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Moringa oleifera lam. Leaf Extract Preserves Spatial Memory and Hippocampal Microstructure in Aluminium Chloride-Induced Neurotoxicity in Adult Wistar Rats

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ABSTRACT

Aluminium is a widely available and subtly consumed metal, whose actions on human health include neurotoxicity and cognitive decline, among others. It is important to protect against these aluminium effects through antioxidants, as aluminium utilizes the oxidative pathway to exert its effect. Due to the abundance of antioxidants in *Moringa oleifera (M. oleifera*), this study investigated its neuroprotective potential against aluminium chloride ($AlCl_3$)-induced hippocampal intoxication in Wistar rats. The phytochemical screening and toxicity (LD_{50}) of *M. oleifera* leaf ethanol extract (MO) were evaluated. Thirty adult male Wistar rats (150–220 g) were then assigned into six groups (n = 5): control, $AlCl_3$ (100 mg/kg), *M. oleifera* low dose (MOLD, 250 mg/kg), *M. oleifera* high dose (MOHD, 1,000 mg/kg), concomitant $AlCl_3$ + MOLD, and $AlCl_3$ + MOHD. These administrations were oral and lasted for 21 days. On day 22, spontaneous alternation behaviour (SAB) was tested in the T-maze, the animals sacrificed, and the brains processed for histology and immunoreactivity. The phytochemicals of MO included flavonoids, phenols, alkaloids, saponins, and tannins as the major constituents, while its LD_{50} was greater than 5,000 mg/kg. There was significantly ($p < 0.05$) less SAB, hippocampal cornu ammonis (CA) 3 chromatolysis, and decreased neuron-specific enolase (NSE) and glial fibrillary acidic protein (GFAP) expressions in the $AlCl_3$ group. Concomitant treatment with MOLD and MOHD did not significantly improve SAB, and NSE and GFAP expressions, but preserved Nissl distribution. MO protected the hippocampus against $AlCl_3$ intoxication by improving SAB, and modulating Nissl distribution, NSE, and GFAP expressions, which supports its antioxidant potential.

Keywords

Aluminium, Hippocampus, Histology, Immunohistochemistry, Moringa oleifera

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INTRODUCTION

Aluminium is ubiquitous and a known neurotoxic metal with functional and structural neuronal deficits (Taïr *et al*., 2016; Ekong *et al*., 2017; Wang *et al*., 2018). It induces oxidative stress, which is associated with alterations in diverse brain areas, irrespective of the individuals' age or species (Yuan *et al*., 2012; Fulgenzi *et al*., 2014). The hippocampus is particularly susceptible to the effects of aluminium (Çabuş *et al*., 2015; Kuznetsova *et al*., 2017). Continuous exposure to this metal has been reported in diseased organs, including the human brain, as their levels in live and autopsied brains have shown (Exley and Vickers, 2014; Akpanyung *et al*., 2018; Adighije *et al*., 2020; Krupińska, 2020).

Aluminium is easily and unconsciously ingested due to its presence in medicines, drinking water, and many manufactured foods and utensils (Krupińska, 2020; McFarland *et al*., 2020; Bryliński *et al*., 2023). It has also been indicted in the aetiology of Alzheimer's disease (Exley and Vickers, 2014; Virk and Eslick, 2015), although this is still controversial (Virk and Eslick, 2015; Krupińska, 2020). The consumption of aluminium is subtle, and the risk to humans cannot be overemphasised (Kuznetsova *et al*., 2017;

Wang *et al*., 2018), there is a need to intentionally manage its actions in the body, especially in the brain. One possible action is its chelation out from the body using continuous supplies of antioxidant-rich and free radical scavenging agents, which are replete in plants and plant-based extracts such as *Moringa oleifera* Lam (Flora and Pachauri, 2011; Ekong *et al*., 2017; Ademiluyi *et al*., 2018).

Moringa oleifera Lam. (*M. oleifera*) is a plant of the family Moringaceae, and is of particular interest in the present study due to its abundance in Nigeria and other African countries and the reported medicinal, nutritive, and antioxidant properties (Flora and Pachauri, 2011; Lopez-Teros *et al*., 2017; Vergara-Jimenez *et al*., 2017; Ademiluyi *et al*., 2018). This plant has been reported to have several health and protective benefits, making it of high economic value (Villarruel-López *et al*., 2018; Zhou *et al*., 2018). Several antioxidant compounds, including vitamins A and C, beta-carotene, quercetin, and chlorogenic acid, have been identified in the leaf of *M. oleifera* (Lopez-Teros *et al*., 2017; Vergara-Jimenez *et al*., 2017). Due to its abundance, it is an important dietary vegetable, although its medicinal actions are not fully known. Exploring its antioxidant constituents with the aim of counteracting or limiting the bioavailability of heavy metals, especially aluminium, is essential since the possible mechanism of aluminium action is through the reactive oxygen species pathway (Yuan *et al*., 2012; Fulgenzi *et al*., 2014). With its rich antioxidant contents, can the extract of *M. oleifera* leaf protect or ameliorate aluminium-induced hippocampal neurotoxicity? Although this question has already been answered to some extent by various studies, its role in hippocampal protein activities is still lagging. This study therefore investigated the action of *M. oleifera* leaf extract on aluminium chloride-induced hippocampal protein toxicity in adult Wistar rats.

