Moringa stenopetala)

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Moringa is widely cultivated and distributed in Southern Ethiopia mainly in Gamo Gofa, Wolayita, Konso, Sidama, Bale, Keffa, Borana, South Omo, Dherashe areas. The demand for moringa currently increased in Ethiopia due to its nutritional and medicinal values. However, there is no as such information about their chemicals and antioxidant properties in dried form. The purpose of this study was to evaluate the chemical properties of dried moringa leaves and antioxidant properties of its infusion. The dried leaves of moringa were characterized for their proximate (moisture, crude protein, crude fat, total ash and carbohydrate), minerals (sodium, magnesium, calcium, potassium, zinc, iron, copper and manganese contents), phytochemical (total phenolic, total flavonoid, and condensed tannin), and antioxidant (DPPH scavenging capacity, ferric reducing power and total antioxidant activities). This study provides evidence on the dried moringa leaves nutritional profile and its leave infusion antioxidant and phytochemicals properties.

Keywords: Antioxidant activity, Moringa, phenolic content, Infusion.

Moringa (*M. stenopetala*) is often named as African Moringa tree because it is native to Southern Ethiopia, North Kenya and Eastern Somalia (Abay *et al*. 2015; Mataka *et al*. 2006). Moringa is widely cultivated and distributed in Southern Ethiopia mainly in Gamo Gofa, Wolayita, Konso, Sidama, Keffa, Borana, South Omo, Dherashe areas and the adjoining provinces (Hamza and Azmach, 2017; Bedane *et al*. 2013). Moringa plants are among the high value and multi-purpose trees with medicinal, nutritional, and socio-economic values (Habtemariam and Varghese, 2015; Hamza and Azmach, 2017). Moringa leaves have been found to have potential health benefits which include removing different kinds of intestinal worms, exhibit anti-tumor, anti-inflammatory, anti-ulcer, anti-atherosclerotic, increasing food appetite, protecting abdominal constipation, a cure for different kinds of respiratory diseases such as bronchitis and influenza, due to the fact that the leaves being rich in biologically active compounds such as phytochemicals, antioxidants, carotenoids, tocopherols, and vitamin C (Habtemariam and Varghese, 2015; Coppin, 2008). The fresh and dried leaf of moringa is widely sold for vegetable and herbal use and consumed in cities of Ethiopia, due to its

perceived awareness of health and nutritional benefits (Hamza and Azmach, 2017; Habtemariam and Varghese, 2015; Hegde and Hegde, 2013). The dried moringa leaf is eaten fresh, cooked, or stored as dried powder for further uses (Coppin, 2008). The leaves of the moringa tree are potential sources to improve nutrition, boost food security and foster rural development, due to its immense nutritional value such as minerals, antioxidants, protein, and vitamins (Arise *et al*. 2014; Glarum, 2012). However, there is no as such compiled information in Ethiopia about dried moringa leaves chemical properties and its infusion antioxidants properties. Therefore, evaluation of dried moringa leaves; their chemicals and antioxidants content are important to compile information on their nutritional and health benefits.

Fresh moringa leaves were obtained from Arba Minch Agriculture Research Center (Ethiopia) and the samples were packed in polyethylene (plastic) bags and transported to Natural Product and Food Science and Technology laboratories of Wondo Genet Agricultural Research Center and Hawassa University, respectively. The fresh leaves of uniform shape, color and size were selected and subjected to shade drying at ambient temperature for about one week according to Killedar et al. (2017). The leaves were spread thinly on paper-lined wooden trays and protected from direct sunlight to prevent the loss of volatile aroma compounds and also photo oxidation.

The dried samples were separately milled using an electric Blender (Model BLG401, Zhejiang YiLi Tool Co., Ltd., China). The milled samples were sieved using 2 mm sieve size to separate the milled leaves and the large size sample. The sieved samples were kept in an air-tight container and stored at room temperature until further analysis.

