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BIOCHEMICAL AND PHYTOCHEMICAL ANALYSIS OF Moringa oleifera LEAVES AT THREE STAGES OF MATURITY

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ABSTRACT

Moringa oleifera leaves at three stages of growth – 2, 4 and 6 years, were analyzed for their Saponin, Tannin, Alkaloid and Oxalate contents. The results of saponins average contents were 3.217± 0.035, 3.605± 0.035 and 2.907± 0.035 (g/100gdm) at 2, 4 and 6 years respectively. Tannin levels were 1.583 ± 0.010, 1.373± 0.010 and 2.133±0.010 (g/100gdm) at 2, 4, and 6 years, respectively. Alkaloid content averaged, 0.807± 0.010, 1.420± 0.010 and 1.603± 0.010g/100gdm at 2, 4 and 6 years respectively; and the oxalates content were 3.283± 0.030, 3.567± 0.030 and 4.502 ± 0.030mg'100gdm at 2,4 and 6 years respectively. The concentrations of oxalate and alkaloid increased as the age of the plant increased. Saponin concentrations increased from 2 to 4years and reduced at 6 years while tannin concentrations reduced from 2 to 4 years and then increased at 6 years.

INTRODUCTION

Moringa oleifera belongs to the family *Moringaceae*. It is a fast-growing, drought-resistant plant, native to the southern foothills of the Himalayas in India. It is the most widely cultivated species of Moringa genus in the tropics and subtropics (Jahn, 1984). Common names include the *drumstick tree*, because of its long triangular seed pods; the *horseradish tree*, due to the taste of its roots; the *benzoil tree*, which derives from the oil it produces (Olson,2010); or the *miracle plant*, due to its impressive range of nutritional and medicinal values (Bukar *et al*., 2010). Virtually all structural components of the plant are considered edible, just as it is used for a variety of purposes, including water purification and hand-wash (Leone *et al*, 2015). There have been reports that both fresh and dried leaves bear a unique combination of vitamins, minerals and amino-acids; at levels 10 times more than the concentrations found in food products such as milk, carrots and spinach (Janick *et al*, 2008; Bukar *et al*, 2010).

However, there are reports, that *M. oleifera* leaves also contain phytochemicals, non-nutrient components, which have varying bioactive properties (Charles *et al*, 2014); and the concentrations of the phyto-compounds in the plant tend to vary with age of the plant and possibly vegetation zone (Akinyeye *et al*, 2013). This study sought to analyze the approximate composition and determine the variation in concentrations of four bioactive substances (saponins, tannins, oxalates and alkaloids) in the leaves of the *Moringa* plants cultivated in Edo State, Nigeria at different stages of growth.

MATERIALS AND METHODS

Location of the study

This study was undertaken in the Central Analytical laboratory, Faulty of Agriculture, University of Benin, Benin City, Edo state, which lies between latitude 5˚45´ and 7˚3´N, and longitude 5˚0´and 6˚5´E, within the equatorial rainforest belt of Nigeria. Average weather conditions are temperature of 23-32^{0}C,

relative humidity of 79-90% and annual rainfall-2000mm. The soil is lateritic and well drained.

Collection and identification of plant materials

Young leaves of *M. oleifera* were collected from the moringa orchard, in the University demonstration Farms. Three different sets of moringa trees, according to their ages (2, 4, and 6years) were sampled; fresh wholesome leaves unencumbered by the environment were harvested from the trees, and transferred to the Central Analytical laboratory.

Sample Preparation

Leaf samples were dried by open-air method, thus avoiding loss of active compounds. The leaves were spread on open clean table in a ventilated room and left to dry gradually at room temperature for seven days. Following, dried leaves were milled using a Tecator sample mill, model. The powdered samples were transferred into airtight sample pouches, sealed and labeled; then kept in cold storage, 4^0C, until required for analysis.

Chemical analyses

Proximate analysis of leaf samples was performed for crude protein, ether extract, crude fiber, ash and nitrogen-free-extract, using standard analytical methods recommended for each parameter (AOAC, 2005). The phytochemicals in the leaves were determined, following specific procedures described for each endogenous substance by previous workers. Laboratory work was undertaken using equipment, laboratory wares and reagents provided in the Central Analytical Laboratory in the Faculty of Agriculture, University of Benin. Nigeria.