MATERIALS AND METHODS

Animal Handling

Wistar rats and CD-1 mice were obtained from the Animal Facility of the Faculty of Basic Medical Sciences, University of Uyo, Uyo, Nigeria, and acclimatised for two weeks. The animals were humanely cared for in accordance with the guidelines for laboratory animal care and use (National Research Council, 2011), and the Akwa Ibom State Ethical Committee gave the ethical approval with reference ID AK/MHS/RC/084. The rats were assigned to six groups of five animals each and were allowed standard chow and water *ad libitum*.

M. oleifera Extract Preparation

Fresh samples of *M. oleifera* were harvested from a local farm in Nduetong Oku within the Uyo Metropolis of Nigeria in the month of June and authenticated at the Department of Pharmacy with voucher number u.uH61/18. The leaves of *M. oleifera* were air-dried for two weeks, pulverised using a manually operated blender, and macerated in 70% ethanol for 72 h at room temperature (26–28 ℃). To eliminate the ethanol, the *M. oleifera* extract was concentrated using a rotary evaporator, and the concentrate was dried in a Plus 11 Gallenkamp oven at 45 ℃. The concentrated extract was weighed and refrigerated at 4 °C until use.

Chemical Preparation

Aluminium chloride (Sigma) was the preferred heavy metal compound over other aluminium compounds due to its mild neurotoxic effect (Çabuş *et al*., 2015). It was dissolved in distilled water, which was also the vehicle for the *M. oleifera* leaf extract.

Phytochemical Screening of *M. oleifera* Leaf

Phytochemical screening of *M. oleifera* leaf extract was carried out in the Department of Biochemistry laboratory of the institution. The plant extract was screened qualitatively (using colour reaction) and quantitatively for the presence of phenols, flavonoids, tannins, steroids, saponins, and alkaloids. As illustrated by (Santhi and Sengottuvel, 2016): Alkaloids were screened using Mayer's and tannic acid tests; flavonoids were screened using lead acetate and H_2SO_4 tests; phenols were screened using ferric chloride and lead acetate tests; terpenoids were screened using Salkowski's test; saponins were screened using Froth's test; amino acids were screened using Ninhydrin and Xanthropic tests; proteins were screened using Biuret's test; and sugar was screened using Benedict's and Fehling's tests. Tannins, steroids, and anthraquinones were also screened following standard procedures. Quantitatively, the alkaloids, flavonoids, phenols, saponins, and tannin contents of *M. oleifera* leaves were estimated (Harborne, 1984; Evans, 2009; Santhi and Sengottuvel, 2016).

Evaluation of Acute Oral Toxicity of *M. oleifera* Leaf

The acute oral toxicity of the leaf extract was evaluated using the "limit-dose", which is also known as the "up-and-down" method (Bruce, 1985; OECD, 2002). With this method, 20 CD-1 mice (23–24 g) were randomly assigned into five groups (n = 4). The mice were fasted overnight prior to dosing on each occasion but were allowed access to water ad libitum. Mice from the first group were dosed orally with 1,000 mg of body weight (mg/kg) of *M. oleifera* leaf extract and allowed for 48 h. With no sign of toxicity among the mice, this process was repeated for groups 2–5, administering, respectively, 2,000, 3,000, 4,000, and 5,000 mg/kg body weight of *M. oleifera* extract. Since there was no mortality or adverse behavioural changes at 5,000 mg/kg body weight, the dosing was discontinued. The mice were nevertheless observed for 14 days to monitor any delayed toxic effects.

Animal Treatment

The thirty male Wistar rats (150–220 g) were assigned into six groups (n = 5). Group 1 was the control and administered distilled water (5 mL/kg), while group 2 was administered 100 mg/kg aluminium chloride based on Ekong *et al*. (2017). Taking the estimated LD50 of 5,000 mg/kg, 5% (low dose) and 10% (high dose) of the LD50 amounted, respectively, to 250 mg/kg of *M. oleifera* and 1,000 mg/kg. Groups 3 and 4 were respectively administered 250 mg/kg and 500 mg/kg of *M. oleifera*, while groups 5 and 6 were administered concomitant 100 mg/kg aluminium chloride and 250 mg/kg *M. oleifera*, and 100 mg/kg aluminium chloride and 1,000 mg/kg *M. oleifera*. The administrations were oral and once daily for 21 days.

Spontaneous Alternation Behaviour Test
Twenty-four hours after the last administration (day 22), spontaneous alternation behaviour was tested in the T-maze. Briefly, rats were placed at the base of the T-maze and allowed to explore the apparatus for 60 sec while their choice of goal arms was recorded. If the rat did not choose any arm, it was scored zero. Five trials were performed for each rat, and between trials, the maze was cleaned with 70% alcohol. Their percentage alternations were thereafter calculated (Deacon and Rawlins, 2006).

