

Phytochemical and Acute Toxicity Study of *Moringa Oleifera* Root

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ABSTRACT

Majority of Africans today depend either totally or partially on medicinal plants for the healing of their ailments which was used by their ancestors. This form of treatment, which is referred to as ethno medicine is sometimes the only kind of health care available to the rural populations. As part of the efforts to ascertain the healing capability credited to *Moringa oleifera* by the general public and some traditional practitioners, this work aimed at determining the phytochemical and acute toxicity of *M. oleifera* was embarked on. The study aimed at establishing a safety profile, evaluating phytochemical constituents. Qualitative and quantitative phytochemical analysis, thin layer chromatography and acute toxicity study was carried out using standard methods. Acute toxicity study was carried standard procedure in rats. Phytochemicals which include alkaloids, flavonoids, tannins, glycosides and triterpenes were detected in both hexane, ethyl acetate and methanolic extracts. Anthraquinones, carbohydrate, phenol, steroid and saponins were detected in the ethyl acetate and methanolic extract but absent in hexane extract of *Moringa oleifera* root. The quantitative phytochemical analysis showed that tannins (64.0 mg/g) was the highest phytochemical detected while the lowest was phenol (4.0 mg/g). Eight spots were detected with *p*-Anisaldehyde spray and the R_f values were shown alongside the spots in methanol extract. LD₅₀ was above 5000 mg/kg and did not cause mortality in all the tested rats. The results of this investigation may be useful for deriving doses that are safe for human consumption of *Moringa oleifera*.

Key words: Phytochemicals, Thin layer Chromatography

1.0 INTRODUCTION

Medicinal plants contain active variables which are responsible for conferring its bioactive functionalities (Okoronduet *al.*, 2015). Due to this varied composition of phytochemicals in plants they are regarded as a blessing from nature. Plant extracts have been widely used for therapeutic purposes including combating

antibiotic resistance. *Moringa oleifera*, one of the most widely cultivated species of a monogeneric family, is a type of local medicinal Indian herb which has turn out to be familiar in the tropical and subtropical countries (Khawaja *et al.*, 2010). All parts of the *Moringa* tree are edible and have long been consumed by humans. Extracts from different parts of the plant have been widely used for therapeutic purposes. Extracts have been

proven to be antimicrobial, anti-asthma, and aids cancer therapy/protection, circulatory/endocrine disorders, detoxification, digestive disorders, inflammation, immunity, nervous disorders, nutritional, reproductive health, skin disorders, general disorders/conditions (Fuglie, 1999; Fahey, 2005). Despite the many available applications of the plant, more research is ongoing to ascertain its effect on selective functionality or physiology. More so, mode of extraction, solvent for extraction and part of the plant analysed play a role in determining the amount of each phytochemical extracted (Okorondu *et al.*, 2015). Phytochemicals are, in the strictest sense of the word, chemicals produced by plants. However, they are commonly referred to only those chemicals which may have an impact on health, or on flavor, texture, smell, or color of the plants, but are not required by humans as essential nutrients (Okorondu *et al.*, 2015). *Moringa oleifera* is rich in compounds containing the simple sugar, rhamnose called glucosinolates and isothiocyanates (Fahey *et al.*, 2001; Bennett *et al.*, 2003). The stem bark contains two alkaloids, namely Moringine and moringinine (Kerharo, 1969). Vanillin, sitosterol, β -sitostenone, 4-hydroxymellin and octacosanoic acid have been isolated from the stem of *M. oleifera* (Faizi *et al.*, 1994a). Purified, gum exudate from *M. oleifera* has been found to contain Larabinose, galactose, -glucuronic acid, and L rhamnose, mannose, xylose and degraded gum polysaccharide consisting of L-galactose, -glucuronic acid and Lmannose has been obtained on mild hydrolysis of the whole gum with acid (Bhattacharya *et al.*, 1982). Flowers contain nine amino acids, sucrose, Dglucose, traces of alkaloids, wax, quercetin and kaempferat; the ash is rich in potassium and calcium (Ruckmani *et al.*, 1998). They have also been reported to contain some flavonoid pigments such as alkaloids, kaempferol, rhamnetin, isoquercitrin and kaempferitrin (Faizi *et al.*, 1994a; Siddhuraju and Becker, 2003). Thiocarbamate and isothiocyanate glycosides have been isolated from the acetate phase of the ethanol extract of *Moringa* pods (Faizi *et al.*, 1998).

