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Acute toxicity, phytochemicals, and nutrient composition of *Moringa oleifera* leaves, a plant used as a food supplement in the tropical region of Nigeria

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ABSTRACT

Introduction: The *moringa oleifera (MO)* plant is popularly known as the "miracle tree". The uses of *MO* leaf extract in controlling high blood pressure and as a food supplement with high nutritional value have been documented scientifically. Some studies have also shown that dosages appear to be safe from tested toxicity but a relative increase such as 3-4 times the recommended doses is known to cause genotoxic damage. However, there is a paucity of human evidence of the potency of MO presently, most studies use animal models such as rats and ethanol extract. **Objective:** The study aims to assess the acute toxicity lethal and sublethal doses (LD_{50}) of MO using Swiss albino mice and its phytochemical constituents in our locality where consumption is very high. **Methods:** The ethanol extraction method was used to obtain the concentrates of 100 g, and thirty (30) adult mice (30 - 40g) were used for Acute Toxicity (LD_{50}). Phytochemical analyses were carried out to determine the major Bio-constituents. **Result:** Showed that LD_{50} of 3900 mg/kg produced death in mice. Piloerection amongst others and weight gain were observed in sublethal doses. Carbohydrates (36.6%), Calcium, Phenol, Ascorbic acid, and Methyl Octadecenoate (30%) recorded the highest constituents. **Conclusion:** it is safe to consume MO to promote health in the right doses.

Keywords: *Moringa oleifera*; Toxicity; Phytochemicals; Nutrients and Food supplement.

INTRODUCTION

The *moringa* plant is popularly known as the "miracle tree" or "tree of life". It is identified by various names, such as Drumstick tree or Horseradish plant in English, and locally in Nigeria, it is known as Zogale in Hausa, Okwe oyibo in Igbo, Ewe Ile in Yoruba, and Gawara in Fulani [1]. It is highly consumed in the tropical region of the country where it is sold in the open market as dried leaf powder in colored small bottles. The people in the locality believe it is good for consumption for health but do not know the constituent. Recent medical research supports the use of *Moringa* leaf extract in controlling high blood pressure. Anwar et al. [2] indicated that *Moringa* leaf extract had a significant effect in reducing blood pressure levels of guinea pigs and rabbits under laboratory conditions. Rolim et al. [3] also revealed that *Moringa Oleifera* is referred to as a "nontoxic" and non-side-effect" plant, but some studies have discovered that genotoxicity has been recorded with high doses of *Moringa* leaf and seed extracts [4].

For usage as a food supplement, *moringa oleifera* is recommended mostly as a highly nutritious antioxidant. Some studies have shown that supplemental dosages appear to be safe from tested toxicity but a relatively small increase such as 3-4 times the recommended doses is known to cause genotoxic damage and may promote cancer formation whereas doses higher than that cause overt organ damage mostly liver and kidneys. Beyond that, very reasonable supplemental dosages appear to be able to induce abortions in pregnant rats and thus supplementation is contraindicated in pregnant women [4].

Ugwu et al. [5] studied and analyzed the acute toxicity and phytochemistry of ethanol leaf extract of *Moringa oleifera* and reported that the phytochemical analyses showed the presence of tannins, carbohydrates, saponins, glycosides, reducing sugars, terpenoids, steroids, flavonoids, and alkaloids. They discovered that phytochemicals such as resins, proteins, and fat oils were absent and the lethal dose (LD_{50}) of the ethanol leaf extract in mice was found to be less than 5000mg/kg body weight. They concluded that ethanol leaf extract of *Moringa oleifera* is rich in phytochemicals and at the same time safe for consumption and opined that this could be the reason why ethanol leaf extract of *Moringa oleifera* has been in use for numerous ethnomedicinal practices.

Since MO is used both as a vegetable and medicinal herb, as such, a proper analysis should be carried out to assess its macronutrients, phytochemicals, and nutritional profile in the locality where

consumption is very high. However, there is a paucity of human evidence of the potency of MO presently. Few clinical trial studies on humans have been published recently, the major issue is the standardization of the MO as a product for human consumption [34], hence the need to make further studies on MO constituents with more advanced analytical techniques for human suitability [35]. The majority of animal model evidence uses rats as the model and uses ethanol extract from the MO leaves. The Swiss mice are similar in physiologic composition such as circulatory system, muscles, and skeletal structures to humans [38]. The study aims to assess the acute toxicity lethal dose (LD_{50}), phytochemical constituents and supplemental dosage of *moringa oleifera* leaves in our locality, the tropical region of Nigeria where there is very high dietary consumption of the leaves as vegetables, tea, and medicinal consumption for hypertensive heart diseases.

