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ETHANOL LEAF-EXTRACT OF *MORINGA OLEIFERA* PROTECT AGAINST HYPERGLYCAEMIC-INDUCED NEURONAL IMPAIRMENT IN ALBINO WISTAR RATS

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ABSTRACT
This study was designed to investigate the effect of ethanol leaf-extract of *Moringa oleifera* on hyperglycaemic-induced neuronal impairment in albino Wistar rats. Thirty-five (35) adult albino Wistar rats were used for the study. The rats were divided into 7 groups of 5 animals each. Group 1 was the control and was administered with 10ml/kg of distilled water. Hyperglycaemia was induced in Group 2 – Group 7 following the administration of 150mg/kg of aloxan. Group 2 – Group 6 were treated respectively with daily doses of moringa 500mg/kg, moringa 1000mg/kg, moringa 1500mg/kg, metformin 14.29mg/kg and moringa 1000mg/kg + metformin 14.29mg/kg. Treatment lasted for 28days. Group 7 had no treatment. Behavioural evaluation was carried out using novel object recognition test, T-maze simple alternation test and elevated plus maze. Furthermore, immuno-histochemical assessment of the cerebral cortex, and hippocampus were also carried out. The results showed no significant difference in the percentage (%) alternation (T-maze) and transfer latency (elevated plus maze) between control and the experimental groups. The discrimination ratio (novel object recognition test) was positive in all the groups but negative in the non-treated group. Immunohistochemical assessment showed severe and mild increase in the GFAP (Glial Fibrillary Acid Protein) expression respectively in hippocampus and prefrontal cortex of the non-treated animals. The immunohistochemical changes were, however, ameliorated by low and moderate doses of *Moringa oleifera*. In conclusion, ethanol leaf-extract of *Moringa oleifera* is neuroprotective.

KEYWORDS: Hyperglycaemia; Moringa oleifera; alloxan; neuronal-impairment.

INTRODUCTION
Diabetes mellitus is one of the most common chronic diseases that affect humankind. It is a metabolic disorder that results from dysfunction of beta cell of the pancreas or failure of insulin to perform its biological functions at the level of the muscle or the liver, leading to hyperglycaemia (Ojo, 2013).

It was estimated in 2010 that approximately 285 million individuals aged 20-79 years were affected with diabetes around the world, and this number may progressively increase to 439 million by 2030 (Shaw *et al.* 2010). In this regard, reducing the burden of diabetes remains to be a major challenge for researchers.

There is convincing evidence that diabetes mellitus increases the risk of cognitive dysfunction and memory deficit. Adverse effects of diabetes on cognitive abilities and perturbations of memory capacity have been observed by scientists (Leibson *et al.*1997). Diabetes often involves disturbances in the metabolism of carbohydrate, fat and protein resulting in chronic complications. These complications insult the physiological systems and organs of the body and may result in cerebrovascular dysfunction and neurodegeneration leading to dementia.

Convincing evidence have revealed that continuous intake of foods that contain saturated fats and sugar, without engaging in regular exercise, would result in insulin resistance and obesity, leading to metabolic disorders such as diabetes mellitus (DM) (Kraegen *etal*, 1991). These disorders of metabolism have a negative impact on life expectancy and are correlated with increase incidence of high blood pressure, diseases of the heart, dyslipidemia, hypercholesterolemia, and proinflammatory states (Lind and Lithell, 1992). Currently, a lot of studies showed that patients with type II DM are prone to cognitive deficit and are highly susceptible to Alzheimer's disease (Leibson *et al.* 1997). Recent evidence also revealed a strong correlation between the pathology observed in the brains of AD and DM patients. In the brain, insulin has been shown to modulate energy metabolism, survival, proliferation and differentiation through insulin signaling transduction mechanism (Lacroix *et al.*2008). Thus dysfunctional