Moringa leaves act as a good source of natural antioxidant due to the presence of various types of antioxidant compounds such as ascorbic acid, flavonoids, phenolics and carotinoids (Anwar *et al.*, 2005; Makkar and Becker, 1996). The high concentrations of ascorbic acid, oestrogenic substances and β -sitosterol, iron, calcium, phosphorus, copper, vitamins A, B and C, α -tocopherol, riboflavin, nicotinic acid, folic acid, pyridoxine, β -carotene, protein, and in particular essential amino acids such as methionine, cystine, tryptophan and lysine present in *Moringa* leaves and pods make it a virtually ideal dietary supplement (Makkar and Becker, 1996). The composition of the sterols of *Moringa* seed oil mainly consists of campesterol, stigmasterol, β -sitosterol, Δ 5-avenasterol and clerosterol accompanied by minute amounts of 24-methylenecholesterol, Δ 7-campestanol, stigmastanol and 28-isoavenasterol (Tsaknisi *et al.*, 1999; Anwar and Bhangar, 2003; Anwar *et al.*, 2005). Despite the fact that the root of *Moringa oleifera* have several medicinal properties, no standardization parameter has been assessed. Due to lack of standard parameters, proper identification and ascertaining quality and purity of the root of *Moringa oleifera* in the events of adulteration has been thwarted.

1.1 Plant Materials

The root of *Moringa oleifera* was collected in March, 2017 from Botanical Garden of Afforestation, Gwale Local Government of Kano state. The plant was identified and authenticated in the Herbarium of the Plant Biology Department of Bayero University, Kano by Malam Bahawuddeen and was compared with a voucher specimen number of BUKSAN11.

1.2 Preparation of Plant Extract

The roots collected were air-dried for two weeks and size reduced with mortar and pestle. Root powder (100g) of *Moringa oleifera* was extracted successively in 1 Liter of hexane, ethyl acetate and methanol for 72hrs using successive cold maceration and the concentrate was evaporated to dryness on a water bath and stored in desiccator for further use.

1.3 Phytochemical Screening

Phytochemical screening was carried out on the dried extract using simple chemical tests to detect the presence of alkaloids, saponins, tannins, flavonoids, terpenoids, anthraquinones, and cardiac glycosides. The following methods were used:

1.4 Tests for carbohydrates

1.4.1 Molish's (General) Test for Carbohydrates

To 1 ml of the filtrate, 1 ml of Molish's reagent was added in a test tube, followed by 1 ml of concentrated sulphuric acid down the test tube to form a lower layer. A reddish colour at the interfacial ring indicates the presence of carbohydrate (Evans, 2009).

Tests for Saponins

Frothing test

About 10ml of distilled water was added to a portion of the extract and was shaken vigorously for 30seconds. The tube was allowed to stand in a vertical position and was observed for 30mins. A honeycomb froth that persists for 10-15mins indicates presence of saponins.

Test for Flavonoids

Shinoda Test

A portion of the extract was dissolved in 1-2ml of 50% methanol in the heat metallic magnesium chips and few drops of concentrated hydrochloric acid were added. Appearance of red color indicates presence of flavonoids (Evan, 1996).

Test for Alkaloid

❖ Wagner's Test

Few drops of Wagner's reagents were added to a portion of the extract, whitish precipitate indicates the presence of alkaloid (Evans, 1996).