MATERIALS AND METHODS

Study Site

The study involved *Moringa oleifera* plant and animal model was carried out at the Department of Physiology, Pharmacology, Biochemistry and Animal Health, College of Veterinary Medicine, Michael Okpara University of Agriculture, Umudike, Abia State, Nigeria. The institution was chosen because it has all the equipment and resources to embark on the study with a high level of accuracy.

Study Design

An experimental study design was adopted.

Data Collection Procedure

Plant Collection and Identification

Fresh leaves of *Moringa oleifera* (MO) were collected from a MO plantation at a local settlement in Umuakwela, Obodo-Chiara in Ahiazu Mbaise Local Government Area of Imo State, Nigeria. The leaves were authenticated by Prof M.C. Dike, a Botanist at the Department of Forestry, College of Natural Resources and Environmental Management, Michael Okpara University of Agriculture, Umudike, Abia State, Nigeria. The Voucher specimen was deposited with the College with reference no. SET/DFCNREM/2015-015.

Figure 1: *Moringa oleifera* tree and leaves branches[6]

Preparation of leaf extract of Moringa Oleifera

The leaves were stripped off the stalks, washed, allowed to drain, and spread in a well-ventilated room to dry. The ethanol extraction method was used to obtain the concentrates. This method is better than the water extraction method because it does not cause growth mold on the extracts and will preserve the extract required to complete the study [6]. The ethanol extract of MO leaves was prepared using the Soxhlet method reported by Jensen[7] and Akah et al [6]. In this method, the fresh leaves were air dried at room temperature for 8 days after which they were pulverized to powdered form using a manual blender and then sieved with a muslin cloth. Fifty (50) grams of the powdered material was introduced into the extraction chamber of the Soxhlet extractor and extraction was done using ethanol 98% as solvent. The extraction temperature was maintained at 70^0C for 48 hours. At the end of the period, the ethanol was evaporated low-temperature turn at 40^0C in an electric oven to obtain a crude extract that weighed 11.90 g and represented a yield of 23.8%. The solvent used for reconstitution of the MO extract was Dimethylsulfoxide (DMSO), it allows the extract to be stable for a longer period. This procedure was conducted by Laboratory Scientists at the Department of Veterinary Physiology and Pharmacology Laboratory, MOUA, Umudike.

Phytochemical test of MO leaf extract

Two different phytochemical analyses were carried out namely:

1. Qualitative analysis
2. Quantitative analysis

Gas chromatography-mass spectrometry (GCMS) analysis was carried out by Laboratory Scientists at the National Research Institute for Chemical Technology (NRICT), Federal Ministry of Science and Technology, Zaria, Nigeria.

Qualitative analysis

A phytochemical qualitative study was carried out using the methods of Trease et al [9]; Harborne and Sofowora [10].

Tests for Major Bio-Nutrients and Bio-Organic Constituents of MO Leaves

Test for Proteins: Few drops of nitric acid were added by the sides of a test tube very gently to 1ml of M.O. Formation of yellow color indicates the presence of protein in the sample.

Test for Carbohydrate: 1ml each of Fehling A and Fehling B was added to dilute M.O. and heated for 30 minutes and observed for the formation of a brick red color which indicates the presence of carbohydrates.

Test for Resins: 5ml of distilled water was added to a little amount of M.O. in a test tube and observed for turbidity which indicates the presence of resins.

Test for Tannins: 5 ml of 45% ethanol was added to 2g of M.O. and boiled for 5 minutes. The mixture was then cooled and filtered; 3 drops of lead sub-acetate solution were added to 1 ml of the filtrate. A gelatinous precipitate was observed which indicates the presence of tannins. Another 1ml of the filtrate was added to 0.5ml of bromine water. A pale brown precipitate confirms the presence of tannins.

Test for Saponins: 0.5ml of M.O. was added to 5ml of distilled water in a test tube. The solution was shaken vigorously and observed for a persistent froth. The frothing was mixed with 3 drops of olive oil and shaken vigorously. The formation of an emulsion indicates the presence of saponin.

Test for Flavonoids: 0.5ml of M.O. was introduced into 10ml of ethyl acetate and heated in boiling water for 1 minute and filtered. Four milliliters of the filtrate were shaken with 1ml of 1% aluminum chloride solution and kept. The formation of yellow color in the presence of 1ml dilute ammonia solution indicates the presence of flavonoids.

Test for Alkaloids: 5g of M.O. extracted with 10 ml of ammoniacal chloroform was mixed with 5 ml of chloroform. The mixture was filtered and the filtrate was shaken with 10 drops of 0.5M H_2SO_4. The observation of a cream-white precipitate indicates the presence of alkaloids.

