

The Journal of Phytopharmacology

(Pharmacognosy and phytomedicine Research)

Research Article

ISSN 2320-480X
JPHYTO 2019; 8(5): 210-215
September- October
Received: 30-07-2019
Accepted: 11-10-2019
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doi: 10.31254/phyto.2019.8501

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Phytochemical and Neurotoxicity Evaluation of Methanolic Leaf-Extract of *Moringa oleifera*

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ABSTRACT

Objective: As a plant with a host of medicinal properties used for treating various ailments, the research set out to investigate the neurotoxic effect of methanol leaf-extract of *Moringa oleifera*, as well as explore its phytochemical constituents. The amphiphilic property of methanol as a solvent was exploited to extract non-polar and medium polar phytoconstituents. **Methods:** Forty (40) male Swiss white mice were randomly grouped into four (n=10 per group). The control animals receive normal saline (p.o.) while two other groups received low dose (500mg/kg) and high dose (2500mg/kg) of the leaf-extract of *M. oleifera* for 21 days. The forth group received a low dose (500mg/kg) of the extract thirty minutes before the animals were sacrificed for histological studies. **Results:** The phytochemical screening and quantitative analysis showed that the methanol leaf-extract of *M. oleifera* contains alkaloids (1.80±0.2%), glycosides (1.37±0.1%), saponins (1.47±0.3%), tannins (0.48±0.01%), flavonoids (8.23±0.2%), polyphenols (20.47±0.3%) and reducing compounds (7.05±1.0%). Acute toxicity evaluation using Lorke's method showed the LD₅₀ to be 5,477.226mg/kg. Histopathological evaluation shows no toxicity at 2500mg/kg in the hippocampus, amygdala, cerebral cortex and the cerebellum. **Conclusion:** The methanol leaf-extract of *M. oleifera* has no neurotoxic effect even at high doses; and so, it very safe for use in the treatment regimes.

Keywords: neurotoxicity, methanolic leaf-extract, *Moringa oleifera*.

INTRODUCTION

There are so many therapeutic properties attributed to this medicinal plant, so called 'miracle tress' that not having even a passive interest in the properties of this plant is inconceivable. The generic name of *M. oleifera* comes from the Sinhalese name '*Morunga*'. In English the plant is called *Moringa* tree, ben-oil tree, drumstick, and West Indian ben. It is called '*zogallagandi*' in Hausa and '*ewe-igbale*' in Yoruba [1]. Its value lies in its parts: the fruits, roots, leaves and seed. This plant is used extensively in ethno-medicine [2].

Traditionally *M. oleifera* is used to stimulate production of phlegm, to control spasms and as a diuretic. Its freshy root has a strong unpleasant smell and vesicant (causes blisters). Its gum is moist and sticky (mucilaginous). Its bark is abortifacient. It also has antibacterial and antifungal properties. Its flowers are taken to 'lift the spirit' (tonic). It is also a diuretic, an antiseptic, and is often used as a cardiac circulatory tonic [3]. Its pods are used in the treatment of fever. The root is antiparalytic. *M. oleifera* is used in treatment and management of ailments which include. It is due to these numerous therapeutic uses of all the parts of this *M. oleifera* that earned it the name 'Miracle tree'.

It is interesting to note that many of the claims about the properties of this medicinal plant have scientific experimental backing. Many research works have been carried out to ascertain the various medicinal properties of *M. oleifera*. It was reported that *M. oleifera* could help control grand mal and petit mal epilepsy [4]. It was also reported that it has analgesic property [5]. Yet another research work reported that it reduced urine sugar as well as the level of protein in the urine [6] which justifies the ethno-medical use of *M. oleifera* in diabetes management. It was also found to decrease the intensity of neuropathic pain associated with diabetes by reducing oxidative stress [7].

M. oleifera was also found to possess hepatoprotective as well as anti-inflammatory and antioxidant property [8,9]. The extract could also enhance male sexual desire and performance [10]. *M. oleifera* fruit is rich in phenols, strong reducing power and antioxidant property [11]. In another research, *M. oleifera* possesses anti-ulcer potential [12]. *M. oleifera* also protects against Streptozotocin induced diabetes and

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carbon tetrachloride induced hepatotoxicity [13, 14].

A knowledge of the phytoconstituents of the leaves and their percentage composition as well as their neurobehavioural effect and effect on brain cytology will add to the existing body of knowledge about the plant, and guide its use.

