Journal of Pharmacology and Toxicology

ISSN 1816-496X

Academic Journals Inc.

www.academicjournals.com

OPEN ACCESS Journal of Pharmacology and Toxicology

ISSN 1816-496X
DOI: 10.3923/jpt.2023.17.24

Research Article
Effects of *Moringa oleifera* on 3, 4-Methylenedioxymethamphetamine (MDMA) Induced Neurotoxicity in the Pre-Frontal Cortex of Experimental Wistar Rats

[1,2]Fabiyi Oluseyi Sunday, [1]Ogunbiyi Olubunmi Esther, [1,3]Owolabi Joshua Oladele, [1]Adelodun Stephen Taye, [1]Adetunji Opeyemi Adebola, [1]Olanrewaju John Afees, [1]Lawal Oluwatosin Bukola, [1]Adeniji Kehinde and [1]Sambo Ayuba Jacob

[1]Department of Anatomy, Benjamin Carson Snr, School of Medicine, Babcock University, Ilisan-Remo, Ogun, Nigeria
[2]Department of Anatomy, Olabisi Onabanjo University, Ago-Iwoye, Ogun, Nigeria
[3]Department of Anatomy, University of Global Health Equity, Kigali, Rwanda, Nigeria

Abstract

Background and Objective: Methylenedioxymethamphetamine (MDMA) is used by millions of people worldwide as a recreational drug. Due to the popularity of MDMA use and its potential to create long-lasting or permanent 5-HT axon damage, the need for ways to control its effect on the brain is crucial. The present study aimed at investigating the effect of *Moringa oleifera* aqueous leaf extract on MDMA-induced neurotoxicity in the prefrontal cortex. **Materials and Methods:** Thirty adult male Wistar rats were randomly divided into 5 groups, A, B, C, D and E (n = 6) which were treated every day for 2 weeks. Group A: Control group, Group B: Moringa only (200 mg kg^{-1}), Group C: MDMA only (5 mg kg^{-1}), Group D: MDMA for the first 1-7 days (5 mg kg^{-1}), moringa for the remaining 8-14 days (200 mg kg^{-1}) and Group E: MDMA in the morning (5 mg kg^{-1} b.wt.) and moringa in the evening (200 mg kg^{-1}). Brain samples were collected and weighed and the prefrontal cortex was sliced, sectioned and processed for histological and immunochemical analysis. **Results:** The MDMA caused neuronal and myelin sheath degeneration. Moringa, however, promoted the regeneration of myelin sheaths and neurons to reverse damage caused by MDMA. **Conclusion:** The result showed that *M. oleifera* aqueous leaf extract protected and also ameliorated neurotoxicity caused by MDMA.

Key words: Neurotoxicity, methylenedioxymethamphetamine, *Moringa oleifera*, prefrontal cortex, memory, anxiety, distal axotomy, glycosides

Citation: Sunday, F.O., O.O. Esther, O.J. Oladele, A.S. Taye and A.O. Adebola *et al.*, 2023. Effects of *Moringa oleifera* on 3, 4-methylenedioxymethamphetamine (MDMA) induced neurotoxicity in the pre-frontal cortex of experimental Wistar rats. J. Pharmacol. Toxicol., 18: 17-24.

Corresponding Author: Adetunji Opeyemi Adebola, Department of Anatomy, Benjamin Carson Snr, School of Medicine, Babcock University, Ilisan-Remo, Ogun, Nigeria Tel: +2348038217080