The extract was prepared from the sieved samples according to Koh et al. (2009) and Mingarro et al. (2003). The infusion was boiled for 5 min at 97 ^{o}C as usual for normal tea preparation. The infusion was filtered through a double-layered muslin cloth to get rid of the large particles and filtered through a filter paper (Whatman no.1). The filtered product was then allowed to concentrate at 45^{o}C for three consecutive days by evaporating excess water and obtain the dried. The extract was weighed and its representative percentage yield was recorded. The crude extract 1 g was dissolved in 50 ml of the solvent (methanol) to make a stock solution of 20 mg/mL. The prepared stock solution was kept at 4°C in a refrigerator, to serve as the working solution for all the phytochemicals and antioxidant tests.

The moisture content of milled dried leaves of moringa was determined using the method of Association of Official Analytical Chemists (AOAC, 2005). A dry sample of 2 g was weighed in triplicates and placed in a forced air oven at 105°C for 3 hrs. The samples were removed and cooled in desiccators. The loss in weight was determined and recorded as the moisture content. The percentage of moisture was calculated using the following equation.

$$\text{Moisture (\%)} = \frac{W2-W3}{W1} \times 100$$

Where W1= weight of fresh samples, W2=weight of fresh samples and crucibles and W3= weight of dried samples and crucible

The ash content was determined according to Association of Official Analytical Chemists (AOAC, 2005). A dry sample of 2 g was measured into crucibles of a known weight and incinerated in a muffle furnace at 550°C for 3 hrs. The samples were then cooled in desiccators and weighed. The percentage of total ash was calculated as under:

$$\text{Ash (\%)} = \frac{w3-w1}{w2} \times 100$$

Where; W1 = weight of empty crucible, W2 = Weight of fresh samples and W3=weight of ashed samples and crucibles.

The crude protein was determined using the Kjeldahl method as described in Association of Official Analytical Chemists (AOAC, 2005). Dry Sample of 2g was introduced into the digestion flask. Then, 10mL of concentrated sulphuric acid (H_2SO_4), a mixture of 2.5 g of copper sulphate ($CuSO_4$), potassium sulphate (K_2SO_4), and titanium dioxide (TiO_2) were added into each sample and digested in a Kjeldahl digestion flask (KDN-20C, China) at 380°C for 6 hrs, until the mixture clears. The digest was filtered into 500mL volumetric flask and made up to mark 100mL with deionized water and connected for distillation. Ammonia was steam distilled for an hour to which had been added 20mL of 40% NaOH solution. The distillates 200mL were collected in 250mL conical flask containing 20mL of 0.2 N H_2SO_4 and methyl red indicator. The ammonia that distilled into the receiving conical flask was reacted with 0.2 N H_2SO_4 and the excess acid in the flask were estimated by back titration against 20mL of 0.1 N NaOH with a color change from red to yellow. A blank distilled was collected in 250mL conical flask containing 20mL of 0.2 N H_2SO_4 and methyl red indicator. The distillate was titrated against 20mL of 0.1 N NaOH. Total nitrogen and crude protein content of the sample was then determined and calculated with the following formula, respectively.

Nitrogen (%) = $\frac{(\text{M of N2} \times \text{acid conc.(0.02m)} \times \text{volumemade} \times \text{titervalue})}{(1000 \times \text{W1})} \times 100$

Crude protein (%) = % Nitrogen × 6.25

Where, W1=sample weight, M=molar mass

The crude fat content was determined using the Soxhlet method according to the Association of Official Analytical Chemists (AOAC, 2005). In this method, 2g of the sample was added into a flat bottom flask of known weight with the extractor mounted on it. The thimble was held halfway into the extractor and the weighed sample was transferred into the thimble and the thimble was plugged with cotton wool and extracted with 200mL of petroleum ether for 6hr. The solvent-free fat in the flux was dried in an oven for an hour at 105°C, cooled in desiccators, and fat content was then calculated using the equation below.

Crude fat (%) = $\frac{\text{We} + \text{Wf}) - \text{Wf}}{Ws}$ x 100

Where We =Weight of extract, Wf= Weight of flux and Ws= Weight of sample

The crude fiber was determined according to Association of Official Analytical Chemists (AOAC, 2005). The dried samples of 2g were introduced into the extraction unit, 150mL of hot 0.2N H_2SO_4 was added and digested for 30 min. Then, the acid was drained and the sample was washed with hot deionized water. Finally, the fiber was extracted and dried by moistening with a small portion of acetone which was then allowed to drain. The sample was incinerated at 550^{o}C for 3hr until all carbonaceous matter was burnt. The crucible containing the ash was cooled in the desiccators and weighed. The percentage of crude fiber was calculated using the following equation.