The choice of methanol as an extractant was informed by the fact that methanol is amphiphilic which makes it more suitable for extracting non-polar as well as medium polar phytoconstituents. This is an advantage over ethanol and water [15].

MATERIALS AND METHOD

Experimental animals

Inbred Swiss white mice were bought from the animal house of the Department of Physiology. The mice were kept at room temperature (26°C) and under a 12/12 light/dark cycle. The mice were allowed to acclimatize for a week before extract administration and testing. Ethical approval was duly obtained for the use of these animals for the experiments from the Animal Ethics Committee of the Faculty of Basic Medical Sciences University of Calabar with approval no. 017PY20215.

Preparation of extract

We gathered fresh *Moringa oleifera* leaves from Calabar South LGA of Cross River state and a sample was identified at the Herbarium, Botany Department, University of Calabar. The deposited specimen was given Herbarium number 76. The leaves were thoroughly washed, sun-dried and blended to fine particles. The powdered leaves were then immersed in methanol for 48 hours following which the mixture was filtered using Whatman filter paper No.1. We evaporated the extract at room temperature and the pasty concentrate was stored in a refrigerator until when required for reconstitution and administration.

Study design

Eighty (80) Swiss white mice were randomly grouped into four (20 mice in each group). The control animals received normal saline (p.o.). The other three (3) groups are the test groups. Two (2) of these receive low dose (500mg/kg) and high dose (2500mg/kg) of the leaf-extract of *M. oleifera* for 21 days. The third group received a low dose (500mg/kg) of the extract thirty minutes before analysis.

Phytochemical screening (Qualitative analysis)

The phytochemical screening was done using the methods of Sofowora [16], Culieli [17], Trease & Evans [18] and Harbone [19].

Quantitative analysis

Determination of tannin content

This was done using the Folin Denis colorimetric method as described by Kirk & Sawyer [20]. About 5g of the extract was mixed in distilled water in the ratio of 1:10 (w/v), shaken for about 30min at room temperature and filtered.

Volumes of 2ml of tannic acid solution and 2ml of distilled water were dispensed into separate 50ml volumetric flasks to serve as standard and reagent blank respectively. Then 2ml of extracts was put in the respective labelled flask, and mixed with 35ml distilled water and 1ml of the Folin Denis reagent and then 2.5ml of saturated Na₂CO₃ solution added. Each flask's was diluted to 50ml mark using distilled water and then incubated for 90min at room temperature. The optical absorbance was measured at 760nm in a spectrophotometer with the reagent blank

at zero. The tannin content was calculated as shown below;

$$\% \text{ Tannin} = \frac{100}{w} \times \frac{au}{as} \times C \times \frac{Vt}{Va}$$

w = weight of sample

au = absorbance of test sample

as = absorbance of standard tannin solution

C = concentration of standard tannin solution

Vt = total volume of the extract

Va = volume of extract analysed

Determination of saponin content

The double solvent extraction gravimetric method as described by Harborne [19] was used. Five grams of extract was added to 50ml of 20% ethanol and incubated for 12hrs at 55°C and agitated constantly. Whatmann's filter paper 42 was then used to filter the mixture and the residue re-extracted with 50ml of the ethanol solution for 30min. Evaporating the extract reduced it to about 40ml. It was transferred to a separating funnel and an equal volume of diethyl ether added and mixed to form a partition. After discarding the other layers, the aqueous layer was re-extracted with ether and then its pH was reduced to 4.5, by dropping NaOH solution.

The saponin component of the extract was taken up in successive extraction with 60ml and 30ml portion of named butanol. The precipitate was washed with 5% NaCl solution and evaporated to dryness in a previously weighed evaporation dish. The saponin fraction was then oven-dried at 60°C (to remove any residual solvent), cooled in a desiccator and reweighed. The saponin content was calculated as follows;

$$\% \text{ Saponin} = \frac{W_2 - W_1}{W}$$

W = weight of sample used

W₁ = weight of empty evaporation dish

W₂ = weight of dish + saponin extract

Determination of alkaloid

The method used as described by Harborne [19] was the alkaline precipitation gravimetric method which determined the alkaloid content of the extract. The extract (5g) was added to 100ml of 10% acetic acid in ethanol solution, shaken vigorously and then allowed to incubate for 4 hours at room temperature while shaking at intervals of 30min. The mixture was filtered using Whatmann's filter paper 42. The filtrate was concentrated by evaporation to a quarter of its original volume. Alkaloid was precipitated by drop-wise addition of concentrated ammonia solution. This dilution was done till ammonia was in excess. The alkaloid precipitate was removed by filtration using Whatmann's filter paper 42. The precipitate in the filter was then washed with 1% ammonium hydroxide solution, dried at 60°C and weighed after cooling in a desiccator. The alkaloid content was calculated thus;

$$\% \text{ Alkaloid} = \frac{W_2 - W_1}{\text{Wt of sample}} \times \frac{100}{1}$$