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Methylenedioxymethamphetamine (MDMA) is a common recreational drug of abuse. Central serotonergic neurotoxicity of MDMA to distal axotomy has been attributed to several studies, in which only the raphe nucleus of the fine serotonergic axons descending are lost without damage to their cell bodies but *in vivo*, this axotomy has never been visualised[1]. Methylenedioxymethamphetamine has both stimulatory and hallucinogenic properties which make its psychoactive effects unique and different from those of typical psychostimulant and hallucinogenic agents[2]. Immune-histological studies show evidence for MDMA-induced serotonergic damage through profound loss of fine serotonergic axon terminals throughout the forebrain, swollen varicosity and fragmentation were visualised in the Serotonergic axons of the cortex and no morphological aberrant changes in the raphe cell bodies under the electron microscope analysis, therefore, MDMA specifically induce distal axotomy of serotonergic neurons in the brain[2]. The prefrontal cortex is part of the brain located at the front of the frontal lobe. It is a structural and functional heterogenous group of cortical areas located anterior to the motor and premotor regions of the frontal lobe. The anterior part of the frontal lobe is occupied by the prefrontal cortex on its medial, lateral and orbital surfaces. Its relative size is about 30% of the cerebral mantle[3]. Imaging studies have shown that in humans, prefrontal areas do not attain maturity until full adolescence. Behavioural evidence shows that cortical areas are critical for those higher cognitive functions that develop late, such as propositional speech and reasoning[4]. Due to the multimodal association of the prefrontal cortex, it involves information processing from various sensory modalities like decision-making, personality expression, planning complex cognitive behaviour and moderating social behaviour[5]. In vain, it involved executive functions like the ability to differentiate conflicting thoughts, determine bad and good, social control and current and future action consequences[6]. *Moringa oleifera* is a tropical deciduous perennial dicotyledonous tree. In recent years, plant extracts and other natural products have been tested for their antioxidant properties[7]. Antioxidants quench, scavenge and suppress the formation of reactive oxygen species and free radicals or oppose their actions[8]. The ethnopharmacological use of *Moringa oleifera* leaf was supported by the reported presence of rich antioxidants and phytochemicals[8]. Phytochemical analysis of *Moringa oleifera* leaves reveals the presence of alkaloids, flavonoids, steroids, tannins, saponins and glycosides as major components. The petroleum ether extracts of *Moringa oleifera* contain high contents of bioactive compounds such as phenol and flavonoids[9]. Reports from recent studies suggested that *Moringa oleifera* has ameliorative potential against neurotoxicity and oxidative stress in rodents[10-12]. Therefore, this investigates the effect of *Moringa oleifera* aqueous leaf extract on MDMA-induced neurotoxicity in the prefrontal cortex.

MATERIALS AND METHODS

Study area: The study was carried out from May to June, 2022.

Animal sampling: Thirty male adult Wistar rats (*Rattus norvegicus*) weighing between 150-200 g were used for this experiment. They were gotten from the animal housing facility, at Babcock University, Ilishan-Remo, Ogun State. They were housed under well-maintained environmental conditions (12:12-hrs light:dark cycle) and controlled humidity and temperature. The rats were allowed a 1 week (14-21 May, 2022) acclimation period before treatment and experimental design followed Table 1 (self-developed). The rats were weighed with a Camry Electronic Kitchen Scale EK5055 weighing balance every 2 weeks. The MDMA was administered to induce neurotoxicity.

Ethical clearance: Research was carried out following all the necessary rules and regulations in animal research and the teaching approved by the Institute of Laboratory Animal Resources, National Research Council DHHS, pub No. NH86-23, 1885.

Experimental protocol: According to Table 1, the 30 Wistar rats were divided into 5 groups, 6 animals in each group to avoid overcrowding and for easy accessibility and identification of the animals in the course of the research.

Methylenedioxymethamphetamine (MDMA) was purchased from Sigma Aldrich UK while the *Moringa oleifera* leaves were gotten from Ikorodu. The leaves were authenticated by the Department of Plant Biology and Taxonomy at the Department of Botany, University of Ibadan. After this, a voucher specimen was deposited at Ibadan Herbarium with the reference number: 110265. A total of 27.0 g of *Moringa oleifera* leaves were gotten. The leaves were washed and air-dried under hygienic conditions. Sun drying process was not used to prevent the loss of nutrients from the leaves, rather the leaves were air dried. The leaves were grounded to powdered form and put in 4.5 L of distilled water and left in distilled water for 3 hrs. The mixture was then filtered and put into a water bath at 40°C for evaporation,

Table 1: Experimental design of the study

Groups	No. of animals	Doses	Rationale
Group A	6	Control group, fed *ad libitum* (14 days)	Control group
Group B	6	Moringa extract only (200 mg kg^{-1} b.wt.) 1-14 days	To observe the sole effects of moringa
Group C	6	MDMA only (5 mg kg^{-1} b.wt.) 1-14 days	To induce neurotoxicity and observe its sole effects
Group D	6	MDMA (5 mg kg^{-1} b. wt.) for days 1-7, Moringa extract (200 mg kg^{-1} b.wt.) for days 8-14	To observe possible therapeutic effects of moringa
Group E	6	MDMA in the morning (5 mg kg^{-1} b.wt.), Moringa extract in the evening (200 mg kg^{-1} b.wt.) (14 days)	To observe possible protective effects

leaving powdered *Moringa oleifera* extract. The administration of MDMA and *Moringa oleifera* was administered orally using the orogastric tube. At the end of the experiment, the Wistar rats were sacrificed after the experimental protocols by cervical dislocation. The skulls of the rats were opened up using a bone crusher instrument and the whole brain was extracted and weighed. Thereafter, the prefrontal cortex of each brain was separated. Some were fixed in sample suitable fixatives (formol saline) for further tissue processing.