Animal Sacrifice and Tissue Processing
The rats were anaesthetised with 50 mg/kg ketamine hydrochloride (Rotex Medica, Germany) intraperitoneally and sacrificed through intracardiac perfusion, initially with phosphate-buffered saline, then subsequently with 10% buffered formalin. Immediately after, the brains were collected and post-fixed in 10% buffered formalin for 48 hours, trimmed appropriately, and processed for histological and immunohistochemical studies. Tissue blocks were sectioned at 10 µm and mounted on slides. Serial sections were then deparaffinised and taken to the water.
A set of sections were stained in 0.1% cresyl violet for 15 minutes and rinsed in tap water to remove excess. The sections were then dehydrated in ascending grades of alcohol at 2 min each, cleared in two changes of xylene, and mounted in dibutylphthalate polystyrene xylene (DPX) (Suvarna *et al*., 2019). Antigen retrieval was performed on sections for immunolabelling; sections were incubated in citric acid solution (pH 6.0) in a microwave at 100 W for 5 min. Thereafter, they were equilibrated by gently displacing hot citric acid with distilled water. The sections were pretreated with 3% hydrogen peroxide for 10 min and preincubated in normal rabbit serum. They were then incubated either with anti-neuron-specific enolase (NSE) or glial fibrillary acidic protein (GFAP) for an hour at room temperature (27℃) in a humidified chamber and then incubated with biotinylated anti-immunoglobulin-G secondary antibodies for 30 min. They were then incubated in the avidin and biotin complex for 30 minutes, and diaminobenzidine (DAB) was used as a chromogen for 5 min. Sections were washed with distilled water, counter-stained for 2 min in haematoxylin, washed, dehydrated, cleared, and mounted in DPX.

Photomicrography and Cell Count
Cover-slipped tissue sections were viewed under the light microscope, and photomicrographs were obtained using the microscope camera linked to a computer. The cell count of tissue sections was performed by the same experimenter blinded to the experimental groupings. Photomicrographs were uploaded to the ImageJ software, thresholded, and manually counted.

Statistical Analyses
One-way analyses of variance were used to compare the means for all groups for spontaneous alternation and cell count data. Thereafter, a student Newman-Keuls post-hoc test was carried out with GraphPad Prism (5.1) software. Data were presented as mean ± standard error of mean. A probability level of $p < 0.05$ was regarded as significant.

RESULTS

Phytochemicals of *M. oleifera* Leaf Ethanol Extract
The phytochemicals in the *M. oleifera* leaf showed flavonoids (4.34 g/100g) and phenols (4.10 g/100g). Others include alkaloids (2.62 g/100g), saponins (1.65 g/100g), and tannins (1.05 g/100g). Amino acids, terpenoids, anthraquinones, and sugar were in trace amounts. However, proteins, cardenolides, and steroids were not observed (Table 1).

Table 1: Phytochemical analyses of the *M. oleifera* leaf extract

Test	Inference	Intensity	Quantity (g/100g)
Alkaloids			
(1) Meyer test	Present	++	2.62
(2) Tannic acid test	Present	+++	
Flavonoid			
(1) Lead acetate test	Present	+++	4.34
(2) H_2SO_4 test	Present	+++	
Amino acid			
(1) Ninhydrin test	Present	+++	-
(2) Xanthropic test	Present	++	
Protein			
Biuret's test	Absent	-	-
Phenolics			
(1) $FeCl_3$ Test	Present	+++	4.10
(2) Lead acetate test	Present	+++	
Terpenoids			
Salkowski's Test	Present	++	-
Tannins	Present	+++	1.05
Saponins			
Froth's Test	Present	++	1.65
Anthraquinones	Present	++	
Phlobatannins	Absent	-	
Sugar			
(1) Benedict's Test	Present	+	-
(2) Fehling's test	Present	++	
Cardenolides	Absent	-	-
Steroids	Absent	-	-

+++ = highly present; ++ = moderately present; + = lowly present; - = Absent

Median Lethal Dose of *M. oleifera* Leaf Extract
There was no mortality in any of the groups, nor was there any sign of toxicity observed with up to 5,000 mg/kg body weight of the *M. oleifera* extract 14 days later. Therefore, the process was discontinued, and the median lethal dose was estimated to be over 5,000 mg/kg body weight (Table 2).

Table 2: Median lethal dose for the extract of *M. oleifera*

Group (n = 4)	Dosage of *M. oleifera* Leaf Extract (mg/kg)	Mice Mortality
1	1,000	0/4
2	2,000	0/4
3	3,000	0/4
4	4,000	0/4
5	5,000	0/4

LD_{50} is over 5.000 mg/kg body weight of *M oleifera*

Spontaneous Alternation Behaviour

Spontaneous alternation behaviour measures spatial memory. In the present study, there was a significant decrease ($p < 0.05$) in spontaneous alternation behaviour in the aluminium chloride group (37.50 ± 16.14%) compared with the control (90.00 ± 6.124%). However, there was no significant difference ($p > 0.05$) between the test groups administered 250 mg/kg of *M. oleifera* (62.50 ± 16.14%), 1,000 mg/kg of *M. oleifera* (60.00 ± 10.00%), aluminium chloride and 250 mg/kg of *M. oleifera* (58.33 ± 8.33%), and aluminium chloride and 1,000 mg/kg of *M. oleifera* (50.00 ± 10.21%) compared with the control and the aluminium chloride group (Fig. 1).