W₁ = weight of empty filter paper

W₂ = weight of filter paper + alkaloid precipitate

Polyphenol content determination

Folin-ciocalteu spectrophotometric method of Ekwenye & Okorie [21] was used to determine the polyphenol content of the extract. One gram of the sample was extracted in 10ml of pure methanol and filtered with Whatmann's filter paper 1 and 1.0ml of the filtrate was then mixed with equal volume of folin-ciocalteu reagent in a test tube, followed by addition of 1.0ml of standard solution which was also treated in the same way. Thereafter, 1ml of sodium bicarbonate was added to both tubes. Absorbance of both mixtures was read and their respective content was calculated as

$$\% \text{ Polyphenols} = \frac{AE \times C \times VE}{AP \times WS} \times \frac{100}{1}$$

Determination of glycosides

This was done using the method of Horwitz & Latimer [22]. About 1g of the extract was dissolved in 200ml of distilled water contained in a 250ml flask and allowed to stand for 2 hours. A volume of 150-170ml of distillate was obtained in a 250ml conical flask containing 20ml of 2.5% NaOH. An anti-foaming agent (tanic acid) was added before distillation. Then 100ml of the distillate was measured into a fresh 200ml flask and 8.0ml of 6N NH₄OH and 2.0ml of 5% KI added, mixed and titrated with 0.02N AgNO₃ using a micro burette against a black background. Permanent turbidity indicated end points. The process was repeated and the average titre volume calculated. Glycoside content of the sample was then calculated using the formula;

$$\text{Glycosides (mg/g)} = \frac{\text{Titre volume (ml)} \times 1.08 \text{ (g)}}{\text{weight of sample (g)}}$$

Determination of flavonoid content

This was done using the method of Harborne [19]. Five grams of extract was heated in 100ml of 2M HCl solution under reflux for 40 minutes. The extract mixture was cooled before filtration. The filtered extract was mixed with equal volumes of ethyl acetate and the moisture and transferred to a separation funnel. The flavonoid extract in the ethyl acetate portion was separated by filtration. The weight was obtained after drying in the oven and cooling in a desiccator. The weight was expressed as a percentage of the weight analyzed. It was calculated as shown below;

$$\% \text{ Flavonoid} = \frac{W_2 - W_1}{\text{wt of sample}} \times \frac{100}{1}$$

W₂ = weight of filter paper × flavonoid precipitate

W₁ = weight of filter paper alone

Determination of reducing compounds

This was carried out using Benedict's quantitative test as described by Horwitz & Latimer [22]. About 10ml of plant extract was distilled in 90ml distilled water. This solution was then transferred to a burette and titrated against 20ml of standard Benedict's reagent in a 100ml conical flask placed on electric hot plate with anti-bump chips placed inside the conical flask. Titration was continued till the blue colour of the reagent changed and the end point was recorded. The process was repeated three times and the average volume of titre calculated. Result obtained was then computed against that of a glucose standard and using the formula;

$$\frac{18.9\text{mg standard} \times \text{average volume of titre}}{10\text{ml Benedict's reagent}}$$

Acute toxicity evaluation (LD₅₀)

Acute toxicity is the unwanted effect(s) that occurs within twenty-four hours of consuming a substance [23]. The LD₅₀ was done using a modification of Lorke's method [24]. Animals are given different doses

of the extract and observed for 24 hours. Then the LD₅₀ is calculated using the formula;

$$LD_{50} = \sqrt{(D_0 \times D_{100})}$$

Where; D₀ = Highest dose that gave no mortality,

D₁₀₀ = Lowest dose that produced mortality.

Extract administration

The test groups of mice received 500mg/kg and 2500mg/kg of the extract respectively (orally) for 21 days. Another group received acutely (30 minutes before the animals were sacrificed) a low dose (500mg/kg) of the extract.