insulin signaling transduction mechanism causes a change in the signaling pathway, resulting in an AD-like pattern of decreased cerebral glucose metabolism in the brain (Baker *et al.* 2010). Several convincing histopathological evidence supports this suggestion. The experimental induction of DM modulates the pathogenesis of AD, such as Senile and Neurofibrillary tangles development, in several animal models mimicking DM (Ho *et al.* 2004). Type 2 diabetes may be present in up to 80% of individuals with dementia who are aged 65 years or older (Acee, 2012). Convincing evidence have shown that type 2 diabetes may increase the chances of developing Alzheimer's disease from the "pre-dementia" condition of mild cognitive impairment (Carlsson, 2010). 35.6 million People are living with dementia worldwide. This number is projected to double every 20 years (Acee, 2012; Sanz *et al.*2012). In the UK 700,000 people are estimated to have dementia and this figure is expected to rise to over 1.7 million by 2051 (Strachan *et al.* 2009), with one in 14 people over the age of 65 years and one in six over the age of 80 years living with dementia (Phillips and Phillips, 2011). Risk factors for dementia include diabetes mellitus, age and genetic predisposition (Parikh *et al.* 2011). The association between glucose, insulin, lipid metabolism and the underlying neurobiological changes observed in patients with Alzheimer's disease has also been established.

MATERIALS AND METHODS

Animal care

Adult albino Wistar rats weighing between 100-130 g were obtained from the Animal House, Department of Pharmacology, University of Uyo, Uyo, Akwa Ibom State. The animals were kept in standard cages in a well ventilated room under standard conditions (12-hour/12-hour dark cycle). Feed and water were provided *ad libitum*. Ethical approval was obtained from the College of Health Science Committee on Health Research Ethics.

Collection and Identification of *M. oleifera* Leaves

The fresh leaves of *M. oleifera* were collected within Uyo, Uyo local government area of Akwa Ibom State, Nigeria. The plant was identified and authenticated by a Taxonomist in the Department of Botany and Ecological Studies, University of Uyo and an herbarium specimen voucher number UUPH A50(i) was assigned and deposited in the same Department.

Extraction of *M. oliefera* Leaves

The plant leaves were sun dried, pulverized, macerated in absolute ethanol for 72hours. The extract was then filtered, and dried in water bath. The resultant extract was preserved in a refrigerator until when needed.

Induction of Hyperglycaemia

The basal glucose level of all the animals was determined before induction of hyperglycaemia. Hyperglycaemia was induced by rapid infusion of aloxan intraperitoneally at a dose of 150mg/kg. 72 hours after administration of aloxan, fasting blood glucose was again determined. Rats with glucose level above 180mg/dl were selected for the study. Blood glucose estimation was performed using glucometer.

Experimental Design

Animals were arranged into seven (7) groups as shown in Table 1.

Table 1: Experimental Design.

Group	Treatment
Group 1 (Normal Control)	Normal Saline
Group 2	Diabetic Treated with 500mg/kg of Moringa
Group 3	Diabetic Treated with 1000mg/kg of Moringa
Group 4	Diabetic Treated with 1500mg/kg of Moringa
Group 5	Diabetic Treated with 14.29mg/kg of Metformin
Group 6	Diabetic Treated with 1000mg/kg of Moringa + 14.29mg/kg of Metformin
Group 7 (Diabetic Control)	Normal feed and water

Treatment was done for 28 days and the final blood glucose was determined on day 28.

Neurobehavioral assessment

Memory test were carried out after day 28 of treatment. Memory test employed included the following: T – Maze simple alternation test, Novel object recognition test and Elevated plus maze.

T – Maze Simple Alternation Test

The T-maze is made up of the base, the left arm and the right arm of length of about 50cm and 10cm width. The different tasks, such as left-right discrimination and forced alternation, are mainly used with rodents to test reference and working memory. The natural tendency of rats in a T-maze, however, is to alternate their choice of goal arm. Animals were introduced from the base of the T-maze and allowed to choose one of the goal arms abutting the other end of the stem. The trial was carried out twice in quick succession. At the second trial, the rodent tended to choose the arm not visited before, reflecting a memory of the first choice. This is called 'spontaneous alternation'. Each trial was completed in less than 2min (Dember and Richman, 1989). The percentage of alternation (number of turns in each goal arm) and total trial duration was recorded.

