicable for the determination of the essential and nonessential metals.

Table 1: Recovery and precision test for the optimized procedure from sample spike (n = 3)

Metals	Sample type	Conc. in sample ($\mu g\ mL^{-1}$)	Amount spiked ($\mu g\ mL^{-1}$)	Conc. in spiked sample ($\mu g\ mL^{-1}$)	Recovery (%)	Precision (%, RSD*)
K	Leaf	129.90±1.572	4.0	133.883±1.815	99.583±6.292	6.318
	Stem	104.467±0.896	4.0	108.273±0.983	95.167±2.673	2.809
Na	Leaf	2.60±0.520	4.0	2.637±0.520	92.583±4.544	4.908
	Stem	6.033±0.153	4.0	10±0.436	99.167±12.583	12.689
Cu	Leaf	28.311±1.263	2.5	30.202±1.366	95.387±6.19	4.524
	Stem	29.483±0.525	2.5	31.515±0.606	99.94±4.117	1.923
Mn	Leaf	10.267±0.467	2.0	12.167±0.382	95±4.38	3.139
	Stem	7.45±0.721	2.0	9.411±0.651	98.033±3.71	6.914
Cr	Leaf	1.487±0.0231	2.5	3.956±0.0311	98.79±1.652	0.786
	Stem	1.065 ±0.117	2.5	3.538 ±0.0544	98.938 ±2.595	1.537
Cd	Leaf	0.022±0.0076	0.5	0.503±0.025	96.267±4.202	4.922
	Stem	0.0133±0.00497	0.5	0.50±0.0312	97.88±6.125	6.245
Fe	Leaf	8.1±1.153	3.0	10.929±1.1325	94.3±2.917	10.362
	Stem	77.733±11.01	3.0	80.604±11.002	95.7±2.079	13.649
Zn	Leaf	10.177±0.0643	3.0	13.128±0.107	98.389±1.484	0.818
	Stem	20.867±2.61	3.0	23.702±2.534	94.513±5.331	10.69

RSD*= Relative standard deviation

Table 2: Mean %R and RPD values of metals from MS and MSD; and %R and RSD values of LCSs analysed along with real samples for method validation.

Metals	*LCS (%R*, n=3)		LCS (RSD*)		MS* and MSD* (%R, n=2)		MS and MSD (RPD*)	
	Leaf	Stem	Leaf	Stem	Leaf	Stem	Leaf	Stem
K	94.17±6.29	96.67±1.44	6.681	1.493	100±8.84	94.88±3.712	12.5	5.534
Na	90.83±7.64	91.67 ±8.04	8.408	8.767	91.88 ±6.19	92.5±7.071	9.524	10.811
Cu	98.55±6.17	97.41±7.01	6.256	7.194	91.95±3.61	103.41±3.75	5.128	1.905
Mn	100.07±5.73	96.65±6.32	5.721	6.537	94.88 ±6.19	97±4.596	6.186	8.978
Cr	98.26±2.5	99.69±1.23	2.55	1.235	98.15±1.74	100.1±2.317	1.556	2.198
Cd	98.6±4.06	100.6±3.86	4.12	3.834	101.3±2.12	96.2±4.243	9.428	12.121
Fe	99.75±3.76	96.71±0.68	3.77	0.71	98.5±8	98.617±7.13	9.839	13.451
Zn	99.72±5.12	96.598±1.68	5.13	1.74	94.75±0.15	92.07±0.632	1.59	4.015

LCS= laboratory control samples, %R= percent recovery, RSD*= relative standard deviation, MS*= matrix spike, MSD*= matrix spike duplicate, RPD*= relative percent difference

Table 3: Instrument detection limit (IDL), method detection limit (MDL), method quantitation limit (MQL) and correlation coefficients of calibration curves for metals determined in stem and leaf samples of *R. prinoides.*

Metals	Correlation coefficient of calibration curves (r^2)	Calibration equation	IDL ($mg\ kg^{-1}$)	MDL ($mg\ kg^{-1}$) for		MQL ($mg\ kg^{-1}$) for	
				Leaf	Stem	Leaf	Stem
K	-	-	0.72	3.97	3.60	7.09	6.28
Na	-	-	0.40	3.11	2.01	7.02	3.57
Cu	0.999	Y= 0.033X+0.003	0.62	3.28	3.00	5.28	4.85
Mn	0.996	Y= 0.008X+0.003	0.15	1.60	1.25	3.95	3.34
Cr	0.999	Y= 0.013X+0.002	0.13	0.73	0.60	1.89	1.59
Cd	0.999	Y= 0.082X+0.002	0.06	0.50	0.51	1.02	1.05
Fe	0.998	Y= 0.015X+0.005	0.59	1.20	2.30	3.08	3.97
Zn	0.997	Y= 0.049X+0.020	0.44	1.24	2.08	3.37	3.69