% Crude fiber = $\frac{W2 - W3}{W1} \times 100$

Where: W1 = weight of sample used W2 = Weight of sample and crucible before ashing W3= Weight of crucible and ash.

Carbohydrate content of the milled leaves of moringa was determined by the difference as described by Ihekoronye and Ngoddy (1985) and calculated using the following equation.

% CHO = 100 - % (ash + protein + fat + crude fiber + moisture)

The mineral contents such as: Sodium (Na), Potassium (K), Magnesium (Mg), Calcium (Ca), Zinc (Zn), Iron (Fe), Copper (Cu) and Manganese (Mn) of moringa were analyzed as described by Marcinek and Krejpcio (2015) using ash method.

The minerals were analyzed using an Atomic Absorption Spectrophotometer (Spectra AA 220, USA Varian). Sample of moringa 2g was put in crucibles and then in a muffle furnace for 3hr. to obtain ash. The residue was dissolved in 10mL of HNO_3: HCl (2:3 v/v) then heated until fumes disappeared. The solution was transferred separately in 250mL volumetric flasks by filtration using Whatman filter paper No 42, then volume made up to 250mL with distilled water. The concentration of mineral was calculated and expressed as mg/100 gas described below.

Element (mg / 100g) = $\frac{C \times V \times df}{W}$

Where C = the concentration of the element in the sample solution in mg/L; V = the volume of the undiluted sample solution in mL; W = the sample weight in grams, and df = is the dilution factor.

Total phenolic content (TPC)

The total phenolic content of the extract was estimated according to the method used by Shan et al. (2005) using gallic acid as a standard. Folin-Ciocalteu reagent (diluted ten times) (1mL) was added to 0.1mL of the extract (1 mg/mL). The mixture was left for 5min and 1mL (7.5% w/w) of sodium carbonate was added. The absorbance of the resulting blue color was measured at 765 nm with a UV-visible double beam spectrophotometer (Spectronic 20, UK) after incubation for 90 min at room temperature. The TPC was estimated from gallic acid calibration curve ($y = 0.0073x - 0.0462$, $R^2 = 0.973$) and results were expressed as milligram gallic acid equivalent/gram of dried extract (mgGAE/g).

Total flavonoid content (TFC)

Total flavonoid content (TFC) of extract was determined according to Ayoola et al. (2008). The extracts (1mg/ mL) were diluted with 1.25mL distilled water and 0.75µL 5% $NaNO_2$ was added to the mixture. After 6min, 150µL 10% $AlCl_3$ was added and after another 5min, 1mL NaOH was added to the mixture. Immediately the absorbance of the solutions was measured using a UV-visible spectrophotometer (JANEWAY, 96500, UK) at 510nm. All the calculations were done using standard equation Catechin obtained from standard calibration curves ($y=0.0014x+0.0192$, $R^2=0.97(p<0.001)$). Results were expressed as milligram of catechin equivalents per gram of dry extract (mgCE/g).

Condensed tannins content (CTC)

The condensed tannins content of the extract was assayed as described by Chew et al. (2011). The undiluted crude extract 0.5mL was first mixed with 3mL of vanillin reagent (4%, w/v, in absolute methanol), and followed by the addition of 1.5mL of concentrated HCl (37%). The mixture was stored in a dark at room temperature for 15min. Blank was prepared by replacing 0.5mL of the undiluted crude extract with 0.5mL of deionized water. The absorbance of the solutions was measured using a UV-visible spectrophotometer (JANEWAY, 96500, UK) at

500nm. Catechin was used for calibration of the standard curve ($y=0.0042x+0.0331$, $R^2=0.995$($p<0.001$) and the results were expressed as mg catechin equivalent per 100 g dry weight sample (mg CE/100 g).