Tissue processing procedure: Brain tissue was fixed in formol saline for 48 hrs. It was then dehydrated in the following order of alcohol: 70% for 1 hr, 80% for 1 hr, 90% for 2 hrs, 95% for 2 hrs and two changes of 100% absolute alcohol for 2 hrs each. It was then cleared in two changes of xylene for 2 hrs each. It was then infiltrated with two changes of paraffin wax for 2 hrs for the first change and 4 hrs for the second chance. The processed tissue was placed in paraffin wax for embedding using a plastic mould. The embedded tissue was sectioned using Cambridge rocker microtome at 5 µm and picked on an albuminized slide in a warm bath at a regulated temperature lower than 58°C which is the regular melting point of the wax.

Haematoxylin and Eosin Staining: Slides were deparaffinized in 3 changes of xylene for 3 min each. Then, they were hydrated in 100% alcohol and 95% alcohol, 2 changes for 3 min each and rinsed in distilled water until ripples disappeared from the slides. They were placed in Haematoxylin for 8 min. They were then rinsed in tap water until the water was clear. They were decolourized in 1% HCl in 70% alcohol, 4 quick dips. Differentiation was checked microscopically to check for distinct nuclei and uncoloured cytoplasm. After which, they were rinsed in tap water until ripples disappeared from slides then dipped in Bluing Agent, (4 long dips). They were washed in lukewarm tap water for 5 min (37-40°C) and stained in Eosin for 30 sec 2 min. They were then dehydrated in 95% alcohol and 100% alcohol, 3 changes each for 2 min and 0020 crfcf0076 cleared in 3 changes of xylene for 2 min each. The cover glass was then mounted on the slides using DPX.

Cresyl fast violet procedure: The slides were placed in xylene for 5 min. Then placed in 95% Alcohol and then 70% alcohol for 3 min each. They were then deionized in distilled water for 3 min. They were placed in cresyl violet, for 10 min at 60°C ovens. Then placed in distilled water for 3 min. Then dehydrated in 70% alcohol for 3 min, 95% alcohol for 2 min and one dip in 100% alcohol. They were then placed in xylene for 5 min. The slides were mounted with glycerol gelatin and a cover slip was used.

Luxol fast blue procedure: The brain tissue was deparaffinized and hydrated in 95% alcohol. And was placed in Luxol fast blue solution at 60°C overnight. Slides were rinsed in alcohol (95%) then in distilled water thereafter placed in lithium carbonate solution (5 sec), then in two changes of alcohol (70%) for 10 sec each. Slides were then washed in distilled water. These steps were repeated until sharp contrast in the grey matter. Then slides were rinsed in alcohol (70%) and placed in eosin (1 min) then rinsed in distilled water. The slides were placed in cresyl violet (1 min) and then rinsed in distilled water. Thereafter, the tissue was dehydrated in 95 and 100% alcohol, cleared in xylene and mounted and coverslip[13].

Glial fibrillary acidic protein (GFAP): Sections were deparaffinized thoroughly in three changes of xylene, 3 min each and hydrated through two changes each of 100 and 95% ethyl alcohols, 10 dips each. Then washed well with distilled water. Sections were rinsed in distilled water and excess water was tapped off. They were circled with a pap pen liquid blocker to reduce reagent usage and ensure tissue coverage. Endogenous peroxidase was blocked with freshly made 3% hydrogen peroxide and then incubated for 5 min. Slides were rinsed gently in distilled water. Then rinse in two changes of Tris buffered saline. The excess buffer was tapped off. Glial fibrillary acidic protein primary antibody was applied and incubated at room temperature for 30 min. Slides were rinsed in two changes of buffer. The excessive amplified buffer tapped off was incubated for 10 min, in two changes of buffer, the slides were rinsed and HRP polymer was applied to the excessive buffer that was tapped off and then incubated for 10 min thereafter slides were rinsed in two changes of buffer.

The excess buffer was tapped off, DAB was applied and incubated for 5 min. Slides were rinsed in four changes of distilled water. Slides were counterstained lightly with Mayer haematoxylin stain for 2 min. Slides were rinsed in warm tap water to blue sections. Slides were dehydrated in 2 changes each of 95 and 100% ethyl alcohol. Slides were cleared in 3 changes of xylene, 10 dips each. Glycerol gelatine was applied and carefully covered with a coverslip[14].