Laboratory Control Samples Result

The percent recovery values of LCS lied in the range 90.833 (Na in leaf)–100.6% (Cd in stem) and their relative standard deviations 0.71 (Fe in stem)–8.767 (Na in stem) (Table 2). And all the values were found under standard control limits 80–120% for LCS recovery, and ≤15% for RSD (Csuros and Csuros, 2002). This showed that the method used for the study has provided the required level of accuracy and precision throughout the analytical process.

Metals in the Leaf Samples

Levels of selected essential and nonessential metals were determined in two 0.5 g leaf samples of R. *prinoides* (of lowland and medium altitude areas) and the data of total concentrations of the metals in the leaf samples studied were summarized in Table 4.

Table 4: Average concentration (mean ± SD*, n = 5, $mg\ kg^{-1}$ air dry weight) of metals in stem and leaf samples of *R. prinoides.*

Metals	Leaf ($mg\ kg^{-1}$ air dry weight)		Stem ($mg\ kg^{-1}$ air dry weight)	
	Lowland	Medium altitude	Lowland	Medium altitude
K	12927.3±346.915	11651.3±333.437	10239.54±386.225	8855.543±269.944
Na	226.214±19.235	320.214±10.368	308.657±7.906	413.657±7.906
Ca	7424±158.997	11120±126.491	6144±190.997	7416±153.883
Mg	352.34±13.143	1526.809±122.978	413.617±12.638	1210.213±103.081
Cu	29.10±3.455	36.978±6.428	46.338±4.745	49.913±5.829
Mn	13.107±0.28	7.357±1.976	9.407±0.259	3.357±0.198
Cr	1.714±0.34	1.714±0.193	2.174±0.296	2.374±0.237
Cd	BMDL*	BMDL	BMDL	BMDL
Fe	8.58±1.23	10.71±3.98	9.387±3.37	10.73±0.101
Zn	3.48±1.54	7.91±4.99	16.33±3.04	18.36±6.12

BMDL* = below method detection limit, SD* = standard deviation

As shown in the table, the mean Potassium concentrations in the leaf samples were 12927.3 $mg\ kg^{-1}$ for lowland and 11651.3 $mg\ kg^{-1}$ for medium altitude. So, K concentrations were relatively higher than other metals, which were still below the FAO/WHO maximum permissible limit of 1–5% K in plant dry matter (FAO, 2006), indicating no exposure risk to it.

The Sodium concentrations of the leaf samples analyzed were 226.214 and 320.214 $mg\ kg^{-1}$ respectively in the lowland and medium altitude areas. These concentrations were below the WHO recommendation on sodium maximum consumption for adults, which is 2 g sodium/day (WHO, 2012).

The average concentration of Calcium in the leaf samples were 7424 $mg\ kg^{-1}$ in the lowland areas and 11120 $mg\ kg^{-1}$ in the medium altitude areas. Ca concentration was greater in the leaf sample of the medium altitude areas which was slightly greater than typical concentration in plant dry matter of 0.2–1.0% (FAO, 2006).

Magnesium concentrations in the leaf samples were found 352.34 $mg\ kg^{-1}$ (lowland) and 1526.809 $mg\ kg^{-1}$ (medium altitude), which was greater in the leaf sample of the medium altitude areas.

Concentrations of Copper in leaf samples were 29.0995 and 36.978 $mg\ kg^{-1}$. The greater concentration of Cu was the one found in leaf sample of medium altitude areas and both concentrations were below the maximum permissible limit of 40 $mg\ kg^{-1}$ dry weight (FAO/WHO, 1995).

Concentration of Manganese in the leaf samples of lowland was 13.107 $mg\ kg^{-1}$ and that of medium altitude areas was 7.357 $mg\ kg^{-1}$, being lower in leaf samples of medium altitude areas. Both Mn concentrations were below the tolerable limit of 500 $mg\ kg^{-1}$, dry weight (Council of US, 2002). The critical concentration of Mn is 300–500 ppm dry weight and the estimated safe and adequate daily dietary intake in adults is 11 mg/day (Khan *et al.,* 2008).