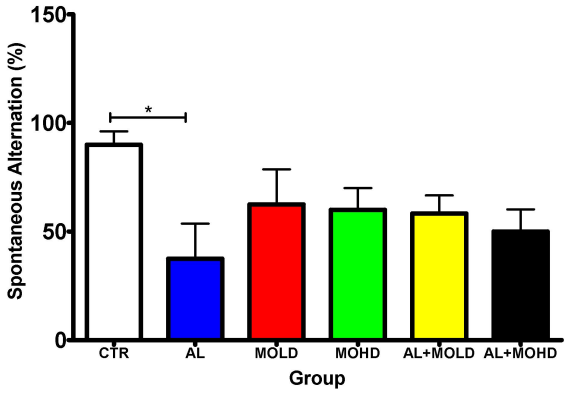


Fig. 1: Spontaneous Alternation Behaviour

* = Significantly different from CTR at $p < 0.05$; n = 5; P value = 0.0694; F = 2.470. CTR = Control; Al = Aluminium chloride; MOLD = *M. oleifera* low dose (250 mg/kg); MOHD = *M. oleifera* high dose (1,000 mg/kg)

Nissl Substance Distribution

The cornu ammonis (CA) 3 region of the hippocampus of the control group showed Nissl substance distribution in the three layers: outer molecular, middle pyramidal, and inner polymorphic. These three layers showed well-stained Nissl substance, although the pyramidal layer contained a dense population unlike the molecular and inner polymorphic layers (Fig. 2a).

The CA3 region of the hippocampus of the test groups also showed Nissl substance distribution in the three layers. In the aluminium chloride group, some of the pyramidal cells had less Nissl staining compared with the control group (Fig. 2b). In the low (250 mg/kg) and high (1,000 mg/kg) *M. oleifera* dose groups, the Nissl substance was well-stained in most of the pyramidal cells compared with the control (Fig. 2c and d). In the concomitant aluminium chloride and low (250 mg/kg) and high (1,000 mg/kg) *M. oleifera* doses, Nissl substance was well-stained throughout the layers compared with the control (Fig. 2e and f).

The population of Nissl-stained cells in the aluminium chloride group was significantly than in the control, low (250 mg/kg) and high (1,000 mg/kg) *M. oleifera* dose groups, as well as the concomitant aluminium chloride and high (1,000 mg/kg) *M. oleifera* dose ($p = 0.0001$, $F = 48$) groups. However, the population of Nissl-stained cells in the high (1,000 mg/kg) *M. oleifera* dose group was significantly higher than the low (250 mg/kg) *M. oleifera* and concomitant aluminium chloride and low (250 mg/kg) *M. oleifera* dose groups (Fig. 2g).

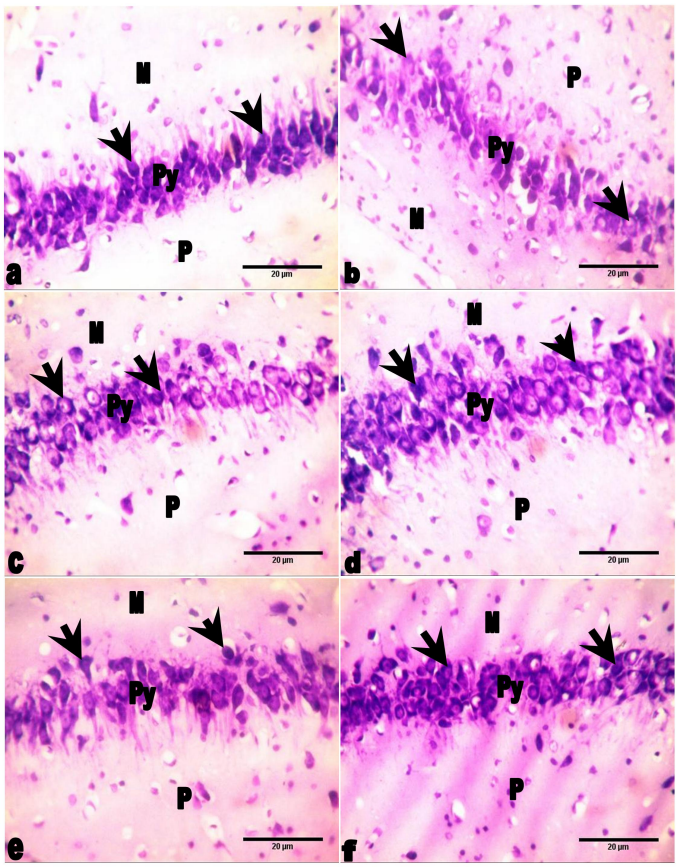


Fig. 2: The Sections and Nissl count estimates of hippocampal CA3. a. Control group having well-stained Nissl substance (arrows) in the three layers; b. Aluminium chloride group having less Nissl staining (arrows); c. Low dose *M. oleifera* group having well-stained Nissl substance (arrows); d. High dose *M. oleifera* group having well-stained Nissl substance (arrows); e. Aluminium chloride and low dose *M. oleifera* group having well-stained Nissl substance (arrows); f. Aluminium chloride and high dose *M. oleifera* group having well-stained Nissl substance (arrows). M = molecular layer; Py = pyramidal layer; P = polymorphic layer; Cresyl fast violet, ×400); g. Nissl Count Estimate; ** = Significantly different from CTR at $p < 0.01$; *** = Significantly different from CTR at $p < 0.001$; b = Significantly different from AL at $p < 0.05$; d = Significantly different from MOHD at $p < 0.05$; n = 5; P value = 0.0001; F = 48; CTR = Control; Al = Aluminium chloride; MOLD = *M. oleifera* low dose; MOHD = *M. oleifera* high dose