RESULTS AND DISCUSSION

The brain is very important in the human body because it is the seat of cognition and thinking. The prefrontal cortex which was the section of interest of this research plays a very important role in planning complex cognitive behaviour, personality expression, decision making and moderating social behaviour. The potential restorative effects of *Moringa oleifera* have been demonstrated on several neurotoxic, neurodegenerative and related conditions in laboratory animals[12,15-17].

Brain weight and body weight: At $p<0.05$ there was no statistical significance in the brain and body weight differences when Groups B, C, D and E were compared to Group A. However, the bar chart in Fig. 1 showed that when compared to Group A (control group) which had the highest organ weight, there was a slight difference in Group B and then Group C followed by Group E. Group D recorded the lowest organ weight. There was no significant difference in the body weight of the animals across the groups when compared to group A (Control). The bar chart in Fig. 2 showed that Group B had the highest weight, followed by Group A (control group), then Group C followed Group D and lastly Group E which had the lowest body weight. This may be suggested that *M. oleifera* increased the body weight of rats in Group B due to the rich phytochemical components contained in *M. oleifera*.

Haematoxylin and Eosin: The histological demonstration of the prefrontal cortex of the experimental rats using the H&E staining technique (Fig. 3a-e) provided knowledge on the general histoarchitecture and cell morphology and distribution. This helped to observe the structural integrity of the neurons in the prefrontal cortex. Normal general histoarchitecture was demonstrated in Group A (control). The cells were observable and well demonstrated. No deviation from the normal basic histological and cytomorphological features was observed. Aberrations in the experimental rat's brain could be observed in the various groups to determine the effect of the treatments on the prefrontal cortex and the consequent changes. Group B showed a normal structure of

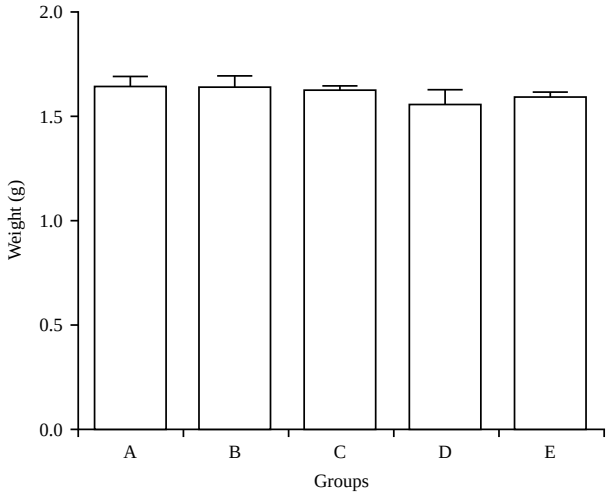

Fig. 1: Bar chart representing brain weight difference in rats

Brain weight of Group A (control) was 1.644±0.04781. The brain weight of Group B showed a slight decrease when compared to the control group at 1.640±0.05394. The brain weight of Group C showed a reduction in brain weight when compared with the control group 1.626±0.01806. Brain weight of Group D showed a decrease in weight when compared with the control group at 1.554±0.07481. The brain weight of Group E decreased when compared with the Control group at 1.590±0.02881

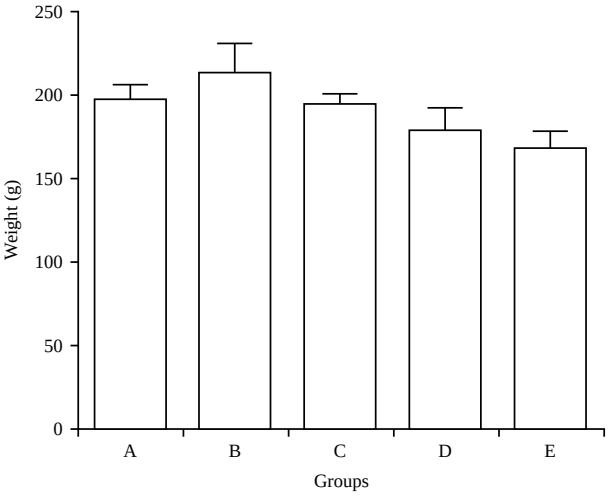

Fig. 2: Bar chart showing body weight difference in the experimental rats

Body weight of Group A (control group) was 197.2±9.292. There was an increase in body weight in Group B when compared with the control group at 212.8±18.51. There was a slight decrease in the body weight in the experimental rats in Group C when they were compared with the control group at 194±6.726. There was a decrease in body weight in Group D when compared to Group A at 178.6±14.13. There was also a decrease in body weight of Group E when compared with the control group at 167.8±10.66