Iron was determined in the leaf samples and was found to be 8.58 and 10.71 $mg\ kg^{-1}$ in leaf samples of lowland and medium altitude areas, respectively. In the UK, the Guidance Level for supplemental intake of iron is 17 mg/day for adults. The WHO's thresholds for iron adequacy ranges between 0 and 6% according to age and sex (SACN, 2010). Thus, the concentrations of Fe obtained by this research were in this recommended threshold for Fe.

Zinc, analyzed in both leaf samples was 3.48 and 7.91 mg kg^{-1} in the case of lowland and medium altitude areas, respectively. The amounts of Zn were below the US Recommended Daily Allowance (RDA) which is 15 mg/day for males and 12 mg/day for females and estimated maximum intake of 77 mg/day (http://www.google.com.et/search?output=search&sclient=-psyab&q=Risk+Assessment%2C+Zinc+pdf).

Chromium was detected in both the leaf samples although at very low concentrations. Its concentration was observed to be 1.714 mg kg^{-1} in leaf samples of both lowland and medium altitude areas. The daily intake of Cr 50–200 µg has been recommended for adults by US National Academy of Sciences (Khan *et al.,* 2008) and the minimum reporting limit for Cr in tissue is 1 mg kg^{-1} (US EPA, 2012).

The mean Cadmium concentrations were below method detection limits in leaf and were below the acceptable concentration for food stuff which is around 1 ppm (Khan *et al.,* 2008), indicating no exposure risk to Cd.

Generally, when the distribution of the selected essential and nonessential metals over each leaf sample of *R. prinoides* was observed, they were found to vary in the order: K > Ca > Mg >Na > Cu > Mn > Fe > Zn > Cr > Cd (BMDL) in leaf samples of lowland areas, K > Ca > Mg> Na > Cu > Zn > Fe > Mn > Cr > Cd (BMDL) in leaf samples of medium altitude areas.

Metals in the Stem Samples

The levels of ten metals (four major and six trace) were determined in two stem samples and the results were summarized in Table 4. This data indicated the concentrations of K, Ca, Mg, Na, Cu, Mn, Fe, Zn and Cr to be 10239.54, 6144, 413.617, 308.657, 46.338, 9.407, 9.387, 16.33 and 2.174 mg kg^{-1} respectively in the lowland stem samples of *R. prinoides*; and 8855.543, 7416, 1210.213, 413.657, 49.913, 3.357, 10.73, 18.36 and 2.374 mg kg^{-1} respectively in the medium altitude stem samples of *R. prinoides*. The concentrations of all metals were greater in the medium altitude stem samples of *R. prinoides* except that of K which was greater in the lowland stem samples of *R. prinoides* and of Fe, Zn and Cr which were comparable in both samples. And all the concentrations were under the FAO/WHO maximum permissible limit of typical concentration in plant dry matter 1–5% for K, 0.2–1.0% for Ca, 0.1–0.4% for Mg, 20–500 µg g^{-1} for Mn, 5–20 µg g^{-1} for Cu, 50–250 µg g^{-1} for Fe, 21–150 µg g^{-1} for Zn (FAO, 2006) and the minimum reporting limit 1 mg kg^{-1} for Cr in tissue (US EPA, 2012).

Generally, the distributions of the selected essential and nonessential metals in each stem samples of *R. prinoides*, they were in the order of: K > Ca > Mg > Na > Cu > Zn > Mn > Fe > Cr > Cd (BMDL) in lowland stem samples and K > Ca > Mg > Na > Cu > Zn > Fe > Mn > Cr > Cd (BMDL) in medium altitude stem samples.

Metal Levels of Lowland Leaf and Stem

The analyzed selected metal levels of lowland stem and leaf samples of the *R. prinoides* indicated that they were irregularly distributed. The concentration of K was highest in leaf and lowest in stem. Na, Mg, Cu, Fe and Cr levels were highest in stem, lowest in leaf; Ca and Mn were highest in leaf, lowest in stem; Zn levels were lowest in leaf and highest in stem samples; and Cd levels were below method detection limits in both sample items (Table 4).