Neuron Specific Enolase Immunolabelling

The hippocampal CA3 region of the control group showed cytosolic expression of neuron-specific enolase (NSE) in the three layers (Fig. 3a). In the aluminium chloride group, there was less NSE expression, with significantly ($p < 0.05$) fewer NSE-positive cells compared with the control (Fig. 3b and 3g). The low-dose *M. oleifera* group showed similar NSE expression and significantly ($p < 0.05$) fewer NSE-positive cells compared with the control (Fig. 3c).

The hippocampal CA3 region of the high-dose *M. oleifera* group showed deep NSE expression and significantly ($p < 0.05$) fewer NSE-positive cells compared with the control (Fig. 3d). There were fewer NSE expressions and significantly ($p < 0.05$) fewer NSE-positive cells in the concomitant aluminium chloride with low and high doses of *M. oleifera* groups compared with the control (Fig. 3e and 3f).

The NSE-labelled cells were not significantly different ($p > 0.05$) in the aluminium chloride group compared with the other test groups, with the exception of the concomitant aluminium chloride and high dose group (Fig. 3g).

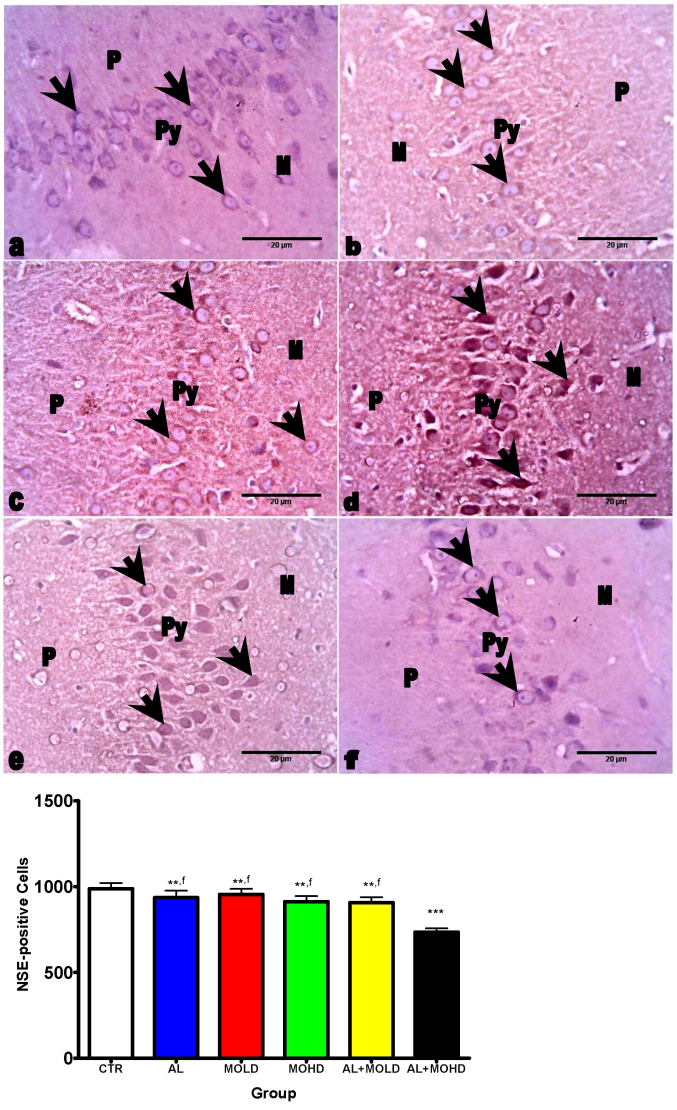


Fig. 3: The Sections of the NSE-labelled cells and cell count in the hippocampal CA3. a. Control group having neuron specific enolase (NSE) positive neurons (arrows) in the three layers; molecular, pyramidal and polymorphic; b. Aluminium chloride group having less NSE-positive expression (arrows); c. Low dose *M. oleifera* group having NSE expression (arrows); d. High dose *M. oleifera* group having deep NSE expression (arrows); e. Aluminium chloride and low dose *M. oleifera* group having less NSE expression (arrow); f. Aluminium chloride and high dose *M. oleifera* group having less NSE expression (arrow). M = molecular layer; Py = pyramidal layer; P = polymorphic layer; NSE, ×400). NSE-labelled cell count; *** = Significantly different from CTR at $p < 0.001$; f = Significantly different from AL+MOHD at $p < 0.05$; n = 5; P value < 0.0001; F = 67.90 CTR = Control; Al = Aluminium chloride; MOLD = *M. oleifera* low dose; MOHD = *M. oleifera* high dose

Glial Fibrillary Acidic Protein Immunolabelling

The CA3 region of the hippocampus of the control group showed expression of GFAP in cells of the three layers; GFAP expression was observed mostly in the processes, although some were also expressed in the soma (Fig. 4a). In the aluminium chloride group, the GFAP-positive cells were significantly ($p < 0.05$) less compared with the control (Fig. 4b). In the low-dose *M. oleifera* group, the GFAP-positive cells were significantly ($p < 0.05$) less compared with the control (Fig. 4c).