Saponins

The method used was that of Obadan and Ochuko (2001). Portion (5g) of each replicate was put into a conical flask and 25ml of 5% aqueous ethanol was added. The samples were heated over a hot water bath for 4 hours with continuous stirring at temperature set at 55^0C. The mixture was filtered and the residue re-extracted with another 50ml of 20% ethanol. The combined extracts were reduced to 40ml over water bath at about 90^0C. The concentrate was transferred into a 250ml separator funnel and 10ml of petroleum ether was added and shaken vigorously. The aqueous layer was recovered while the ether layer was discarded. The purification process was repeated. 10ml of propan-2-ol was added. The combined propan-2-ol extracts were washed twice with 20ml of 5% aqueous sodium chloride. The remaining solution was heated in a water bath. After evaporation, the samples were dried in the oven until a constant weight was obtained. The saponin levels were calculated as g/100g of dried leaves.

Alkaloid

The Harborne (1973) method was used. Three replicates of the sample, each weighing 2.5g were measured into 250ml beaker and 100ml of 10% acetic acid in ethanol was added covered and allowed to stand for 4 hours; and then filtered. The extract obtained was concentrated to one-quarter of the original volume, on a hot water bath, set at 70^0C. Drops of concentrated ammonium hydroxide solution was added to the extract until the precipitation was completed. The whole solution was allowed to settle and then precipitate was collected and washed with dilute ammonium hydroxide and then filtered. The filtrate was discarded and the residue was dried and weighed as the extracted alkaloid and the weighted recorded as g/100gdm of dry leaves.

Tannin

Prussian blue method described by Egedege (2012) was used to determine the tannin content. Portions (500mg each) of the dried moringa leaves were weighed in replicates and placed in a 50ml plastic bottle. 50ml of distilled water was added and shaken for one hour, in a Contech mechanical shaker, model CB-5; and then filtered into a 50ml volumetric flask and made up to the mark. 5ml of the filtrate was removed with pipette and transferred into a test

tube and mixed with 2 ml of 0.1M $FeCl_3$ in 0.1N HCl and 0.008M potassium thiocyanate. The absorbance was measured within 10min, on Atomic absorption spectrophotometer AAS, model SPC260, set at 120nm. Mean tannin concentration was computed and presented as mg alkaloid/100gdm of moringa leaf sample.

Oxalate

The oxidase enzyme method described by Egedege (2012) was used for oxalate determination. Oxalate was first extracted from the leaves by homogenizing 2g portion of each leaf sample with 1.6ml of 0.5M HCl. Then this was diluted with 1ml distilled water. Each homogenate was transferred into 10ml graduated tube and heated in a water bath set at 100^0C for 20 minutes. Thereafter tube was removed from water bath, allowed to cool to room temperature; and then distilled water was added to bring the volume of homogenate up to 10ml mark; and then left to stand for 12hrs. Following, 1ml of each homogenate was clarified by centrifugation, using the Uniscope Centrifuge, model SM902B, set for 10min, at 12,000rpm and temp. 4^0C. Thereafter, 0.5 ml of the supernatant was collected, and 0.016 ml of 2M NaOH (2mol/L) was added, accurately to obtain the oxalate extract.

The enzyme - oxalate oxidase - was prepared from dried moringa leaves. 20mg of milled leaf was weighed out and transferred into 2ml sample tube. Then about 0.1ml distilled water, 0.8ml color reagent and 0.04 ml of horseradish peroxidase (50µl/ml distilled water), were added in succession and left to stand for a few minutes, to obtain the oxalate oxidase. Then 0.1 ml oxalate extract was added to the mixture to initiate the reaction. After incubation for 90 min, spectrophotometry was performed using the SPC-AAS, model 260. The absorbance was set at 555nm for reaction mixture. The color reagent used (pH 4.0) was prepared by adding 10mg 4-aminoantipyrine, 25µl of N,N-dimethylaniline into 100ml of 125mmol L succinate-NaOH buffer in 100ml 75% ethanol.