Metal Levels of Medium altitude Leaf and Stem

The distributions of selected essential and non-essential metal levels analyzed in the medium altitude stem and leaf samples of *R. prinoides* were observed to be irregular. The concentration of K was highest in leaf and lowest in stem. Na, Fe, Zn and Cu levels were highest in stem, lowest in leaf. Ca was highest in leaf, lowest in stem. Concentrations of Mg, Mn and Cr were highest in leaf, lowest in stem and Cd levels were below method detection limits in both sample items (Table 4).

Metal Levels of Leaf and Stem

The findings of this study were compared with some other related published reports conducted in some other parts of the world with regard to the essential and nonessential metal levels in leaf and stem samples.

The mean levels of Na and K in leaf and stem of *R. prinoides* of this study were lower than the one reported by Idris *et al.* (2011) on nutritional composition of the leaves and stems of *ocimum gratissimum* in Nigeria. The amounts of Ca and Mg in leaf and stem of *R. prinoides* of present study were higher than that obtained by Idris *et al.* (2011) in Nigeria where as that of Zn in leaf and stem were slightly lower and Fe were lower in leaf and greater in stem than the value reported by Idris *et al.* (2011); and Ca in leaf was comparable; Mg, Mn, Fe, Zn and Cr in leaf was lower and Cu was slightly greater compared to the results reported by Minaleshewa (2010) on khat here in Ethiopia. The levels of Mn, Fe, Zn and Cd were lower and that of Cu were higher in leaf than the values in leaf reported by Khattak and Khattak (2011) in Pakistan.

A study conducted here in Ethiopia, investigated the levels of essential and nonessential metals in the leaf and stem of *R. prinoides* cultivated in Ethiopia (Afewerk and Chandravanshi, 2012). The study was confirmed concentrations of Ca, Mg, Cu, Mn, Fe, Zn, Cr and Cd to be 6304–22236, 3202–5706, 6.5–73, 8.12–17.9, 47.9–187, 12.2–43, 5.08–20.6 and 0.81–3.1 respectively in leaf and 3601–5675, 2635–5528, 16.8–233, 2.6–3.98, 22.0–124, 17.4–28.2, ND–16.3 and ND–1.56 respectively in stem. The same trend has been observed in the present study except that of Ca and Mn in stem which were somewhat greater; and Mg and Fe in both and Zn in leaf were below this range.

CONCLUSIONS

A wet acid-digestion method using the mixture of HNO_3, $HClO_4$ and H_2O_2 for preparation of leaf and stem samples were optimized, validated and used. The determinations of selected essential and non-essential metal levels (K, Na, Ca, Mg, Cr, Cu, Zn, Fe, Mn and Cd) in the samples were made by flame photometer, flame atomic absorption spectrophotometer and complexometric titration with EDTA. The quality of the analytical data generated and general laboratory performance were continuously monitored and verified through the analyses of several quality control parameters including CCS, LCSs, MS, MSD, method blank and laboratory solvent/reagent blanks. The analyses results ensured that the laboratory systems such as instrumentation, sample preparation, analysis and data acquisition were all operating within acceptable guidelines.

T-test was performed among the leaf and stem samples of the two sites separately to assess whether the essential and nonessential metals were distributed in significantly different concentrations or not. The test results showed that there were significant differences ($p < 0.05$, at 95% CL) in the concentrations of all metals of interest among the analyzed leaf and stem samples except Fe and Zn (in leaf and stem) and Cu and Cr (in stem). The metals such as K, Ca, Mg, Na and Cu were generally found in higher concentrations than the rest metals in all of the samples. However, in comparison with the recommended maximum permissible limits, the levels of all metals of interest were in the similar range of the FAO/WHO or EPA tolerable limits which means that they were found under the safe limits. This indicated that there was no risk of exposure through the use of this plant in the case of the two sampling areas. The metal levels investigated in leaf and stem samples were also found to be comparable with other similar studies conducted over different parts of the world.

The variations of the metal concentrations in the leaf and stem may be ascribed to: nature of the leaf and stem (i.e., the differences in physiological properties of metal uptake that varies from site to site, exposure surface area and plant age); the physical and chemical nature of the soil where the plant grew (cation exchange capacity, organic matter content and soil pH); atmospheric deposition of the metals (which may be influenced by innumerable environmental factors such as temperature, moisture and pH) (Kalra, 1998). In short, leaves and stems are good sources of a healthy diet; and they are not exposure routes to some metal contaminations under the current existing situation.

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