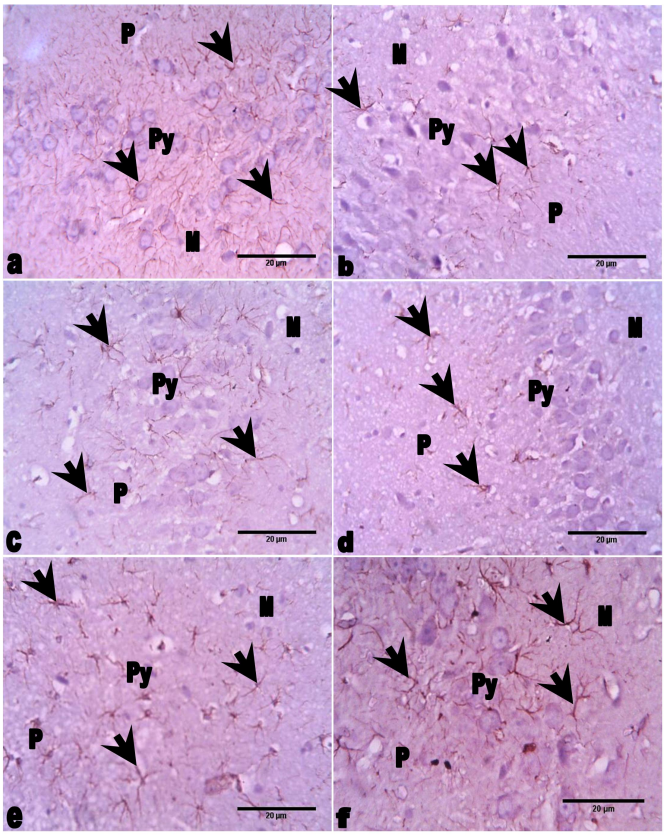
The hippocampal CA3 region of the high-dose *M. oleifera* group showed the GFAP-positive cells were significantly ($p < 0.05$) less compared with the control (Fig. 4d). In the concomitant aluminium chloride and low-dose *M. oleifera* group, the GFAP-positive cells were significantly ($p < 0.05$) less compared with the control (Fig. 4e). In the concomitant aluminium chloride and high-dose *M. oleifera* group, there were deeper-expressing GFAP-positive cells, which were significantly ($p < 0.05$) less compared with the control (Fig 4f).

The GFAP-positive cells were significantly ($p < 0.05$) less in the aluminium chloride group compared to the other test groups (Fig. 4g).

DISCUSSION

This study investigated the hippocampal neuroprotection potential of *M. oleifera* leaf extract against aluminium chloride-induced toxicity. Phytochemical screening of the extract from *M. oleifera* leaves showed a moderate presence of alkaloids, amino acids, flavonoids, tannins, phenolics, saponins, terpenoids, anthraquinones, and sugar. It is believed that the various biological activities of *M. oleifera* are influenced by their phytochemical constituents (Jayasree *et al*., 2012; Adedapo *et al*., 2015). Alkaloids, anthraquinones, flavonoids, phenolics, saponins, tannins, and terpenoids protect cells against oxidative stress (Vermerris and Nicholson, 2006; Dinkova-Kostova and Kostov, 2012; Jayasree *et al*., 2012; Adedapo *et al*., 2015; Nimse and Pal, 2015; Cho *et al*., 2017; Lobo *et al*., 2018). Some other roles include free radical scavenging, metallic ion chelators, and oxidation inhibitors, among others (Ashfaq *et al*., 2012; Nimse and Pal, 2015; Cho *et al*., 2017; Chu *et al*., 2018). The combination of these phytochemicals and their activities in the *M. oleifera* leaf makes the plant very essential for a wide spectrum of health activities (Vergara-Jimenez *et al*., 2017; Mahdi *et al*., 2018; Villarruel-López *et al*., 2018), including nutrition (Ferreira *et al*., 2008; Falowo

et al., 2018; Lin *et al*., 2019). The present phytochemical constituents are similar to those from previous studies (Vergara-Jimenez *et al*., 2017; Lin *et al*., 2019).