Test for Steroids: Two ml of acetic anhydride was added to 0.5g of M.O. and 2 ml of sulphuric acid was added by the sides of the test

tube. A violet or blue-green coloration indicates the presence of steroids.

Test for Phenols: A little quantity of M.O. was mixed with distilled water in a test tube and warmed, followed by the addition of 2ml of Ferric chloride solution. The formation of green or blue color indicates the presence of phenols.

Test for Glycosides: About 0.5ml of M.O. was added to 1ml of glacial acetic acid containing traces of Ferric chloride in a test tube. To this solution, 1ml of concentrated sulphuric acid was added and observed for the formation of reddish-brown color at the junction of the two layers with the upper layer turning bluish-green. This indicates the presence of glycosides.

Quantitative analysis

Tests for Minerals and Vitamins

Bioactive mineral constituents of MO were also tested using the Instrumental Neutron Activation Analysis (INAA). The MO extract sample is placed into a neutron flux analyzer and radioactive nuclides of varying energies are produced. Gamma rays were emitted during the decay of the radioactive nuclides with characteristic energies for each nuclide. The elements found include Copper (Cu), Manganese (Mn), Iron (Fe), Zinc (Zn), Magnesium (Mg), Potassium (K), Sodium (Na), Phosphorus (Ph), and Calcium (Ca).

Atomic Absorption Spectrophotometer (AAS) was used for determining the presence and quantity of Vitamins in the MO extract by making use of the absorption of light by the elements in the extract to measure their concentration. The following were obtained, Carotene (vitamin A), Riboflavin (vitB2), Niacin (Vit B3), Ascorbic acid (Vit C), Thiamin (Vit B1), Vitamin B12, and Vit K using 100 grams of MO extract.

Gas chromatography-mass spectrometry (GCMS) analysis and Identification of Phytocomponents of Moringa oleifera

The extract (sample) was taken to the National Research Institute for Chemical Technology (NRICT), Federal Ministry of Science and Technology, Zaria for GCMS analysis. The characterization of the Phytochemicals in MO was done using GC-MS QP2010 Plus (Shimadzu, Japan). The identification of the phytochemicals in the sample was carried out using a QP2010 gas chromatography with Thermal Desorption System, TD 20 coupled with Mass Spectroscopy (Shimadzu).

Identity of the active components in the extract was done by comparison of their retention indices, peak area percentage, and mass spectra fragmentation pattern with those stored on the National Institute of Standards and Technology (NIST) digital library data and also with published literature[11, 12]. The name, molecular weight, formula, structure, and bioactivities of the compounds were ascertained.

Pilot Study Toxicity

A pilot study was first carried out using 5 adult Mice (30 – 40g). Each Mouse was assigned a particular dose level of the MO leaves extract in the order 1000, 2000, 3000, 4000, and 5000 mg/kg. All treatments were done via the oral route using a gavage. The animals were thereafter observed for 24 hours for toxicity signs and death. The result obtained from this pilot study was used for the acute toxicity (LD_{50}) study proper.

Acute Toxicity (LD_{50}) Study

Thirty (30) adult mice (30 - 40g) of either sex, 15 male and 15 female were used for the study. The mice were divided into six groups of 5 mice each. Each group was assigned a particular dose level of MO leave extract. Groups 1, 2, 3, 4, 5, and 6 received oral doses of 500, 1000, 2000, 3000, 4000, and 5000 mg/kg body weight of the extract respectively for 7 days. The mice were kept in aluminum cages at room temperature and humidity, under a naturally illuminated environment. They were fed on standard diet and water according to the National Institute on Health Publication (NIH), (1985). They were observed for toxicity signs and the number of deaths within a period of 7 days. The determination of LD_{50} value was determined using Kaber's method as reported by Enegide et al [8].

Calculation of the Lethal Dose LD_{50}

Lethal Dose formula

$$LD_{50} = LD_{100} - \frac{\sum (Dd \times Md)}{N}$$

Where LD_{50} = Dose that killed 50% of a given population

LD_{100} = Dose that killed 100% of the population

$\sum (Dd \times Md)$ = Sum of all products of Dose difference and mean death.

N = Number of animals in each group

$$LD_{50} = 5000 - \frac{5500}{5}$$

$$= 5000 - 1100$$

LD_{50} = 3900mg/kg

Figure 2: Swiss albino mice for LD_{50}

Ethical clearance

Ethical clearance was obtained from the research ethics committee, Department of Physiology, Pharmacology, Biochemistry and Animal Health, College of Veterinary Medicine, Michael Okpara the University of Agriculture, Umudike, Abia State. The animals were handled in line with the principles of Replacement, Reduction, and Refinement (3Rs) [33]. They were fed a standard diet and water according to the National Institute on Health Publication (NIH) for 7 days.