Test for Steroid and Triterpenes

Liebermann-Burchard's test

To a portion of the extract, equal volume of acetic acid anhydride was added and mixed gently. 1ml of concentrated sulphuric acid was added down the side of the test tube to form a lower layer. A colour change observed immediately and later indicates the presence of steroid and triterpenes. Red, pink or purple colour indicates the presence of Triterpenes while blue or blue green indicates steroids (Trease and Evans, 1996).

Test for Cardiac Glycoside

Kella-killiani's test

A portion of the extract was dissolved in 1ml of glacial acetic acid containing traces of ferric

chloride solution. This was then transferred into a dry test tube and 1ml of concentrated sulphuric acid was added down the side of the test tube to form a lower layer at the bottom. Observed carefully at the interphase for purple-brown ring, this indicates the presence of deoxy sugars and pale green colour in the upper acetic acid layer indicates the presence of cardiac glycosides (Evans, 1996).

Test for Tannins

Ferric chloride test

To a portion of the extract, 3-5 drops of ferric chloride was added. A greenish black precipitate indicates presence of condensed tannins while hydrolysable tannins give a blue or brownish blue precipitate (Evans, 1996).

Test for Anthraquinones

Bontrager's test

To a portion of the extract in a dry test tube, 5ml of chloroform was added and shaken for at least 5mins. This was filtered and the filtrate shaken with equal volume of 10% ammonium solution, bright pink colour in the aqueous upper layer indicates the presence of free anthraquinones (Evans, 1996).

Quantitative Phytochemical screening of the root extract of *Moringa oleifera*

Alkaloid Determination using Haborne (1973) Method

5g of the sample was weighed into a 250ml beaker and 200ml of 10% acetic acid in ethanol were added and covered and allowed to stand for 4hours. This was filtered and the extract is concentrated on a water bath to one quarter of the original volume. Concentrated ammonium hydroxide was added drop wise to the extract until the precipitation is completed. The whole solution was allowed to settle and the precipitates were collected and wash with dilute ammonium hydroxide and then filtered. The residue is the alkaloid, which was dried and weighed.

Flavanoid Determination by the Method of Bohm and Kocipal – Abyazan (1994)

10g of the plant sample was extracted repeatedly with 100ml of 80% aqueous methanol at room temperature. The whole solution was filtered through Whatman filter paper No. 42 (125mm). The filtrate was later be transferred into a crucible and evaporated into dryness over a water bath and weighed to a constant weight.

Saponin Determination

The method of Obadoni and Ochuko (2001) was used. Out of the grinded samples 10g was weighed for each and put into a conical flask and 100ml of 20% aqueous ethanol were added. The samples were heated over a hot water bath for 4hours with continuous stirring at about 55°C. The mixture was filtered and the residue re-extracted with another 200ml, 200% ethanol. The combined extracts were reduced to 40ml over water bath at about 90°C. The concentrate was transferred into a 250ml separatory funnel and 20ml of diethyl ether was added and shaken vigorously. The aqueous layer was recovered while the ether layer was discarded. The purification process was repeated and 60ml of n – butanol was added. The combined n-butanol extracts were washed twice with 10ml of 5% aqueous sodium chloride. The remaining solution was heated in a water bath. After evaporation the samples were dried in the oven to a constant weight; the saponin content was calculated as percentage.

Tannin Determination by Van-Burden and Robinson (1981) Method

500mg of each sample was weighed into a 50ml plastic bottle and 50ml of distilled water was added and shaken for 1hour on a mechanical shaker. This was filtered into a 50ml volumetric flask and made up of the mark. Then 5ml of the filtrate was pipetted out into a test tube and mixed with 2ml of 0.1M FeCl₃ in 0.1M HCl and 0.008M potassium ferrocyanide. The absorbance was measure at 120mm within 10min.