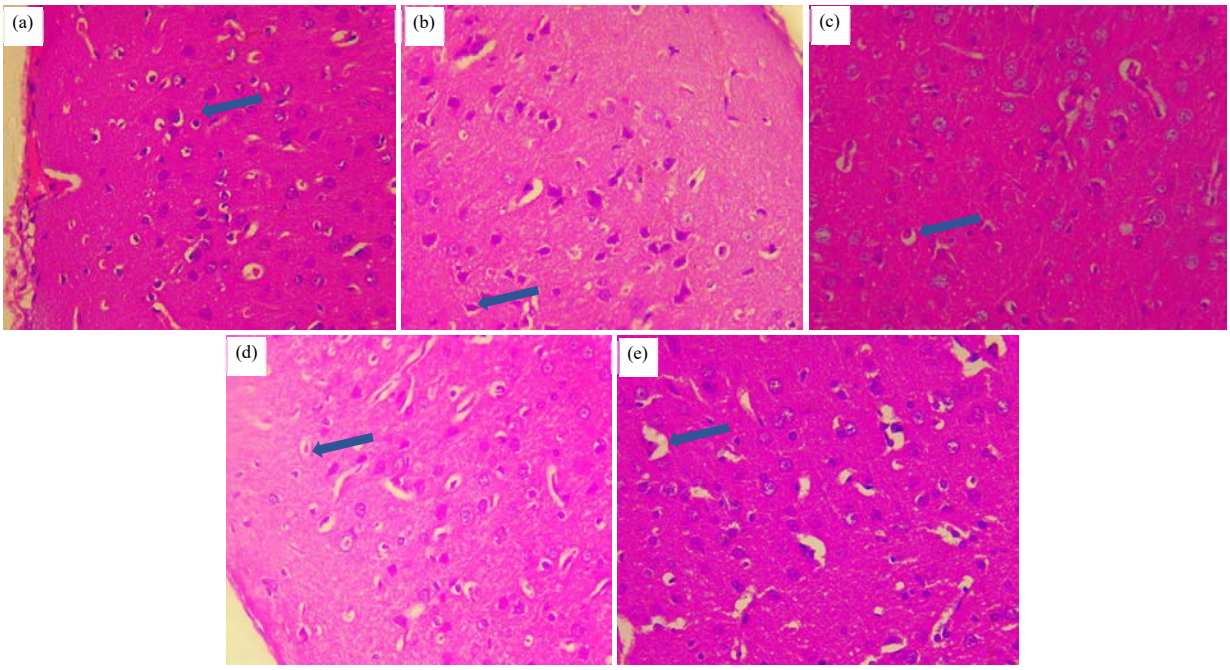

Fig. 3(a-e): Photomicrographs of the prefrontal cortex of experimental Wistar rats, (a-b) Cortical structures intact, normal neuronal and glial cells, (c) Presence of neuronal degeneration and vacuolation, (d) Presence of neuronal degeneration and vacuolation with an increase in glial cells and (e) Presence of neuronal degeneration and vacuolation with cells showing thickening and pyknosis. Heterogeneity is also present
Groups A-D (H&E, X400) showing the different cells: Neurons (blue arrows)

the cell bodies similar to the control group. Group C however, showed neuronal degeneration, vacuolation and abundance of glial cells compared to the control. Group D showed neuronal degeneration and some vacuolation around the neurons. Group E showed higher neuronal degeneration, heterogeneity (different sizes of the neuron), pyknosis and shifting of the cell body compared with the other groups and with the control group. MDMA significantly enhanced neuronal degeneration and apoptosis, similar to reports from[18].

Cresyl fast violet staining technique: This technique is used to show the cytological presence of the Nissl bodies. The Nissl bodies are rough endoplasmic reticulum being demonstrated because of the rRNA materials associated with them. It provides information on the cellular morphology and size but more importantly the functional status of the cell. The level of activities at the level of the rough endoplasmic reticulum is an important indication of the protein synthesis of neurons (Fig. 4a-e). Group B showed fewer Nissl substances when compared to the control group. Group C showed loss of Nissl substance, few neurons and more glial cells. Group D showed pyknosis, vacuolation and Nissl substances pushed aside, more glial cells. Group E showed a loss of Nissl substances compared to the control group, with pyknosis and Nissl bodies pushed aside.