DPPH radical scavenging activity

The 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity of the extract was determined as described by Brand-Williams et al. (1995). Different concentrations (50 to1000 µg/mL) of the extracts were taken in different test tubes. Freshly prepared DPPH solution (2mL, 0.06%, and w/v) in methanol was added in each of the test tubes containing 1mL of the extract. The reaction mixture and the reference standards (ascorbic acid and BHT) were vortexed and left to stand at room temperature in the dark for 30min. The absorbance of the solutions was measured using a UV-visible spectrophotometer (JANEWAY, 96500, UK) at 520nm. Methanol (100%) was used as a blank. The ability to scavenge the DPPH radical was calculated using the following equation.

$$\text{Radical scavenging effect (\%)} = \frac{Ac-As}{Ac} \times 100$$

Where Ac = Absorbance of the control; As = Absorbance of the sample

The antioxidant activity of the extract was expressed as IC_{50} (Inhibitory Concentration 50%) and value is the concentration in (µg/mL) of extracts that scavenges the DPPH radical by 50%.

This assay was carried out according to Safdar et al. (2016). One milliliter of the extract with a concentration of 1mg/mL was mixed with 2.5mL sodium phosphate buffer (0.2 M, pH 6.6) and 2.5mL of 1% potassium ferricyanide. Then the mixture was incubated at 50^{o}C for 20min. Trichloroacetic acid (2.5mL, 10%) was added to the mixture. Finally, 2.5mL of the supernatant solution was mixed with 2.5mL of distilled water and 0.5mL $FeCl_3$ (0.1%) and the absorbance of the solutions was measured using a UV-visible spectrophotometer (JANEWAY, 96500, UK) at 700nm. The reducing power was expressed as mg of ascorbic acid equivalents/g of dried extract (mg AAE/g) using the calibration curve ($y=0.0063x+0.148$, $R^2=0.99$ ($p<0.01$)).

The total antioxidant activity of extract was determined by phosphomolybdenum assay according to Prieto et al. (1999). Sample of 0.3mL extract (1 mg/mL) in the solution was mixed with 3mL phosphomolybdenum reagent (28mM sodium phosphate and 4mM ammonium molybdate in 0.6 M sulphuric acid) in capped test tubes. The samples were incubated for 90min in a water bath at 95°C. After cooling to room temperature, the absorbance of the solutions was measured using a UV-visible spectrophotometer (JANEWAY, 96500, UK) at 695nm against a

blank (3mL methanol without plant extract). The total antioxidant activity was expressed as milligram butylated hydroxytoluene equivalent/gram of dried extract (mg BHTE/g) using a calibration curve ($y=0.0094x+0.112$, $R^2=0.99(p<0.001)$).

Figure 1: Fresh, dried and powder moringa leaves

Statistical Analysis

All data were analyzed using one-way ANOVA with traits as an independent variable using SAS 9.3 software and the values are expressed in mean ± SD.

The level of moisture, ash, crude protein, crude fat, crude fiber and carbohydrate in food is of utmost importance for many scientific, technical and economic reasons. The chemical composition of dried leaves of moringa is shown in (Table 1). Dried moringa leaf had moisture level (6 g/100g), ash (11.2 g/100g), crude protein (25.3g/100g), crude fat (6.9g/100g), crude fiber (9.3g/100g) and carbohydrate (41.30g/100g). This finding is similar to the reports of nutritional assessment of dried moringa leaves (moisture, crude fat, and carbohydrate), higher (proteins) and lower (fiber content) by Abuye et al. (2003). The proximate composition of the moringa leaves in the current study was lower (moisture, protein, and fiber) and higher (ash fat and carbohydrate) than that the report by Ilyas et al. (2015). The chemical compositions variability of moringa might be due to climatic, seasonal and processing methods (De-Heer, 2011).

Table 1: The Proximate composition of dried moringa leaves in (g/100g db)

Sample	Moisture	Ash	Crude protein	Crude fat	Crude fiber	CHO_2
Moringa	6.0±0.5	11.2±0.3	25.3±0.2	6.9±0.2	9.3±0.7	41.3±0.7

The minerals of dried moringa leaves results are showed (Table 2). The dried moringa leaves had level of Ca (1300.7 mg/ 100 g), Na (128.9 mg/100g), K (330.9 mg/100 g), Mg (1056.6 mg/ 100 g), Fe (47.7 mg/ 100 g), Zn (1.8 mg/ 100 g), Cu (0.8 mg/ 100 g) and Mn (3.4 mg/100 g). The mineral levels of moringa were lower than that of findings of Abuye et al. (2003) and higher for all minerals than from

the finding of Seifu (2015). The chemical compositions variability of moringa might be due to climatic, seasonal and processing methods (De-Heer, 2011).