Determination of Total Phenols by Spectrophotometric Method

The fat free sample was boiled with 50ml of ether for the extraction of the phenolic component for 15minutes. About 5ml of the extract was pipetted into a 50ml flask, and then 10ml of distilled water was added. About 2ml of ammonium hydroxide solution and 5ml of concentrated amyl alcohol were added also. The sample was made up to mark and allowed to react for about 30 minutes for colour development. This was measured at 505nm.

Thin Layer Chromatography Profile

TLC aluminum sheet of 20 x 20cm silica gel pre-coated plate using the one way ascending

techniques was employed for this analysis. The plates were cut into sizes of 5 x 10 cm. The extract was dissolved in the initial extraction solvents and spots were applied manually on the cut plate using capillary tubes, after which the plates were dried and developed in solvents ratio Ethyl acetate : Hexane (8:2) in the chromatographic tank. Developed plates were sprayed using general detecting reagents (*p*-anisaldehyde/H₂SO₄, 10% H₂SO₄ in methanol). It was viewed under UV 365nm) and heated at 110°C for 2 minutes where applicable. Number of spots, colour and retardation factors (Rf values) for each of the spots were determine and recorded while the chromatograms was scanned accordingly (Gennaro, 2000; Stahl, 2005). naro, 2000; Stahl, 2005).

Acute Toxicity Studies (LD50)

The LD50 of the extract was determined using Lorke's method (1983). The study was carried out in two phases and animals (mice) were deprived of food for 16-18h prior to administration of the extract. In phase 1, three groups of three animals per group were used. The extract was administered orally in geometrically increasing doses (10mg/kg, 100mg/kg and 1000mg/kg). The treated animals were observed for four hours post administration for signs of toxicity. After 24 hours, when no death occurred, phase 2 was initiated. In phase 2, four groups of one animal each were given the extract orally in geometrically increasing doses (1500 mg/kg, 2250 mg/kg, 3250 mg/kg and 5000 mg/kg). The animals were then observed for signs of toxicity for the first 4 hours and mortality for 24hours. The arithmetic mean of the lowest dose that killed an animal and the highest dose that did not kill was taken as the mean lethal dose (LD50) of the extract.

Results

Phytochemical screening of hexane, ethyl acetate and methanol extracts revealed the presence of alkaloid, flavonoids, glycoside, triterpenes and tannins. Steroid, saponins, phenols, anthraquinones and carbohydrate were absent in hexaneextract but present in ethyl acetate and methanol extract.

Table 1. Mass and Percentage Yield for the Extracts of *Moringa oleifera* root

Extract	Mass (g)	Percentage Yield (%)	Extract Appearance
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Hexane	3.75	3.75	Light brown
Ethyl acetate	7.23	7.23	Brown
Methanol	10.12	10.12	Dark brown

Table 2. Qualitative Phytochemical screening of Hexane, Ethyl acetate and methanolic root extracts of *Moringa oleifera*

Metabolites	Inference		
	Hexane	Ethyl acetate	Methanol
Alkaloid	+	+	+
Flavonoid	+	+	+
Saponins	-	+	+
Cardiac glycoside	+	+	+
Tannins	+	+	+
Steroid	-	+	+
Triterpenes	+	+	+
Phenol	-	+	+
Anthraquinones	-	+	+
Carbohydrate	-	+	+

Key: + Present, - Absent

The tannins (64.0 mg/g) was the highest phytochemical detected in the plant while the lowest was phenols (4.0 mg/g).