Luxol fast blue staining technique: This technique is used to demonstrate the conditions of the myelin sheath. The myelin sheath envelopes the nerve fibres providing insulation and facilitating the neuronal function of conduction. It is a white fatty material composed of lipids and lipoproteins that surrounds the axons of nerves. It enables fast transmission of impulses in the nerve. Luxol fast blue technique demonstrated the cytological conditions and integrity of the myelin sheath to observe the effect of each treatment on the myelin sheaths in the experimental rats. Myelination provides important information on the integrity of cellular communication and could also supply information on possible degeneration processes. When compared to the control group (Group A), Group B showed normally myelinated axons (Fig. 5a-e). Group C showed a few reductions and regeneration of the myelin sheath. Group D showed myelin sheath damage with regeneration, more neurons and astrocytes. Excess demyelination was however observed in Group E with some regeneration but few neurons suggesting that MDMA usage increase demyelination, similar to reports from[19].

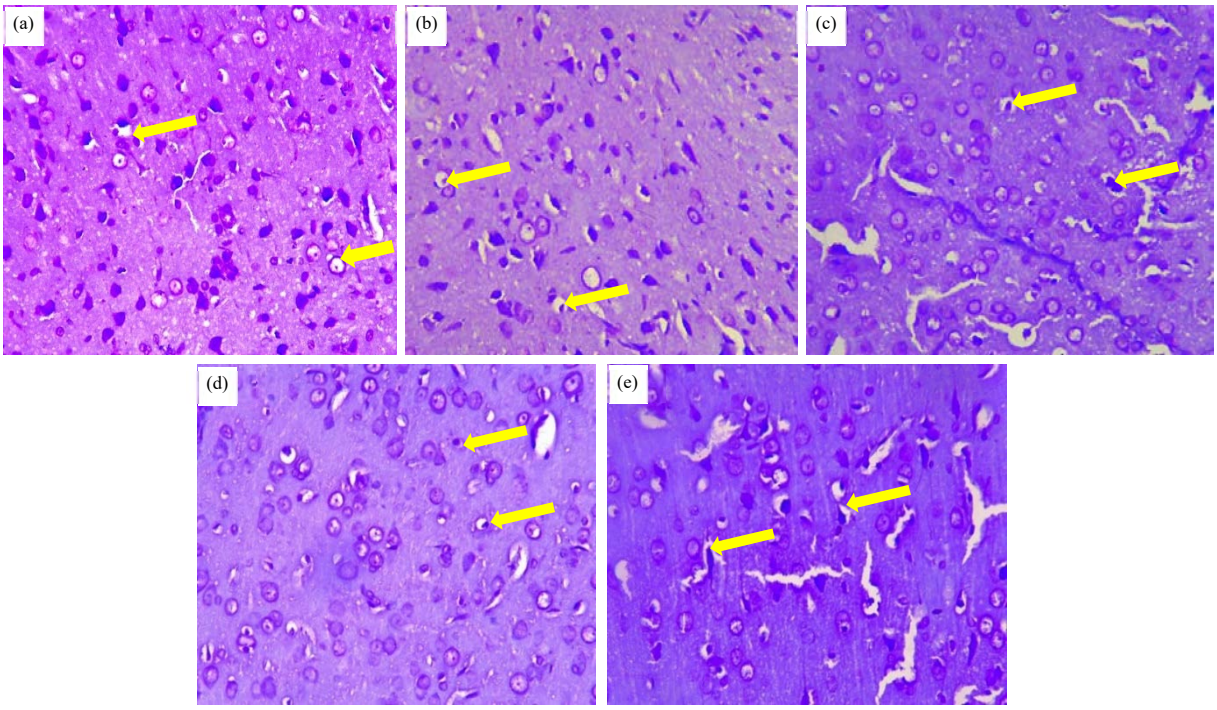

Fig. 4(a-e): Photomicrograph of the prefrontal cortex of experimental animals, (a-b) Intact cortical morphology and (c-e) Few neuronal cell bodies with pyknosis and vacuolation pushing aside Nissl substances amidst increased glial cell population

Group A-E shows intensely basophilic neuronal cell bodies indicating the presence of Nissl substances (yellow arrow) Cresyl Violet, X400