Statistical analysis

The results of the study were expressed as mean ± standard error of means (SEM). One-way Analysis of variance (ANOVA) and the post-hoc Newmann Keul's test were the statistical tools used. The computer softwares Microsoft excel 2013 edition and SPSS 16.0 for windows were also used to aid in the analysis. Differences in means was considered significant at P ≤ 0.05.

RESULTS

Phytochemical analysis of methanolic extract of *M. oleifera*

The result of phytochemical screening (Table 1) shows that the methanolic extract of *M. oleifera* contains alkaloids, anthraquinones, glycosides, saponins, tannins, flavonoids, reducing compounds and polyphenols. Phlobatanins, and hydroxymethyl anthraquinones were also tested for but were not present.

Table 1: Phytochemical screening of methanolic extract of *M. oleifera*.

S/N	Chemical Constituent	Content
1	Alkaloids	++
2	Glycosides	+
3	Saponins	++
4	Tannins	+
5	Flavonoids	++
6	Reducing compounds	++
7	Polyphenol	++
8	Phlobatanins	-
9	Anthraquinones	-
10	Hydroxymethyl anthraquinone	-

KEY: + present. ++ present in excess. - absent.

Table 2: Percentage of crude alkaloids, glycosides, saponins, flavonoids, polyphenol and reducing compounds of methanolic extract of *M. oleifera*.

Name of Sample	Quantity
Alkaloids (%)	1.80 ± 0.2
Glycosides (%)	1.37 ± 0.1
Saponins (%)	1.47 ± 0.3
Tannins (%)	0.48 ± 0.01
Flavonoids (%)	8.23 ± 0.2
Polyphenol (%)	20.47 ± 0.3
Reducing compounds.	7.05 ± 1

Each value represents the mean of 3 determinations ± SD.

Toxicity evaluation of methanolic extract of *M. oleifera*

During phase 1 of the toxicity test, at the doses 500mg/kg, 1500mg/kg and 4500mg/kg, no deaths were recorded. In the second phase, no deaths were also recorded for the dose 5000mg/kg. Two deaths (out of 3 animals) were recorded for each of the 6000mg/kg and 7000mg/kg doses, and 3 deaths (out of 3 animals) for the 8000mg/kg dose. The LD₅₀ was calculated as shown below.

$$LD_{50} = \sqrt{(D_0 \times D_{100})}$$

Where; D₀ = Highest dose that gave no mortality,

D₁₀₀ = Lowest dose that produced mortality.

$$\begin{aligned} LD_{50} &= \sqrt{5000 \times 6000} \\ &= \sqrt{30,000,000} \\ &= 5,477.226mg/kg \end{aligned}$$

Photomicrographs showing sections of the hippocampus in the test groups and control

The micrographs in Figure 1 show sections of the hippocampus. In the control slide (C) the superficial molecular, intermediate pyramidal and inner polymorphic cell layers are seen intact. The molecular layer consists of bundles of nerve fibres and scanty population of small neurons. The pyramidal cell layer consists of compactly packed medium to large pyramidal shaped neurons and interspersed neuroglia cells and the inner polymorphic consists of scanty populated fusiform neuronal cell bodies and modified pyramidal cells with triangular or ovoid cell bodies. The morphological integrity as seen in the slide for the low dose group (LD) showed no abnormality. No abnormalities were seen either in the high dose group (HD) and the acute low dose group (ALD).