Percentage and Mean were used as descriptive statistical tools for data analysis

RESULTS

Data Presentation Introduction

The results were presented in tables, graphs, and figures.

***Moringa oleifera* toxicity**

Table 1 showed a pilot study conducted on *moringa oleifera* toxicity using Swiss albino mice with a mean weight of 33.42 grams. Death was recorded in group 5 at the MO dose of 5000 mg/kg. Doses less than 5000 mg/kg did not result in the death of the Swiss albino mice in groups 1 to 4.

Table 1: Pilot study: Stock concentration (100mg/kg)

Group	Dose (mg/kg)	Body weight (g)	Volume Adm (ml)	Death
1	1000	31.50	0.315	0
2	2000	33.00	0.66	0
3	3000	30.20	0.906	0
4	4000	38.80	1.552	0
5	5000	33.60	1.68	1

The acute toxicity study of MO ethanol extract is presented in Table 2. The study revealed that LD_{50} of 3900 mg/kg (see appendix ii) produced death in adult Mice of 30 – 40 grams body weight. Following the administration of MO ethanol extract to the mice, death did not occur in groups 1 to 3 at a dose range of 500 to 2000 mg/kg (sublethal dose). At a dose increase from 3000 to 5000 mg/kg, there was an increase in the number of deaths with the highest mortality in group 6 which received 5000 mg/kg.

Acute clinical signs were observed in the groups of mice as the doses of MO are administered. Table 3 showed Sublethal doses that produced acute effects of clinical significance which were documented such as diarrhea, vomiting, micturition, salivation, sedation, agitation, piloerection, convulsions, spasms, and irritation. Group 6 mice showed almost all the acute signs before their demise. The majority of the mice had Piloerection (73.3%) while the sedation effect is noted in 10 mice (33.3%). Weight gain was observed in 36.6%.

Table 2: Acute toxicity study of MO ethanol extract

Group	Dose (mg/kg)	Number of Death	Mortality %	Dose Difference (Dd)	Mean Death (Md)	Dd × Md
1	500	0	0	500	0	0
2	1000	0	0	1000	0	0
3	2000	0	0	1000	0.5	500
4	3000	1	20	1000	1.5	1500
5	4000	2	40	1000	3.5	3500
6	5000	5	100	-	-	-

Table 3: Clinical signs of toxicity observed in the groups on day 7

	Group/Dose (mg/kg) and Number of Mice affected (N = 30)						
Clinical Signs Observed in the Mice	**Group 1 500 mg/kg n=5**	**Group 2 1000 mg/kg n=5**	**Group 3 2000 mg/kg n=5**	**Group 4 3000 mg/kg n=5**	**Group 5 4000 mg/kg n=5**	**Group 6 5000 mg/kg n=5**	**Total No & % of Mice with Acute Effects**
Diarrhea	0	1	2	2	3	5	13 (43.3)
Vomiting	0	1	1	2	4	4	12 (40.0)
Micturition	1	2	2	3	3	5	16 (53.3)
Salivation	0	1	1	2	3	4	11 (36.6)
Sedation	0	0	0	1	4	5	10 (33.3)
Agitation	1	3	3	4	5	5	21 (70.0)
Piloerection	1	2	4	5	5	5	22 (73.3)
Convulsions	0	0	2	3	5	5	15 (50.0)
Spasms	1	2	2	1	3	4	13 (43.3)
Irritation	1	3	2	3	4	5	18 (60.0)
Weight gain	1	3	4	2	1	0	11 (36.6)

Qualitative/Quantitative analysis test results of the bio-constituents of MO

Major Bio-Nutrients of Moringa Oleifera Leaves

Figure 3 showed the major bio-nutrients of MO leaves of 100gram weight. Carbohydrate is the highest major constituent (36.6%) followed by Dietary fiber (15.92%) while the least is fatty acids which constitute about 9.2% of 100g of MO leaves.