The oxalate content of each sample was thus determined with reference to standard curve which was drawn based on serial dilution (0, 2, 4, 6, 8, and 10µg) of oxalic acid into 1ml reaction medium. The data was presented as mean mg oxalate/100gdm.

Data Analysis

Mean values of replicate samples were computed for all determinations and one-way variance analyses were performed, while statistical differences were determined by f-test.

RESULTS

Phytochemicals in moringa leaves

Oxalate content increased with age (Table1). The highest **oxalate** concentration (mg/100gdm) was found in leaves of 6^{th} year plants (4.50±0.03). This was significantly higher ($P<0.05$) from concentrations obtained for 4^{th} year (3.57±0.03) and 2 years (3.28 ±0.03) old plants.

The concentrations of **tannin** were not age related while significant differences ($P<0.05$) were recorded between the three sets of moringa leaves (Table1). The highest concentration of tannin (mg/100gdm) was recorded for leaves of 6^{th} year plants (2.13±0.01). In comparison, leaves of 4^{th} year plants measured the lowest concentration (1.37±0.01) while 2^{nd} year plants measured 1.58±0.01.

Table1: Tannin and Oxalate levels in Moringa leaves at different ages (mg/100g).

	Age Difference			
Phytochemical	**2 years**	**4 years**	**6 years**	**SEM**
Oxalate	3.283^{c}	3.567^{b}	4.502^{a}	0.030
Tannin	1.584^{b}	1.374^{c}	2.133^{a}	0.010

^{abc} Means within the same row with different superscripts are significantly different (P<0.05).

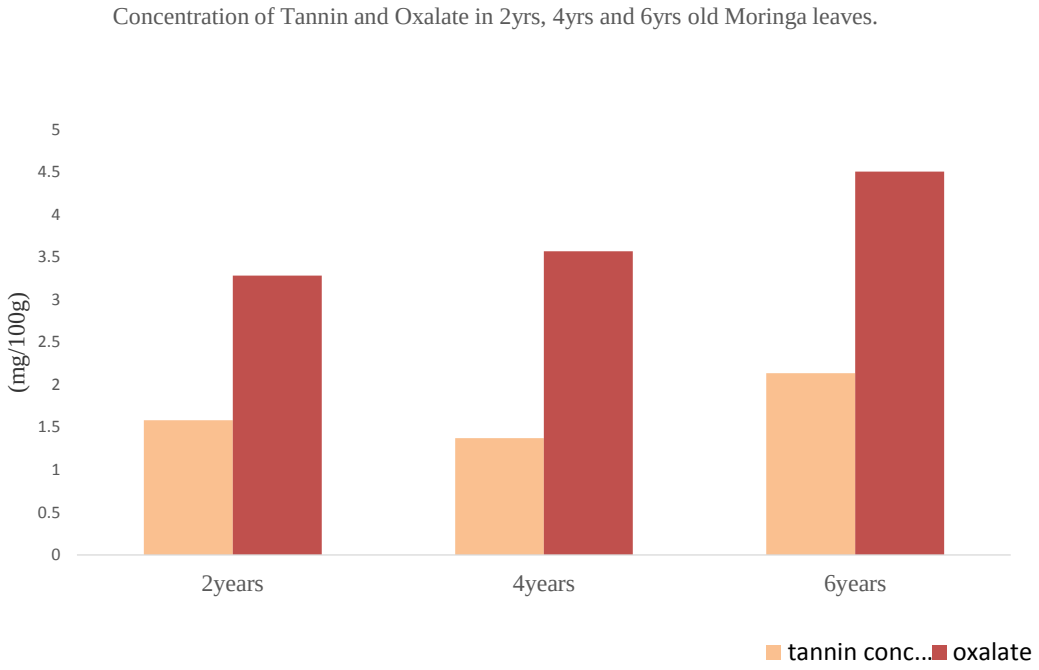


Figure 1: Concentrations of tannin and oxalate 2, 4 and 6 years Moringa (leaves).