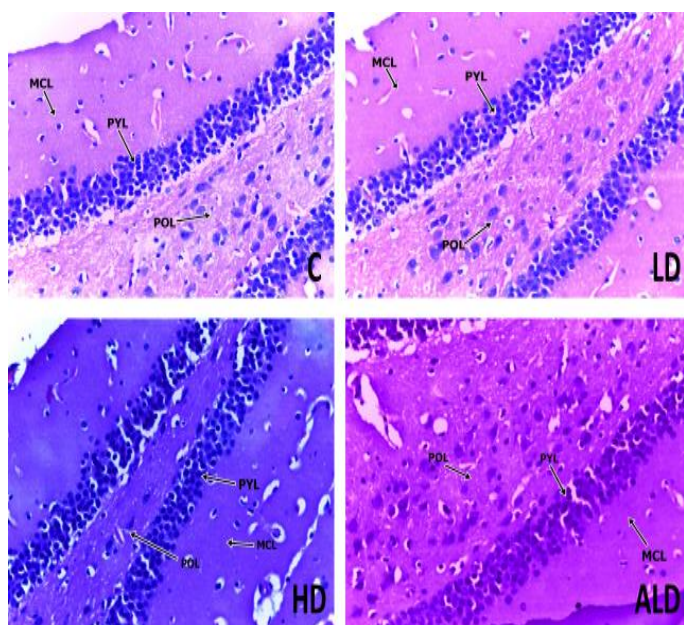


Figure 1: Histological Sections of the Hippocampus in mice fed with low (500mg/kg), high (2500mg/kg) and acute low (500mg/kg) doses of methanolic extract of *M. oleifera* and control (X400). C = Control, LD = Low Dose, HD = High Dose, ALD = Acute Low Dose.

Photomicrograph showing sections of the amygdala in test groups and control.

Sections of the amygdala in the control (C) show meshwork of cell

neuron and glial processes. The neurons have prominent ova to ovoid shaped nuclei with distinct nucleoli and evenly distributed Nissl substances. The glia cells are more numerous than neurons and have smaller nuclei. The glia cells have deeply stained neurons. In the test groups (LD, HD & ALD) no neuronal damage or reactive gliosis were seen. In the acute low dose group, we found moderate eosinophilic cytoplasm and distinct nucleoli with normal mitotic figures of greater than 3 per 10 high power field (Figure 2).

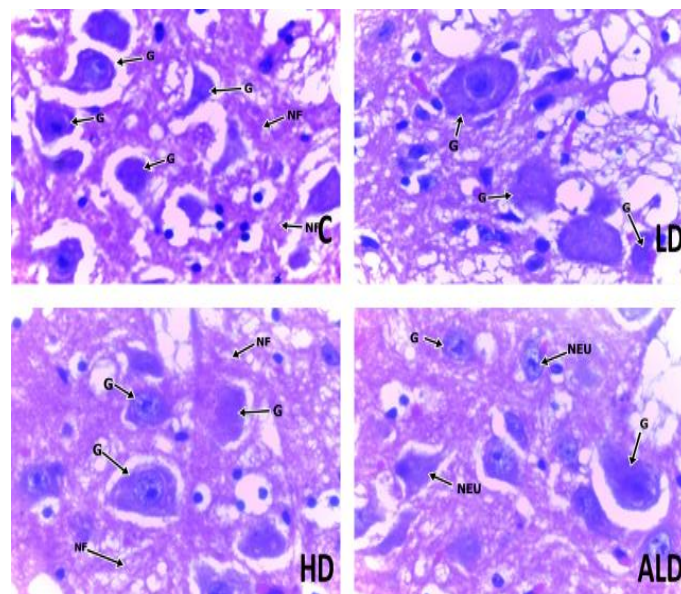


Figure 2: Sections of the amygdala in mice treated with low (500mg/kg), high (2500mg/kg) and acute low (500mg/kg) doses of methanolic extract of *M. oleifera* and control (X1000). C = Control, LD = Low Dose, HD = High Dose, ALD = Acute Low Dose.

Photomicrograph showing sections of the cerebral cortex in test groups and control.

Section of the cerebral cortex showing an intact layer consisting of the molecular cell layer, external granular, external pyramidal, internal granular, internal pyramidal and multiform layer. The nuclei chromatin are evenly distributed with normal mitotic figures in the low dose group. No abnormality is seen in the low dose group (LD) or any other test group (HD & ALD) as shown in Figure 3.