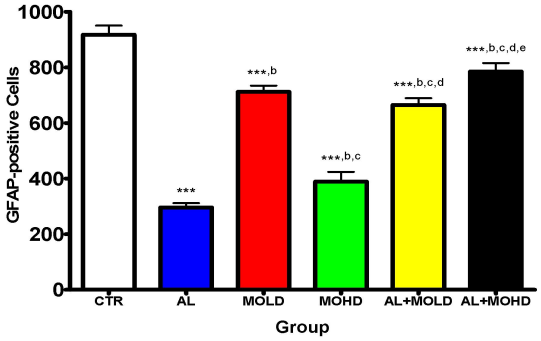


Fig. 4: The Sections of GFAP labelled Cells and Cell Count in the Hippocampal CA3. a. Control group having GFAP-positive cells whose expression is mostly in the processes (arrows); b. Aluminium chloride group shows GFAP-positive cells (arrows); c. Low dose *M. oleifera* group shows GFAP-positive cells (arrows); d. High dose *M. oleifera* group shows GFAP-positive cells (arrows); e. Aluminium chloride and low dose *M. oleifera* group shows GFAP-positive cells (arrows); Aluminium chloride and high dose *M. oleifera* group shows GFAP-positive cells (arrow). M = molecular layer; Py = pyramidal layer; P = polymorphic layer; GFAP, ×400). GFAP-positive cell count;*** = Significantly different from CTR at $p < 0.001$; b,c,d,e = Significantly different from AL, MOL, MOH, AL+MOL respectively, at $p < 0.05$; n = 5; P value < 0.0001; F = 2048; CTR = Control; Al = Aluminium chloride; MOLD = *M. oleifera* low dose; MOHD = *M. oleifera* high dose

The oral acute lethal toxicity test of *M. oleifera* showed no mortality, even up to 5,000 mg/kg, indicating the safety of the plant for oral consumption. The present result agrees with Ekong *et al*. (2017), who reported a similar oral LD50 for ethanol extract of *M. oleifera* leaves.

The present study affected spontaneous alternation, a measure of spatial memory (Deacon and Rawlins, 2006). There was a significant reduction in spontaneous alternation behaviours in the aluminium chloride group compared to the control group, suggesting a cognitive deficit. The present result corroborates a previous report of cognitive dysfunction and negative spatial learning and memory capacities arising from aluminium chloride (Nampoothiri *et al*., 2017). Aluminium chloride affects memory and cognition by interfering with glutamatergic, catecholaminergic, and cholinergic metabolism (Farhat *et al*., 2017; Kaur *et al*., 2022).

There was no significant spontaneous alternation behaviour between the groups given only the extract from *M. oleifera* leaves and the control group, supporting the extract's safety (Stohs and Hartman, 2015; Ekong *et al*., 2017), which could have also related to the brain and spontaneous alternation behaviour. There was also no major difference in the spontaneous alternation behaviour between the groups given both aluminium chloride and *M. oleifera* leaf extract compared to the control, indicating that *M. oleifera* may help protect against the effects of aluminium chloride. The present study supports reports that *M. oleifera* protects against aluminium chloride action and alleviates learning and memory impairment (Ekong *et al*., 2017; Zhou *et al*., 2018).

Nissl is the endoplasmic reticulum of neurons and a site for protein synthesis and release (Byrne, 2014). Histologically, there was a significantly lower Nissl number and staining in the hippocampal CA3 region of the aluminium chloride group compared with the control, indicating neuronal trauma or toxicity. In neurotoxicity or trauma to the brain, Nissl is less stained (Suttie *et al*., 2018), and this condition, known as chromatolysis, has been reported in aluminium neurotoxicity (Ekong *et al*., 2017; Kuznetsova *et al*., 2017; Yang *et al*., 2018; Adighije *et al*., 2020).

The CA3 histology of the groups administered only low and high doses of *M. oleifera* showed mostly well-stained Nissl substance, and that of the high dose of *M. oleifera* was significantly more in population compared with the control. This may indicate the physiological reactivity of the Nissl substance to the *M. oleifera* extract. *M. oleifera* is reportedly safe, as already reported and in previous studies (Ekong *et al*., 2017). However, this does not rule out discrete molecular changes in neurons, as reported by (Abijo *et al*., 1970), which may have influenced the Nissl staining. The concomitant aluminium chloride and low and high doses of *M. oleifera* groups showed mostly well-stained Nissl substance that were significantly more in number in the concomitant aluminium chloride and high dose of *M. oleifera* group compared with the control, indicating a protective effect of *M. oleifera* to chelate the aluminium or antagonised its effect, thereby, preventing its action in this brain area. This result is in line with previous studies (Ekong *et al*., 2017; Abdel-Rahman Mohamed *et al*., 2019).

Neuron-specific enolase is a cytosolic protein, glycolytic isoenzyme, and marker for neuronal metabolic activity and synaptic connections. It evaluates the activity of neurons, promotes neuronal survival (Butterfield and Lange, 2009;

Haque *et al*., 2018), and also serves as a marker for neural dysfunction and pathology (Polcyn *et al*., 2017). Immunohistochemically, there was significantly less NSE expression in the hippocampal CA3 region of the aluminium chloride group, indicating its neurotoxicity. Decreased expression and number of NSE-positive cells may indicate reduced neuronal metabolic activity or damage (Nogami *et al*., 1998; Bharosay *et al*., 2012). These have been reported as adaptive changes in the hippocampus known to be susceptible to neurotoxins (Ding *et al*., 2000; Yardimoğlu *et al*., 2008; Yang *et al*., 2018).