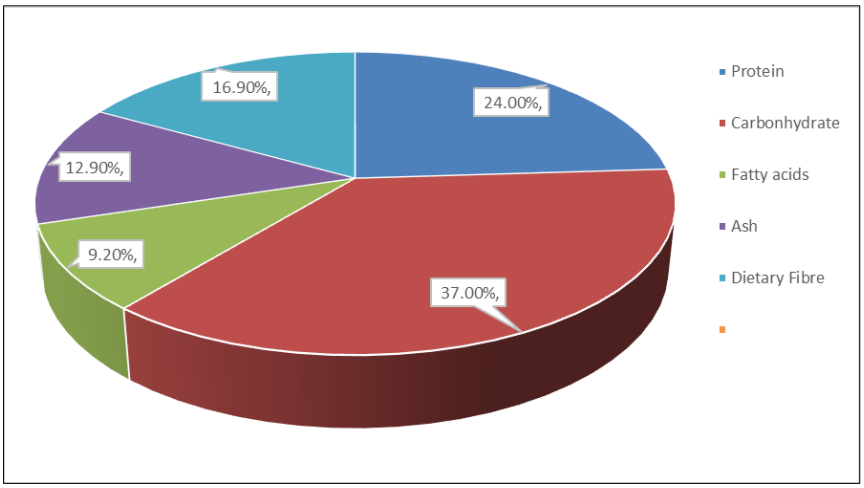

Figure 3: Major Bio-Nutrients found in MO leaves/ 100 grams

Minerals of MO

Figure 4 showed a bar chart of the Mineral constituents of MO leaves in 100 grams weight of the leaves. Calcium showed the highest composition followed by Magnesium while Copper is the least.

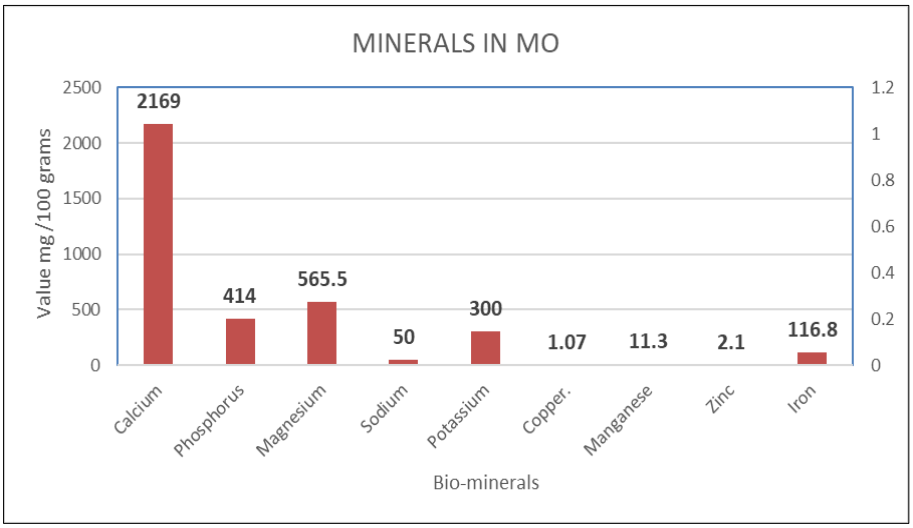

Figure 4: Mineral constituents of *Moringa Oleifera* leaves (mg/100grams)

Phyto-chemicals of MO

Figure 5 showed a horizontal bar chart that presents the Phyto-chemical compounds found in 100 grams of MO leaves alcohol extract. The highest compound present is the Phenol compound, followed by Flavonoid compounds, while the least is the Saponin compound.

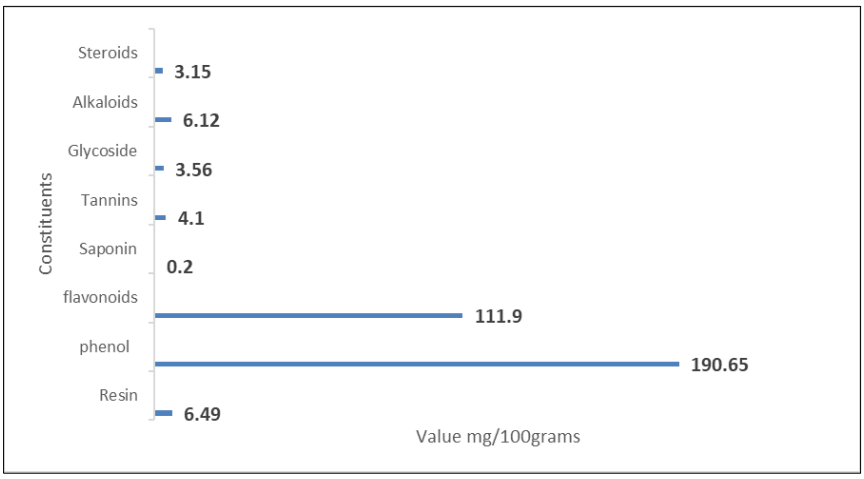

Figure 5: Phyto-chemicals of *Moringa Oleifera* leaves (mg/100g)

Vitamin Composition of MO

Figure 6 depicts a bar chart presenting the vitamin content seen in 100 grams of MO leave alcohol extract. Ascorbic acid (vitamin C) showed the highest while Vitamin B12 is the least present.