Table 3. Quantitative Phytochemical screening of methanolic rootextract of *Moringa oleifera*

Metabolite	Quantity (mg/g)
Alkaloids	50.00±0.0
Flavonoids	40.00±0.0
Saponins	25.00±0.0
Tannins	64.00±0.0
Phenols	4.00 ±0.0

Table 4. TLC results of Hexane, Ethyl acetate and methanolic root extracts of *Moringa oleifera*

Extract	Solvent system	No. of Spots	Distance travelled by Spots	Rf-value
Hexane	HE:EA (8:2)	4	5.8	0.23, 0.47, 0.59, 0.96
Ethyl acetate	HE:EA (8:2)	6	5.9	0.25, 0.38, 0.58, 0.78, 0.87, 0.94
Methanol	HE:EA (8:2)	8	5.9	0.21, 0.36, 0.47, 0.58, 0.68, 0.81, 0.89, 0.94

Key: HE (Hexane), EA (Ethyl acetate) Rf (Retention factor)

No death was recorded in the first phase of the study in rats. In the second phase, doses of 1500, 2250, 3250 and 5000mg/kg were used and no death was also recorded. The oral median lethal dose (LD50) for the methanol root-extract of *Moringa oleifera* was therefore estimated to be greater than 5000mg/kg and no sign of behavioural changes were also observed.

Table 5. Acute toxicity studies of methanolic root bark extract of *Moringa oleifera*

Plant species	Group	Number of Animals	Dose (mg/kg)	Mortality recorded after 24hrs
Phase I	I	3	10	0/3

Phase II	II	3	100	0/3
	III	3	1000	0/3
	I	1	1500	0/1
	II	1	2250	0/1
	III	1	3250	0/1
	IV	1	5000	0/1

DISCUSSION

The percentage yield of different extracts from 100g of powdered root of *Moringa oleifera* includes: methanol (10.12%), ethyl acetate (7.23%) and n-hexane (3.75%). Methanol has the highest yield (10.12%); this is specially credited to the ability of highly polar solvents to extract most of the phytochemical constituents present in plant materials (Kokate *et al.*, 2003). Phytochemical analysis includes qualitative and quantitative analysis, useful for the investigation of the presence and quantification of primary and secondary components of the plant. The investigation should be followed by the standardized extraction procedures, isolation of the secondary components and other studies like efficacy, toxicity etc. The purpose of the present study was to identify the extraction procedure which has the optimum therapeutic portions of the crude drug of the medicinal plant *Moringa oleifera*. Effect of extracted plant phytochemicals depends on: the nature of the plant material, its origin and degree of processing, moisture content, and particle size. The variation in different extraction methods that will affect quantity and secondary metabolite composition of an extract depends on: type of extraction, time of extraction, temperature, nature of solvent, solvent concentration and polarity (Ncube *et al.*, 2008). The percentage yield (mass of extract/mass of dry matter) was used as an indicator of the effects of the extraction conditions. Methanol extract showed maximum percentage yield probably because polarity of the solvent was used here. The best extract should be selected for the further quality standard analysis as per highest diversity of compounds present in it. The present study showed phytochemical variations in results of qualitative and quantitative analysis for all the extracts. Tests were showing results for the presence of secondary metabolites as per their type and quantity. The reactions showed variation in colour intensity also, due to the variation in

quantity of components present in all the extracts. Phytochemical screening of hexane, ethyl acetate and methanol extracts revealed the presence of alkaloid, flavonoids, glycoside, triterpenes and tannins. Steroid, saponins, phenols, anthraquinones and carbohydrate were absent in hexane extract but present in ethyl acetate and methanol extract. This could be as a result of minimal composition of nonpolar phytochemical constituents in the plant and the low polarity of n-hexane extracts (Harborne, 2009). Anthraquinone was absent in hexane extract but present in ethyl acetate and methanol extracts. Similarly, Alhassan *et al.*, 2014, reported it to be present in methanol extract. This is probable due to differences in geographical locations of the plant sources, time of collection and maturity. Evans, (2002) elaborated how geographical locations greatly affects the accumulation and diversity of the phytoconstituents).