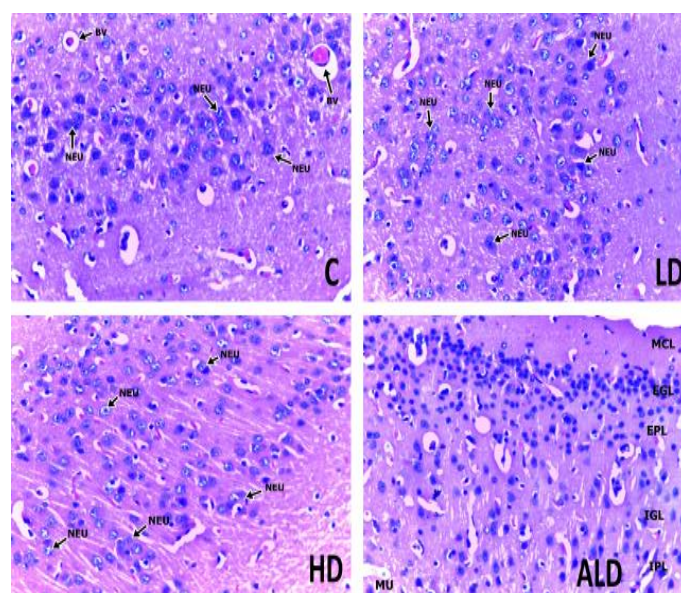


Figure 3: Sections of the cerebral cortex in mice treated with low (500mg/kg), high (2500mg/kg) and acute low (500mg/kg) doses of methanolic extract of *M. oleifera*. (X400). C = Control, LD = Low Dose, HD = High Dose, ALD = Acute Low Dose.

Photomicrograph showing sections of the cerebellum in test groups and control.

Section of the cerebellar cortex shows an outer molecular cell layer, intermediate Purkinje cell layer and innermost granular cell layer. The molecular cell layer consists of scanty population of neuronal cell bodies and abundant nerve processes and scattered neuroglia cells. The intermediate Purkinje cell, consist of a single layer of flask shaped Purkinje cell and the innermost granular layer is made up of closely packed small cells with densely stained nuclei and scanty cytoplasm and scattered neuroglia cells. In the low dose group, the neuronal cell bodies are prominent and those in the granular region are densely stained. The cell processes are prominent. However, no degenerative change or neuronal damage is seen. The same trend persists in the high dose (HD) and acute low dose (ALD) groups (Figure 4).

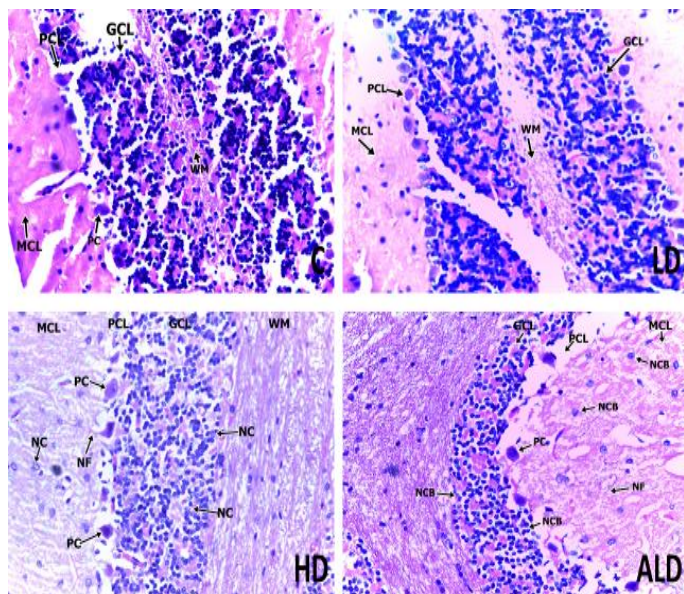


Figure 4: Sections of the cerebellum in mice treated with low (500mg/kg), high (2500mg/kg) and acute low (500mg/kg) dose of methanolic extract of *M. oleifera*. (X400). C = Control, LD = Low Dose, HD = High Dose, ALD = Acute Low Dose.

DISCUSSION

Phytochemical analysis

The result of phytochemical screening (Table 1) shows that the methanolic extract of *M. oleifera* contains alkaloids, anthraquinones, glycosides, saponins, tannins, flavonoids, reducing compounds and polyphenols. Phlobatanins, and hydroxymethyl anthraquinones were also tested for but were not present. The result of phytochemical screening in this study was similar to that of other researchers who did phytochemical screening using ethanolic and aqueous extract [25, 26]. However, this result varies with others in certain aspects. Whereas the ethanolic and aqueous extracts were found to contain hydroxymethylanthraquinone and anthraquinones [27, 25], the methanolic extract obtained in this study did not.