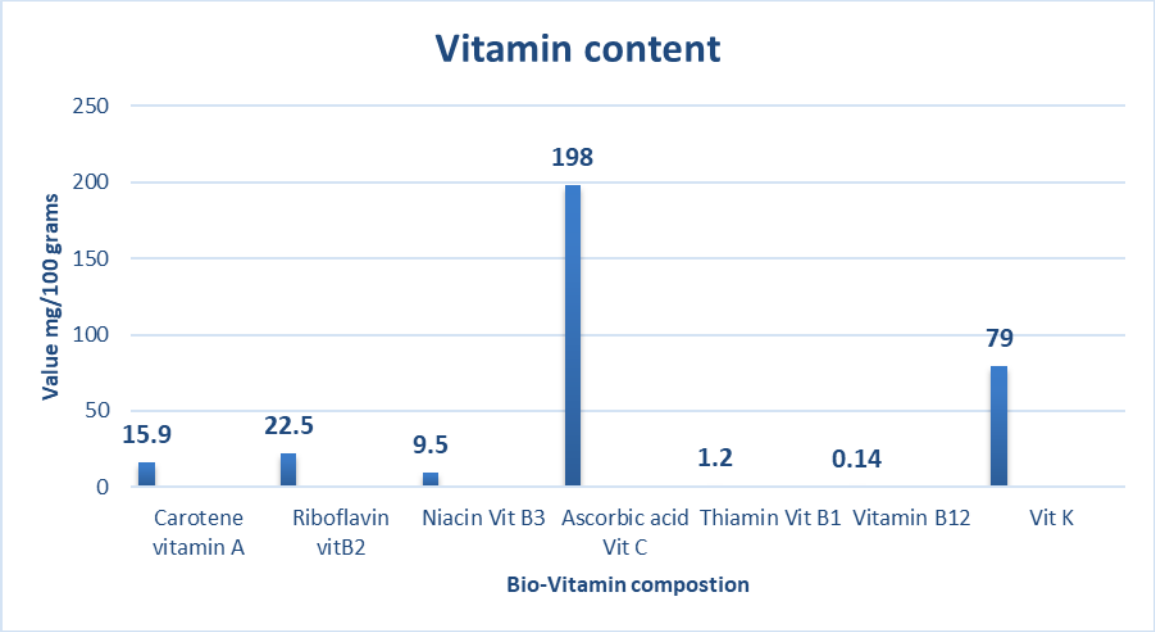

Figure 6: Vitamin content of *Moringa Oleifera* leave mg/100grams

Gas chromatography-mass spectrometry (GCMS) analysis and identification of photo components of Moringa oleifera

Figure 7 showed the result of the GCMS analysis of MO which indicates eleven peaks present as eleven phytochemicals' constituents of MO. In the graph, the intensity of the phytochemicals is plotted against retention time in minutes. The highest peak is recorded at 21.8 minutes with an intensity of 16,697,707. Figure 6 is a Pie Chart showing the 11 peak area percentages of phytochemicals found in MO during GCMS analysis. Methyl Octadecenoate accounted for over 30% of the photo components of MO leaves while Methyl undecanoate (0.57%) recorded the least.