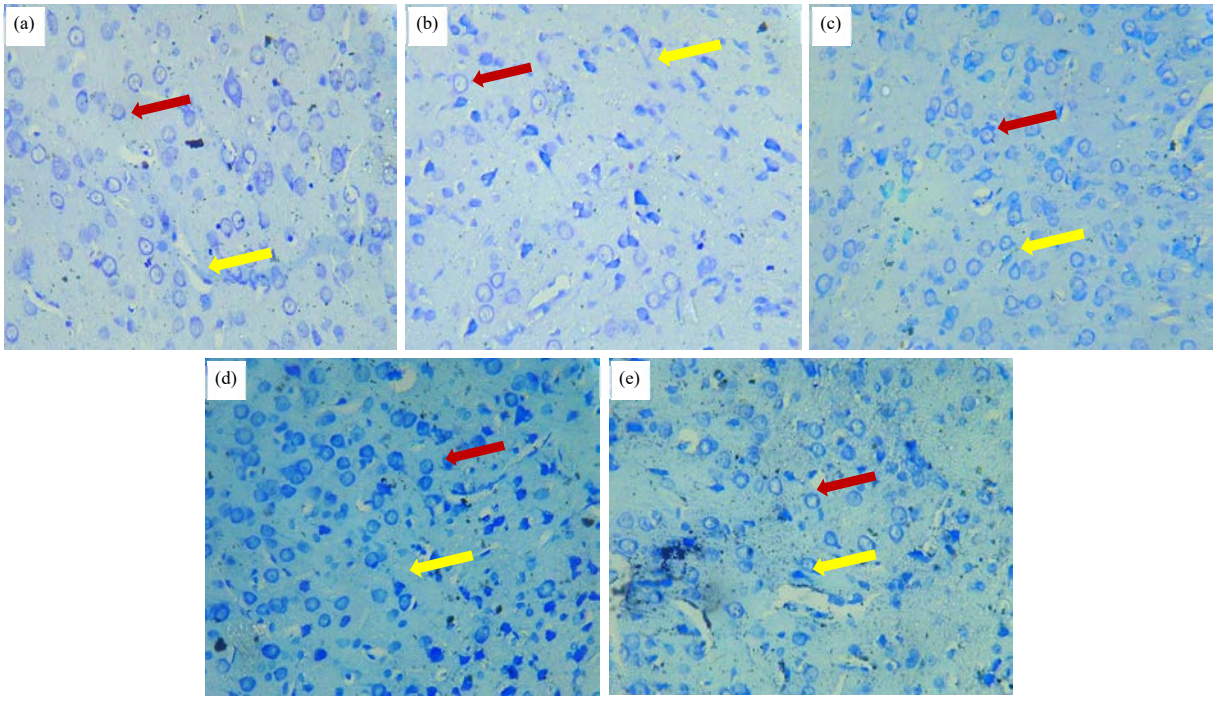

Fig. 5(a-e): Photomicrograph of the prefrontal cortex of experimental animals, (a-b) Intact cortical morphology, (c) Presence of reduced myelin fibres suggests degeneration and (d-e) Increased population of glial cells indicating regeneration

Groups A-E indicating myelination (yellow arrows) and glial cells (red arrows) Luxol Fast Blue, X400