There was significant (P<0.05) increase in **alkaloid** concentration in leaves as plant matured from 2nd to 6th year of growth (Table2). The lowest alkaloid concentration (g/100gdm) was found in the leaves of 2 years old plants (0.81±0.01), while the highest level (1.60±0.01) was recorded in leaves of 6th year plants.

Saponin concentration (g/100gdm) recorded the lowest level (2.91±0.04), in the leaves of 6 years plants; and this was significantly different (p<0.01) from the highest level (3.60±0.04) found in leaves of 4 years plants (Table 2). The leaves of plants in their 4th year measured the median level (3.22±0.035); which was significantly different (p<0.05) from the concentration in 2 years old and 6 years old plants.

Table2: Alkaloid and Saponin levels (%) in Moringa leaves at different ages.

Component	Age of plant			
	2 years	4 years	6 years	SEM
Alkaloid	0.807[c]	1.420[b]	1.603[a]	0.010
Saponin	3.217[b]	3.605[a]	2.907[c]	0.035

[abc]Means along the same row with different superscripts are significantly different (P<0.05).

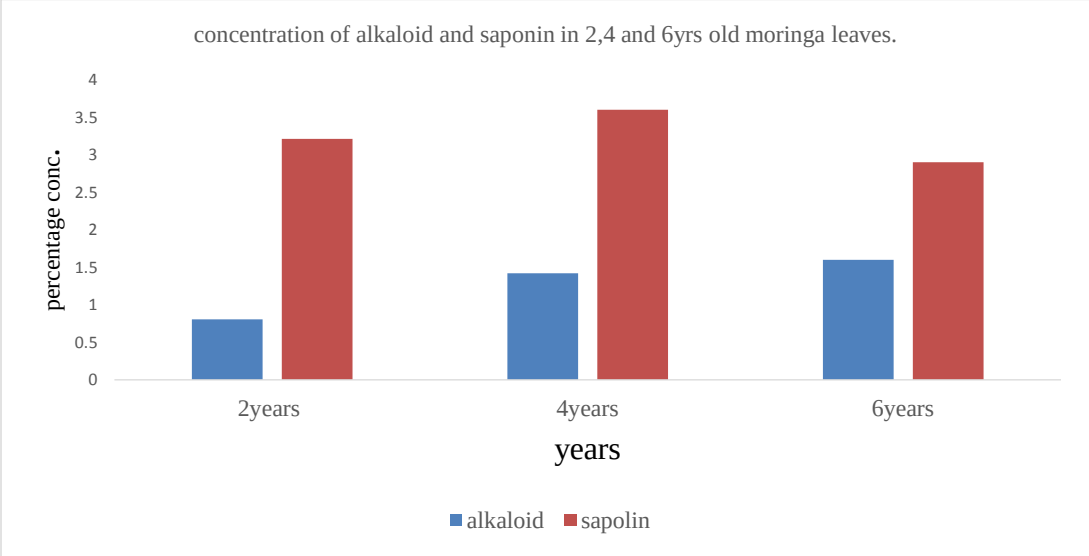


Figure2: Concentrations of alkaloids and Saponins in 2, 4 and 6years old leaves.

Proximate compositions

Moisture content (MC) was highest (84.1%) in the leaves of 4 years old plants. That amount varied significantly ($p<0.05$) from the moisture content levels found in 2nd year and 6th year plants (Table 3). Crude protein (CP) level was highest in 2nd year plants (26.91%dm), and reduced significantly ($p<0.05$) with maturity at 4th year and 6th years. Ether extract concentration increased significantly ($p<0.05$) in 4 year old plants, from the values recorded for the leaves of 2nd year plants; thereafter reduced significantly in 6th year plants. Crude fiber content of 2nd year leaves was significantly lower ($p<0.05$) than 4th and 6th year leaves which were not significantly different in CF contents. Both 2nd and 4th year plants recorded same values in their concentration of ash, which increased significantly ($p<0.05$) to high levels in the 6th year. Carbohydrate (NFE) content was high in leaves of 2nd year plants and reduced significantly with maturity of the plant. However NFE in 4th year plant was non-significantly higher ($p>0.05$) than the concentration measured in 6th year leaves (Table3).