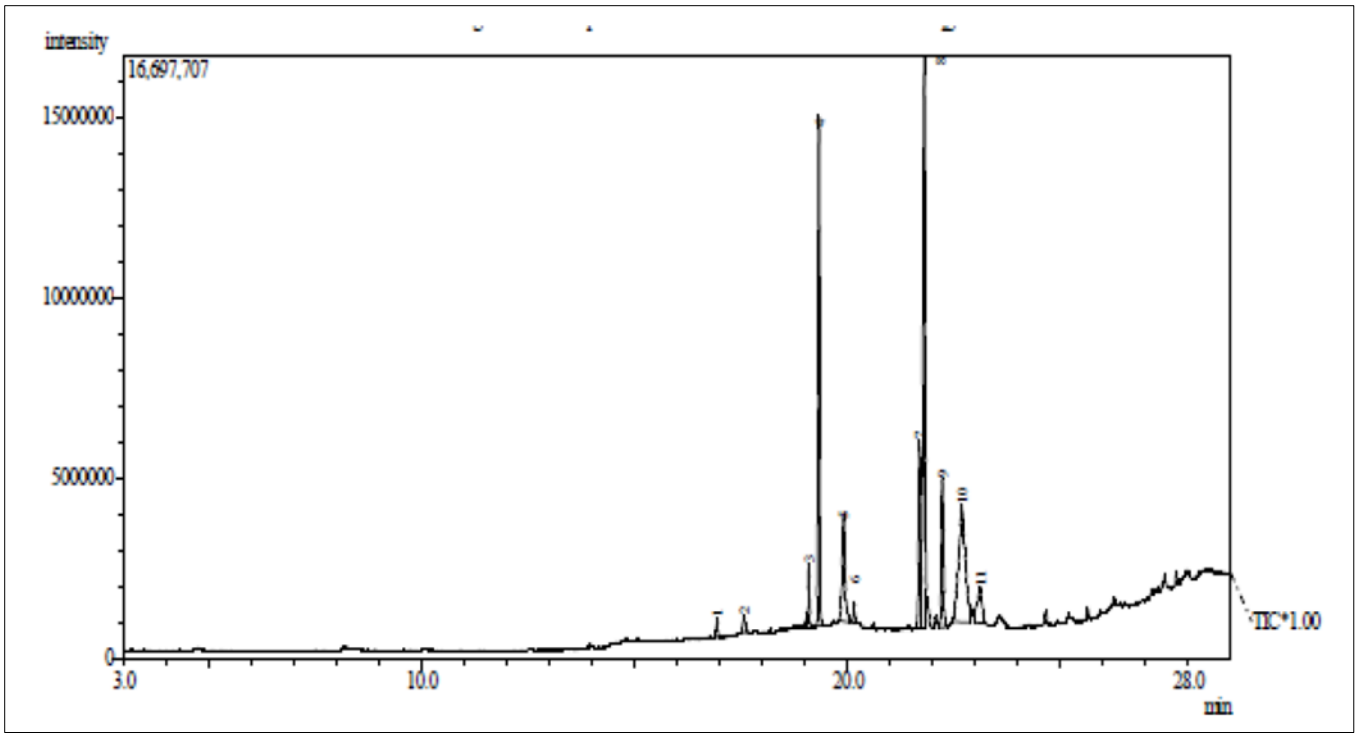

Figure 7: GCMS chromatogram showing 11 peak area percentages of phytochemicals found in MO

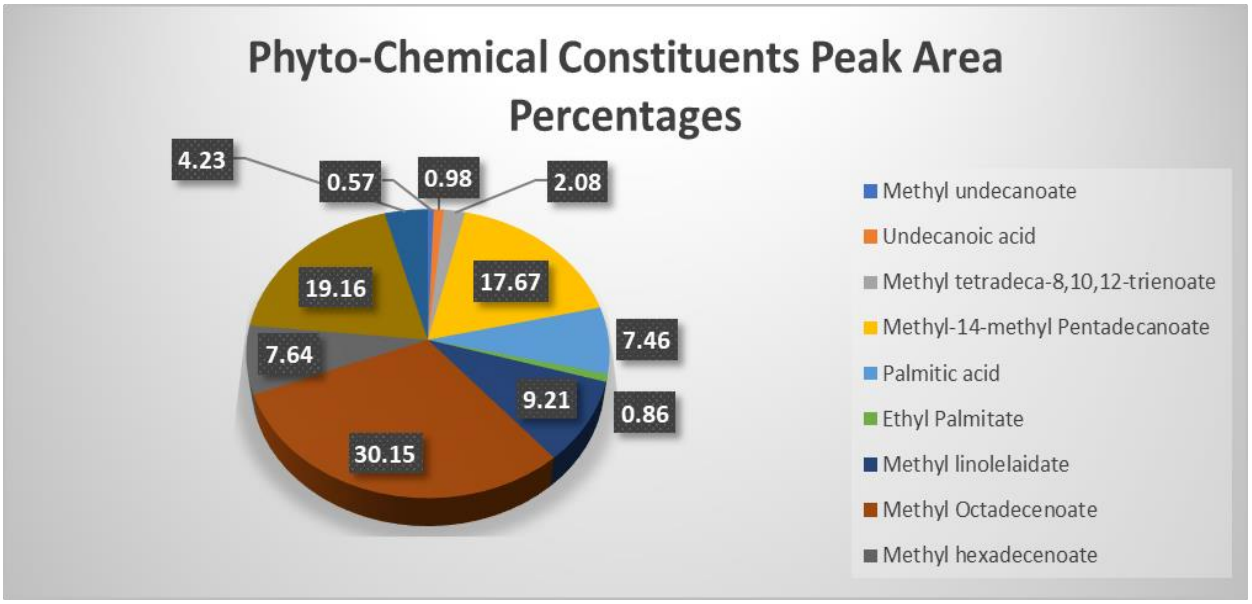

Figure 8: Pie Chart showing 11 peak area percentages of phytochemicals found in MO during GCMS analysis.