The result of quantitative analysis (Table 2) showed that *M. oleifera* contains alkaloids (1.80±0.2%), glycosides (1.37±0.1%), saponins (1.47±0.3%), tannins (0.48±0.01%), flavonoids (8.23±0.2%), polyphenols (20.47±0.3%) and reducing compounds (7.05±1.0%). The quantity of flavonoids in this assay is considerably larger compared to that done using ethanolic extract by Nweze & Nwafor [25]. They found flavonoid content to be 3.56±0.03% while saponin content in their study was approximately equal to the quantity in this study (1.46±0.03%). They also did quantitative analysis using aqueous extract of *M. oleifera*. They had a higher tannin content (9.36±0.04%) in the aqueous extract compared to that found in the methanolic extract (0.48±0.01%). Alkaloid content was also higher in their aqueous extract

(3.07±0.0%) compared to that in this study (1.80±0.2). Apparently, methanol is the best solvent to extract flavonoid content of *M. oleifera* compared to water and ethanol. However, it is not suitable for optimal extraction of tannin and alkaloid content of *M. oleifera*. In decreasing order of magnitude, the methanolic extract of *M. oleifera* contains polyphenols, flavonoids and reducing sugars, alkaloids, saponins, glycosides and tannins.

Toxicity evaluation

In this research work, acute toxicity evaluation using Lorke’s method [24] showed the LD₅₀ to be 5,477.226mg/kg. No mortality was recorded even at 5000mg/kg (p.o.) of the methanolic extract of *M. oleifera*. When compared to the toxicity evaluation using aqueous extract [28] it is observed the mice seem to be more tolerant to the methanolic extract than the aqueous extract. In the said study, mortality was recorded at 1600mg/kg and 2000mg/kg. It is not clear why this is so. However, one may postulate that the difference in phytoconstituents extraction between methanol and water results in an extract composition which renders the aqueous extract more toxic than the methanolic extract. But this is just conjecture. Adedapo *et al.*, [28] also stated that above 2000mg/kg the animals may show some toxicity.

Histology

Histopathological evaluation shows no toxicity at 2500mg/kg in the hippocampus, amygdala, cerebral cortex and the cerebellum. In the hippocampus, the outer molecular, intermediate pyramidal and polymorphic cell layer were intact. There was no pathological change in the morphology of these layers neither in the LD or HD nor in the group that was given an acute low dose of the extract. The trend persists in the histology of the amygdala. The slides show a meshwork of neurons and glial processes with evenly distributed Nissl bodies within the meshwork. No neuronal damage was observed in the groups treated with the extract compared to control. In the cerebral cortex, the six layers were clearly visible in all test groups and control. No abnormality was seen in the slides. The same thing was observed for the histology of the Cerebellum. There was no abnormality in the three layers of the cerebellum. The result of the histological analysis shows that the extract (at 2500mg/kg) has no histologically observable damaging effect on the Hippocampus, Amygdala, Cerebral cortex and the Cerebellum. A similar histological study of the cerebral cortex, hippocampus and cerebellum using the ethanolic extract of *M. oleifera* on rats yielded a similar result [29]. The ethanolic extract of *M. oleifera* had no histologically observable deleterious effect on the brain sections studied. The same result was observed in this study using the methanolic extract of *M. oleifera*.

CONCLUSION

The methanolic extract of *M. oleifera* contains polyphenols, flavonoids, reducing compounds, alkaloids, saponins, glycosides, and tannin (in decreasing order of abundance). Methanol (compared to water and ethanol) is the best solvent for extracting flavonoid content of *M. oleifera*. The LD₅₀ for methanolic extract of *M. oleifera* was obtained as 5,477.226 mg/kg. The extract has no deleterious effects on the histology of the hippocampus, cerebral cortex, amygdala and the cerebellum up to the 2500 mg/kg dose and therefore is not neurotoxic.

Acknowledgement

We, all the authors, here acknowledge the Departments of Physiology and Anatomy University of Calabar, Nigeria for providing the conducive environment within which this research was conducted.

Conflict of interest:

We, all the authors, here declare that there is absolutely no conflict of interest. We also declare that we did not receive funding from any source for this research. All funds used for this research were purely from personal funds committed to this course.

Author contributions

Sunday Agba Bisong conceived the research idea, designed the research and made the final copy of the research article. Imeiba Oluga Ajiwhen conducted most of the laboratory work and made the first draft copy of the research article. Charles Cyprian Mfem proof-read the research article and did some aspect of the research analysis while Clement Oshie Nku conducted the statistical analysis and interpreted the results. Kelechi Chinkata Uruakpa conducted the histological studies and interpreted the figures (photomicrographs).

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HOW TO CITE THIS ARTICLE

Bisong SA, Ajiwhen IO, Nku CO, Uruakpa KC. Phytochemical and Neurotoxicity Evaluation of Methanolic Leaf-Extract of *Moringa oleifera*. *J Phytopharmacol* 2019; 8(5):210-215.