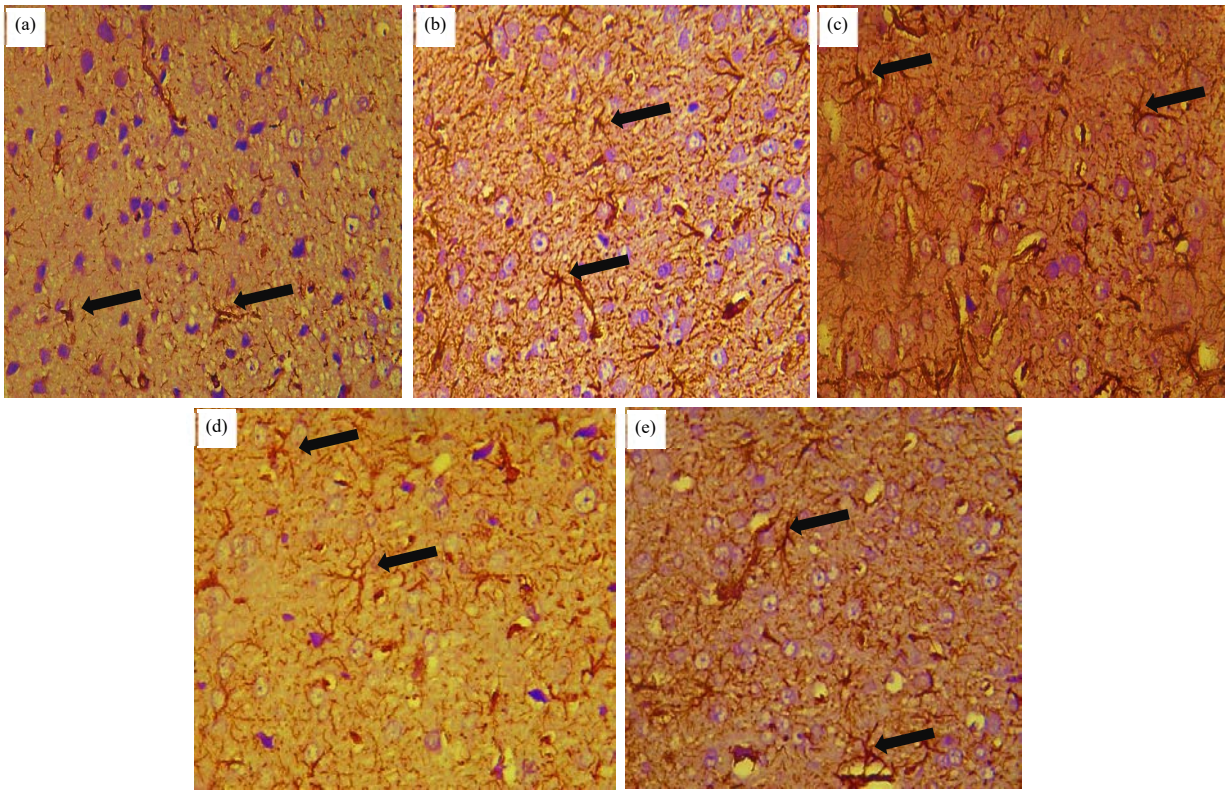

Fig. 6(a-e): Photomicrograph of the prefrontal cortex of experimental animals, (a-b) Intact cortical morphology, (c) Fewer cell bodies and astrocytes suggest degeneration and (d-e) Increased population of astrocytes suggesting regeneration
Groups A-E showing neuronal cell bodies (purple complexes) and positive cytoplasmic astrocytic staining (black arrows) GFAP X400

Glia fibrillary acidic protein (GFAP) immunohistochemistry: This technique is employed in demonstrating astrocytic integrity in the CNS. Astrocytes are one of the glial cell groups, providing support and protection to neurons in the brain. Astrogliosis, an abnormal increase in the number of astrocytes due to the destruction of nearby neurons is a characteristic feature of CNS lesions. The presence of many astrocytes in an area shows the presence of foreign or harmful substances in the brain. The control group showed the presence of few astrocytes (Fig. 6a-e). There were few astrocytes in Group B but more than the control. Group C showed an abnormal increase in astrocytes compared with the control group. Group D showed an increase in astrocytes compared to the control group but fewer than Group C. Group E when compared with the control group showed abundant astrocytes but fewer than Group C. This may suggest that MDMA causes an abnormal increase in astrocytes however Moringa has the potential in reversing the abnormal increase in astrocytes. Astrocytic expression of GFAP was also more prominent in Group B than in the control group (Group A). An increased expression of GFAP indicates astroglial activation and gliosis, often resulting from neurodegeneration. It also suggested that MDMA increased the astrocytic expression of GFAP due to its neurodegenerating properties. There were fewer astrocytic expressions in Group D compared to Group C. Astrocyte hypertrophy suggests the result of neuronal damage, similar to findings from[18].

In groups that received *Moringa oleifera*, there were observable effects on the MDMA-induced neurotoxicity in the experimental rats. *Moringa oleifera* showed significant ameliorative effects on morphological damage caused by MDMA through neuronal regeneration, improved distribution of Nissl bodies and reduction in the level of reactive astrocytes, similar to findings by previous research[12,15-17]. The use of *M. oleifera* leaves phytochemicals can be employed as a therapeutic and ameliorative means in the treatment of MDMA-induced neurotoxicity. More research should be done on Moringa and its effect on neurotransmitters in the brain.

CONCLUSION

Results showed that *Moringa oleifera* has relatively positive effects on the damages induced by MDMA due to its high nutritional and medicinal value which can be sometimes

substituted for food. *Moringa oleifera* promotes the regeneration of myelin sheaths and neurons to reverse damage caused by MDMA. The result from this research showed that *M. oleifera* aqueous leaf extract is qualified to prevent and also ameliorate neurotoxicity caused by MDMA.

SIGNIFICANCE STATEMENT

The use of illicit drugs has been the film of the day and it has been increasing the society with mentally pathological individual, while a readily available supplement to manage this illicit effect of the drug have to be researched and *Moringa oleifera* have in various ways proved to give positive feedback in most pathological symptoms.

ACKNOWLEDGMENT

We appreciate the Benjamin S. Carson (Snr), School of Medicine, Babcock University for the enabling environment to carry out this research.

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