In this study, methanol and ethyl acetate extracts have similar composition of phytochemical constituents (i.e. alkaloids, cardiac glycosides, flavonoid, tannins and triterpenes) but in varied concentrations and is attributable to their closeness in polarity. This is in agreement with the findings of Abubakar *et al.*, 2015 and Alhassan *et al.*, (2014) where similar phytochemicals were reported in the extracts. These primary and secondary metabolites in plants have numerous functions. Crude, pure and isolated alkaloids and their synthetic derivatives have been used as analgesic, antispasmodic and bactericidal agents (Sary, 1998; Okwu and Okwu, 2004). Flavonoids have been shown to provide antibacterial, anti-inflammatory, antiallergic, antimutagenic, antiviral, antineoplastic, anti-thrombotic and vasodilatory activity (Alan and Miller, 1996). Flavonoid also has immense antioxidant and anti-inflammatory activity because of its ability to scavenge hydroxyl radicals, super oxide anions and lipid

peroxy radicals (Okwu, 2004; Okwu and Josiah, 2006). Tannins have been used in the treatment of wounds especially those emanating from varicose ulcers and hemorrhoids (Njoku and Akumfula, 2007) and is able to stop bleeding during circumcision (*Edeoga et al.*, 2005). The phytochemical constituents especially the secondary metabolites could be useful as guide to chemotaxonomic markers (Jonathan and Tom, 2008) that will aid in chemo taxonomical classification system and further phylogenetic studies in Moringaceae family.

The thin layer chromatographic analysis of the n-hexane, ethyl acetate and methanol extracts was carried out on TLC plate precoated with analytical silica gel and developed in suitable solvents systems at different ratios, and it gave distinct and good degree of separations of phytochemicals. The hexane, ethyl acetate and methanol extracts when developed in Hexane: Ethyl acetate (8:2) and sprayed with *p*-Anisaldehyde/H₂SO₄ reagent for visualization, it gave four, six and eight spots of mostly different colours alongside their R_f values (Table 7). The Thin Layer Chromatography chemical screening is usually done to target isolation of new or very important constituent present in the plant extracts which has marked pharmacological activities and also serves as an important tool in recognizing how metabolite for isolation behaved and can be purified; hence, channeling scientific efforts towards the desired compound(s) and prevent waste of resources and time (Patraet *al.*, 2012). The success of separation of bio-molecules by chromatographic technique is markedly influence by the suitability of the separating solvent systems largely influences the successful and also rely upon an ideal range of partition coefficient (k) for each target compound(s) (Ito, 2005).

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- In order to determine the safety margin of drugs and plant products for human use, toxicological evaluation was carried out in experimental animals using Lorke's method to predict toxicity and to provide guidelines for selecting a "safe" dose in animals and also used to estimate the therapeutic index (LD₅₀/ED₅₀) of drugs (Olson *et al.*, 2000; Rang *et al.*, 2012). In this study, median lethal dose (LD₅₀) of the extracts (aqueous and methanol) of the *Moringa oleifera* root was carried out orally in rats. The LD₅₀ was found to be greater than 5000 mg/kg when administered orally in rats and all the animals remain alive and did not manifest any significant visible signs of toxicity at these doses. These studies showed the extracts of *Moringa oleifera* root are practically non-toxic when administered using the oral route. This is based on the toxicity classification which states that substances with LD₅₀ values of 5000 to 15,000 mg/kg body weight are practically non-toxic (Loomis and Hayes, 1996).
- Conclusion**
- The established phytochemical and toxicity standards for the powder of *Moringa oleifera* root could be used as a diagnostic tool for the standardization and identification of this medicinal plant for its purity and quality in the future and hence, inclusion into the pharmacopoeia for official use. *Moringa oleifera* extracts (hexane, ethyl acetate and methanol) have some secondary metabolites namely alkaloids, tannins, flavonoids, cardiac glycosides and triterpenes. These were confirmed by thin layer chromatography profiles. These contribute in the effect of the plant. The Acute toxicity (LD₅₀) of the root extract of *Moringa oleifera* (Methanol) was found to be greater than 5000 mg/kg and is considered safe for use.
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