The low-dose *M. oleifera* group showed similar NSE-positivity, although with significantly fewer ($p < 0.05$) cells compared with the control, indicating less neuronal activity. Neuronal metabolic activity is reflected in NSE expression[55,56], and this process may not have been affected in this group. The high dose *M. oleifera* group showed increased NSE expression, although with significantly ($p < 0.05$) fewer positive cells compared with the control, indicating increased neuronal metabolic activity. NSE is involved in neuronal repair (Haque *et al*., 2018), whose pathway may have been triggered, leading to increased NSE expression. Increased neuronal metabolic activity is also reported with increased NSE expression, and this may lead to excitotoxicity.

The administration of aluminium chloride together with either low- or high-dose *M. oleifera* showed decreased NSE expression, with significantly ($p < 0.05$) fewer positive cells compared to the control, but not different from the aluminium chloride group, indicating less metabolic activity of the neurons. The lower metabolic activities may be due to the antagonising effect of *M. oleifera* on aluminium chloride. This observation corroborates the report of Ekong *et al*. (2017).

Glial fibrillary acidic protein, an intermediate filament protein in astrocytes (Venkatesh *et al*., 2013), also serves as its marker (Zhang *et al*., 2019). Immunohistochemically, there were significantly fewer ($p < 0.05$) GFAP-positive cells in the hippocampal CA3 region of the aluminium chloride group compared with the control, which may indicate neurotoxicity. Decreased GFAP-positivity has previously been reported with aluminium administration (Guo-Ross *et al*., 1999), which may arise due to neurodegeneration or other detrimental conditions (Steffek *et al*., 2008; Bondan *et al*., 2013). The low and high doses of *M. oleifera* groups showed fewer GFAP-positive cells, which were also significantly different from the control group, indicating decreased GFAP activity. Decreased GFAP expression, as in the present study, could have resulted from loss of glial fibrillary acidic protein antigenicity (Liu *et al*., 2012), which Ekong *et al*. (2017) also reported. The groups administered concomitant aluminium chloride with either low- or high-dose *M. oleifera* showed significantly ($p < 0.05$) fewer GFAP-positive cells compared to the control, although significantly more than those of the aluminium chloride group. Such results may indicate decreased GFAP activity, not neurotoxicity. GFAP expression may be up-regulated in traumatised or injured brains brain (Smith and Eng, 1987), but antagonistic action on this intermediate protein may have resulted in increased expression. The present result varies from other studies, which may be due to the doses of *M. oleifera* and the target brain area (Ekong *et al*., 2017).

The hippocampus, a vital memory centre in the brain, is the most affected brain part in aluminium intoxication (Kaur *et al*., 2022). In the present study, aluminium chloride caused cognitive decline, as observed in decreased spontaneous alternation, which is probably a consequence of neuronal protein synthesis disruption and activity, as reported in Nissl substance chromatolysis and decreased NSE expression. The decreased Nissl and NSE expressions arising from aluminium chloride administration could lead to neurotransmitter depletion, which may be responsible for decreased GABA, dopamine, noradrenaline, and serotonin levels reported previously (Kaur *et al*., 2022). The resultant effect is memory impairment, as played out in the present study. Abnormal expression of GFAP occurs in neuroinflammation and neurodegeneration, among others (Li *et al*., 2020), which is implicated in aluminium intoxication (Farhat *et al*., 2017) and may consequently lead to loss of GFAP antigenicity (Liu *et al*., 2012).

M. oleifera may have reversed the cognitive deficit by improving spontaneous alternation through the protection of Nissl, NSE, and GFAP expression. Antioxidation is reported in neuroprotection and neurotransmitter enhancement (Ballaz and Rebec, 2019; Lee *et al*., 2020), thus, in line with the suggested mechanism for *M. oleifera* neuroprotective effect as previously reported (Flora and Pachauri, 2011; Taïr *et al*., 2016; Suttie *et al*., 2018).

Limitations of this Study

This study was limited to spontaneous behavioural alternation, histology, and immunohistochemistry in rats. Firstly, the antioxidant properties of *M. oleifera* were not established, which could have provided insight on its action. Secondly, although percentages of the lethal doses were used to determine *M. oleifera* dosages, it requires optimisation. Thirdly, the study duration may not be sufficient to determine the long-term application and safety of *M. oleifera* extract.

Conclusion

M. oleifera leaf extract contained vital nutritional and medicinal phytochemicals, and was also safe for oral consumption. The administration of aluminium chloride resulted in a decline in spontaneous alternation and hippocampal CA3 chromatolysis, which decreased NSE and GFAP expressions. However, concomitant treatment with *M. oleifera* did improve spontaneous alternation, Nissl distribution, NSE, and GFAP expressions. However, the high dose of *M. oleifera* improved Nissl distribution better.

DECLARATION

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Conflict of Interest
None declared.

Ethical Approval
Ethical approval for the study was obtained from the Faculty of Basic Medical Sciences Research and Ethical Committee of the University of Uyo, with approval number UU_FBMSREC_2024_020.

Consent to Participate and Publish Data
Not Applicable.

Authors' Contribution
MBE - Research conception and design; NKA - Data acquisition; MBE, EIB - Data analysis and interpretation: MBE, NKA - Initial manuscript draft; MBE, GJE, EIB - Substantive revision of manuscript

Conflicts of Interest
The authors declare no conflicts of interest.

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