Table 2: Mineral contents of dried moringa leaves in (mg/100g, db)

Sample	Ca	Na	K	Mg	Fe	Zn	Cu	Mn
Moringa	1300.7±0.1	128.9±0.1	330.9±0.1	1056.6±0.1	47.7±0. 3	1.8±0.1	0.8±0.1	3.4±0. 3

The phytochemical properties of infusion of dried moringa leaves results are showed (Table 3). The dried moringa leaves had level of 2.6±0.1mg GAE*/g total phenolic content (TPC), 11.9±0.2 mg CE/g total flavonoid content (TFC) and 3.3±0.1 mg CE/g condensed tannin content (CTC). The phytochemical contents of the dried moringa leaves infusion in this study is higher than the moringa herbal tea infusion done by Ilyas et al. (2015) and Okiki et al. (2015. The difference could be due to the difference in tea plants, the method used and climatic condition.

Table 3: Total phenolic contents (TPC), total flavonoid contents (TFC) and condensed tannin contents of dried moringa leaves

Infusion	TPC (mg GAE*/g)	TFC (mg CE/g)	CTC (mg CE/g)
Moringa	2.6±0.1	11.9±0.2	3.3±0.1

Values are mean ± SD (n=2), Total Phenolic Content (TPC) expressed as gallic acid equivalent per g of the dried extract; 2: Total Flavonoid Content (TFC) expressed as cateichen equivalent per g of the dried extract.

DPPH scavenging activity, FRAP (Ferric Reducing Assay Power) and total antioxidant activity measured the hydrogen, electron-donating abilities of primary antioxidants and reduction of Mo (Molybdenum) (VI) to Mo (Molybdenum) (V) in the presence of antioxidant compound and subsequent formation of a green phosphate Mo (V) complex at acidic pH and at higher temperature were studied, respectively according to Lim et al. (2007) and the concentration of an antioxidant needed to decrease the initial DPPH concentration by 50% (IC50) is a parameter widely used to measure antioxidant activity (Sánchez-Moreno *et al*. 1998). The lower the IC50 the higher is the antioxidant activity (Brand-Williams *et al*. 1995). The antioxidant properties of dried moringa leaves infusion results are showed (Table 4). The dried moringa leaves had a level of 0.48 ± 0.12 (IC50, g/mL) DPPH scavenging, 2.27±0.02mg AAE/g Ferric reducing power (FRAP) and 1.23±0.02 mg BHTE/g and total antioxidant (TAA). This finding is lower than the finding of Ilyas et al. (2015) and higher than the finding of Tebeka and Libsu (2016) on assessment of antioxidant potential of *Moringa stenopetala* leaf extract.

Table 4: 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) scavenging IC50 values, ferric reducing power, and total antioxidant activities of dried moringa leaves

Sample	DPPH scavenging (IC50, g/mL)	Ferric reducing power (mg AAE/g)	Total antioxidant (mg BHTE/g)
Moringa	0.48 ± 0.12	2.27±0.02	1.23±0.02
AA	0.03±0.02		

Values are mean ± SD (n=2), AAE/g: Ascorbic acid equivalents per gram of dried extract; BHTE/g: Butylated hydroxytoluene equivalents per gram of dried extract; Where AA (Ascorbic acid)

The present study invertigated very usfull information on nutrional and phytochemical compstion of dried moringa leaves of Ethiopia. The result of the study indictaed that the dry moringa leaf is a good source of nutrients and phytochemical required that overcome malnutrion and helath problem of the country. In addition the phytochemical contents of the dried moringa leaves infusion in this study are higher than the moringa herbal tea infusion. This indicates that dried moringa leaves are promising as potential antioxidant to improve human health. However, there is a need to carry out further composition profile of the dried moringa leave infusion using GS-MS, HPLC and UPLC to explore the potential chemicals present in the infusion.

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