Table3: Proximate composition of *Moringa* leaves (g/100gdm) at different ages.

Leaf Component	2 years	4 years	6years
Moisture content (fresh)	78.8	81.8	76.9
Ether extract	9.85	13.37	11.73
Crude protein	26.01	23.92	22.75
Crude fiber	5.67	12.0	12.10
Minerals (Ash	7.00	7.00	12.11

The results of total carbohydrate and fiber fraction determinations, presented in Table 4, indicate that the relative value of neutral detergent fiber (NDF) in moringa leaves was significantly higher ($p<0.05$) in comparison to acid detergent fiber (ADF) and hemicellulose values; while it was significantly ($p<0.01$) lower than the values of total soluble carbohydrates. The trend was similar between the three sets of moringa plants. The concentration of NDF and hemicelluloses increased non-significantly ($p>0.05$) in the 4th year and then was decreased significantly ($p<0.05$) in the 6thyear. ADF was decreased in 4 years old plants; and then increased non-significantly ($p>0.05$) in the 6th year plants.

Table 4: Relative concentrations of carbohydrate and fiber fractions in *M.oleifera* leaves at different ages (%)*

AGE (in yrs)	NDF	ADF	HEM	CHO
2	21.67 [a]	6.67 [a]	15.00 [a]	48.62[a]
4	23.53 [a]	5.10 [a]	18.35 [a]	43.71 [b]
6	13.39 [b]	6.52 [a]	6.67 [b]	41.79 [b]

[abc]Means with different superscripts on the same column are significantly different ($p<0.05$). *values are means of replicate determinations.

DISCUSSION

Nutrient Composition

The results of this study indicate that moringa leaves can be considered a protein source because it contains over 20% crude protein which is the classical definition of a protein source (Egedege, 2012). The leaves contained appreciable amounts of crude protein, ether extract and ash. These components tend to occur at their peak levels in the leaves when the plant is in the 4th year of growth, suggesting that the nutritive value of the leaves is prime and superior at that age. The results also indicate that the approximate nutrient components of moringa leaves reduces with age after the 4year. Thus supporting the suggestion that moringa leaves be included in diets as food supplement to combat malnutrition (Krishnaiah *et al.* 2009), this study suggests that in order to obtain maximum nutritional benefit, the right time to harvest the leaves for this purpose is when the plant is about 4 years old.

Bioactive compounds

There were variations in the concentrations of the four bioactive compounds – tannins, oxalates, alkaloids and saponins, in the moringa leaves examined in this study. We found a relationship between the age of the plant and the concentrations of the phytochemicals in the leaves; corroborating the report by Akinyeye (2013). For oxalates and alkaloids the relationship seemed to be linear, as concentrations increased as plant matured; while tannins and saponins, increased in concentration during the 4^{th} year of plant growth, and then decreased thereafter.

Tannins are not chemically well defined; and the critical level of toxicity has not been ascertained. However, many studies have concluded that excess tannin in animal feed is detrimental to health (Waghorn, 2008). The level of tannin found in most plant tissues, fruits and leaves, is normally in the range of 2.0 – 5.0% of fresh weight (Davis, 1981; Edeoga *et al*, 2005). In this study, tannin concentration ranged from 1.3mg to 2.1mg/100gdm between the three maturity stages of moringa leaves. It cannot be ascertained if these concentrations are detrimental to animals; just as neither has there been any reports of toxicity due to inclusion of moringa in dietary or herbal formulations. However, it would be worthwhile to determine in further investigations whether the tannins in moringa leaves belong to the hydrolysable or the condensed class.

Oxalates are a class of endogenous toxins that are found in a wide variety of foods and they play a supportive role in the metabolism of many plants, animals and humans. In this study, the concentrations of oxalates measured in the moringa leaves at 2, 4^{th} and 6^{th} year of plant growth, were significantly ($p<0.05$) different; The values were below critical levels that were reported to cause toxicity in humans and animals (Jones and Ford, 1972; Holmes *et al*, 2001; Dreosti, 2009; Soetan and Oyewole, 2009; Egedege, 2012). Hence occasional inclusion of *M. oleifera* leaves in food or animal feed may pose no detrimental effects to the consumer.