DISCUSSION

The acute toxicity study of *Moringa Oleifera* leaves ethanol extract in this study revealed that LD_{50} of 3900 mg/kg produced death in adult mice of 30 – 40 grams body weight. This implies that in a human of 50 kg body weight, the LD_{50} would be 3900 × 50 = 195,000 mg (195 grams). The present study shows that 50 g of MO leaves contained 11.9 grams of extract. This can be extrapolated that a human of 50 kg body weight will need to ingest 195 x 50 / 11.9 = 819.3 grams of MO extract at a time to cause death, keeping the dose-response relationships (Pharmacodynamics) constant. This is considered impossible based on local use. Studies have experimented on doses of MO that are safe for consumption and discovered that a relatively small increase in dose such as 3-4 times the recommended preliminary human doses of 1,600 – 3,600 mg for a 70 – 115 kg person is known to cause genotoxic organ damage mostly liver and kidneys; it may promote cancer formation and induce abortions in pregnant rats [13, 14, 4]. The findings of the present study corroborate the findings of Kasolo et al [15]; Prabhadevi et al. [16] and Zahidul et al [17]. The plausible explanation of these reports is that MO is not harmful when consumed by humans at a reasonable quantity or dose but gives adequate nourishment to the human body. Sublethal doses that produced acute effects of clinical significance were observed in the groups, piloerection was generally observed in the majority of the mice (73.3%) before the mice's demise was recorded in groups 4, 5, and 6. The majority of the mice had piloerections (73.3%). Weight gain was recorded in 11 (36.6%) mice with the highest seen in group 3 of 2000 mg/kg. Mortality was observed in the high-dose groups representing a decline in weight gain in those groups. This implies that the MO in addition to the feeds and water may be responsible for the weight gain which declined with the increase in dose. The study corroborates with that of Kim et al [37].

Phytochemical qualitative analysis of MO in this present study consisted of major bio-nutrients which include protein, carbohydrate, fatty acids, ash, and dietary fiber, where carbohydrate has the highest percentage (37%) followed by protein (24%). This present study corroborates with other studies on the major bio-nutrients of MO leaves. The high protein content of MO leaves has shown to be very effective in curbing malnutrition and other related protein deficiency diseases in humans [18]. Protein is an essential macro-nutrient responsible for the overall growth of the human body, while the amino acid present in protein is the building block unit of life [18]. In the present study gas chromatography-mass spectrometer (GCMS) on *moringa oleifera* (MO) revealed four bioactive compounds which are compounds found to be effective against cardiovascular disease [19]. These compounds namely methyl tetradeca-8, 10, 12- tridentate, methyl linolelaidate, methyl (11E)-11-octadecenoate and cis-octadecenoic acid were discovered amongst 11 compounds found in MO. This may suggest the reasons why MO leaves are so much consumed in our locality as an antihypertensive phytotherapy alternative to conventional antihypertensive drugs such as Perindopril [20]. Methyl linolelaidate present is a compound that is found to be protective against metabolic syndrome such as anti-diabetics [21, 22]. Stearic acid is also present which is found to be an anti-inflammatory, anti-androgenic, anti-spermatogenic, and cancer-protective compound [23, 24, 25]. The palmitic acid content of MO leaves also enables it to be used in anti-atherosclerotic activities [21]. Bio-organic constituent of MO leaves such as resins, tannins, saponins, flavonoids, alkaloids, steroids, phenols, and glycosides were also present. These are found to be very high in antioxidant activity more than raw coffee [26, 36] Minerals and Vitamins were also found in MO. Ascorbic acid (Vitamin C) was found to have the highest composition. Studies showed that MO leaves constitute about 10 times more vitamins than that seen in carrots, 7 times more vitamin C than that in oranges, and more than 17 times higher in calcium than that seen in dietary milk also, and 15 times more potassium than that seen in bananas [27].

Moringa Oleifera dried leaves consumption in the tropical region of Nigeria is very high in recent times, they are consumed as vegetables, tea, and medicinal alternative for the prevention and treatment of HHD diseases and cardiac remodeling. This is corroborated by recent studies which reported the efficacy of consumption of medicinal herbal plants in their crude state such as MO for reduction of HHD, food supplements, and health management [28, 29, 30, 31].

CONCLUSION

Moringa oleifera leaves contain a very high source of macronutrients, phytochemicals such as phenol, minerals, and vitamins, which have been proven to promote health, lower blood pressure, prevent cancer cell formation due to their high anti-oxidants, and maintain good metabolic activities when consumed at the right dose or quantity. Malnutrition and other related diseases may be alleviated by the consumption of MO dried leaves as a food supplement since it has a low genotoxic effect when an appropriate dose or quantity is taken.

Data Availability

The authors declare the availability of data for the study.

Conflicts of Interest

The authors declare that there are no conflicting interests.

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