Alkaloids are compounds that are widely distributed among plants; and often tend to interfere with processes in intermediary metabolism; exhibiting a wide range of biological activities (Gouegni and Abubakar, 2013). Animals can suffer from alkaloid poisoning which results from consumption of plants containing indole alkaloids, such as found in poisonous mushrooms (Egedege, 1993). The toxicity of alkaloids is variable and this is dependent on the type and structure of the alkaloids (Egedege, 1993, 2012). In this study, the levels of alkaloid found in leaves of three age groups (2, 4 and 6 years) of moringa plant were significantly ($p< 0.05$) different. There has been no documented report of moringa poisoning from consumption of the leaves in Nigeria, indicating that the alkaloid present in moringa leaves are tolerable.

Saponins are a class of glycosides that are present in many plants and feedstuffs; and tend to have depressing effect on protein digestion and utilization; cause haemolysis of red blood cells and significantly affect growth, feed intake and reproduction in animals (Chummei *et al*, 2010; Ogbe and Affiku, 2011; Egedege, 2012); and predisposing the affected animal to hypo-glycaemia and hypo-cholesterolemia (Price *et al*, 1987; Chummei *et al*, 2010; Egwui *et al*, 2013). Saponin concentration in the leaves of 2years old plants was about thrice the alkaloid concentration. In 4 year plants, saponins were more than twice alkaloid levels, while in the 6 year saponins levels were further reduced to less than twice the alkaloid levels in the leaves of the moringa plant.

Notwithstanding anything, this study has shown that the concentration of saponin in dried leaves of moringa plant, at three stages of growth (2, 4 and 6 years), was lower than 8%, which was the value previously reported by Makkar and Becker (1997). In this regard,

there have been the assertions by Egwui *et al* (2013), that saponin levels in moringa leaves were not critical; just as there has been no established critical level of toxicity of saponin in plants. Nonetheless, we are in agreement with the general suggestion that feeds containing very high levels of saponins should not be fed to livestock (Adesogan *et al*, 2004). However, there is also the assertion that some saponins have positive effects, and health promoting benefits (Price *et al.,* 1987; Blumert and Liu, 2003; Krishnaiah *et al*, 2009; Bukar and Oyeyi, 2010; Oluduro, 2012; Leon *et al*, 2015).

In Nigeria and elsewhere, moringa seeds and leaves are being exploited therapeutically, in traditional medicine, as adjuvant in the management or prevention of degenerative health conditions. There are no secular or scientific reports about moringa toxicity in humans or livestock, to elicit concern. Thus it seems that moringa leaf and perhaps other structural components are endowed with a variety of nutritional and chemical agents (Kawo et al, 2009; Krishanaiah et al, 2009; Amaglo *et al.,*2010; Oluduro, 2012; Saini *et al.,*2014, 2016; Olayinka and Bewaji,2017); and it is the combination and interactions of the various endogenous factors, and likely, the synergy of their biological properties, that could be of significance in endowing the plant with high potentials in pharmaceuticals and nutrition. Research adventures using animal models, to verify or corroborate these assertions would thus be worthwhile.

CONCLUSION

Proximate analyses indicate that moringa leaf is rich in nutrient; and it attains its prime at 4 years when the nutrients components, except CP levels, are most dense. CP content of moringa leaves reduces as plant grows into maturity. All the values of approximate components were significantly reduced in the leaves of 6 years old plants. Phytochemical analysis indicates that tannins, oxalates, alkaloids and saponins were present in the leaves at tolerable levels. The oxalate content of the leaves was about twice the concentration of tannin; and this variation was maintained as the age of plant increased.

In this study, tannin concentrations in moringa leaves ranged from 1.3mg to 2.1mg/100gdm which fell below the range of 2.0 - 5.0% found in most plant tissues, on fresh weight basis. Saponin concentrations in moringa leaves were significantly ($P<0.05$) higher than alkaloid concentration; irrespective of the age of plant. Due to the increasing awareness of the economic value of the plant, further studies aimed at characterizing the nutritional and phytochemical properties of moringa plant parts is highly recommended.

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