Novel Object Recognition (NOR) Test

Novel object recognition test was performed as previously reported (Tang *et al*., 1999, 2001). This test

was performed 2 days after drug treatment. The apparatus for this task consisted of an open field maze (50.8×50.8×25.4 cm). Before the test, mice were habituated in the box for 3 days. During a training session, two objects (various objects differing in their shape and color but similar in size) were placed in the box 35.5 cm apart (symmetrically) and each animal was allowed to explore the box for 5 min. The animals were considered to be exploring the object when the head of the animal was facing the object within a distance of 2cm from the object or any part of the body, except for the tail, was touching the object. The time that mice spent exploring each object was recorded. After training, mice were immediately returned to their home cages, and the box and objects were cleaned with 75% ethanol to avoid any possible instinctive odorant cues. Retention tests were carried out at 1-day intervals following the respective training. During the retention test, each mouse was placed back into the same box, in which one of the objects used during training was replaced by a novel one. The mice were then allowed to freely explore for 3 min and the time spent exploring each object was recorded. Throughout the experiments, the objects were used in a counter-balanced manner in terms of their physical complexity. A preference index, a ratio of the amount of time spent exploring any one of the two objects (training session) or the novel one (retention session) over the total time spent exploring respective to both objects, was used to measure memory performance.

Elevated Plus Maze

The test used an elevated plus-shaped (+) apparatus with two open and two enclosed arms. EPM consisted of two open arms (16 cm × 5 cm) and two covered arms (16 cm × 5 cm × 12 cm) extended from a central platform (5 cm × 5 cm), and the maze was elevated to a height of 25 cm from the floor. The behavioral model is based on the general aversion of rodents to open spaces. This aversion leads to the behavior termed thigmotaxis, a preference for remaining in enclosed spaces or close to the edges of a bounded space. In the EPM, this translates into the animals limiting their movement to the enclosed arms. This assay essentially determines a preference between a comparatively safe and comfortable environment (the closed arms) and a risky environment (elevated open spaces). Though the Elevated Plus Maze (EPM) has been majorly used in anxiety study, it also has memory implication. The natural tendency of the rodent to avoid open space is used to test the memory of the rodent. In this study, we measured the time it took the animals to move from the open arm to the closed arm (Transfer Latency) during the initial exposure to the EPM and after 24 hour.

Immunohistochemical Staining Technique

Sections were deparaffinised using 2 changes of xylene (5 minutes each), followed by dehydration where sections were rinsed twice in 100% alcohol, 95% alcohol, 70% alcohol, 40% alcohol for 5 minutes each and finally rinsed in tap water. Antigen retrieval was performed using citric acid solution with pH 6.0 in a microwave at power 100 W for 15 minutes, thereafter sections were equilibrated by gently displacing hot citric acid with water for 3 minutes. Peroxidase in the tissues was blocked using peroxidase block for 15 minutes. Sections were washed for 2 minutes using phosphate buffered saline (PBS) mixed with tween 20. Protein was blocked using Novocastra protein block for 15 minutes and sections washed in PBS for 2 minutes. Sections were incubated with primary anti GFAP for GFAP immunohistochemical studies, 1 in 100 dilution for 45 minutes each, and sections washed with PBS for 3 minutes. Secondary antibody was added for 15 minutes and section washed twice with PBS. Polymer was added for 15 minutes and section washed twice with PBS. Diamino benzedine (DAB) was added for 5 minutes; sections washed with tap water, counterstained in haematoxylin for 2 minutes, washed, dehydrated, cleared and mounted on DPX.

Statistical Analysis

Data were analyzed with Graph pad 6 version II system package. Results were expressed as mean ± standard error of mean. One way ANOVA and multiple comparison was employed with the significance level at $p<0.05$.

RESULTS

Blood Glucose Assessment

Table 3: Blood Glucose Assessment (mg/dl).

Group/Treatment	Mean Blood Glucose (Baseline)	Mean Blood Glucose (72 Hours)
Group 1 (Normal Control)	90.60	95.40
Group 2 (Moringa500mg/kg)	87.40	192.80
Group 3 (Moringa1000mg/kg)	87.20	201.40
Group 4 (Moringa1500mg/kg)	91.60	181.60
Group 5 (Metformin14.29mg/kg)	90.00	210.80
Group 6 (Moringa1000mg/kg) + metformin 14.29mg/kg)	88.60	221.20
Group 7 (untreated)	90.60	185.20

Assessing the transfer latency of rats after 24 hours

There was no significant difference in the transfer latency between the experimental and the control groups.

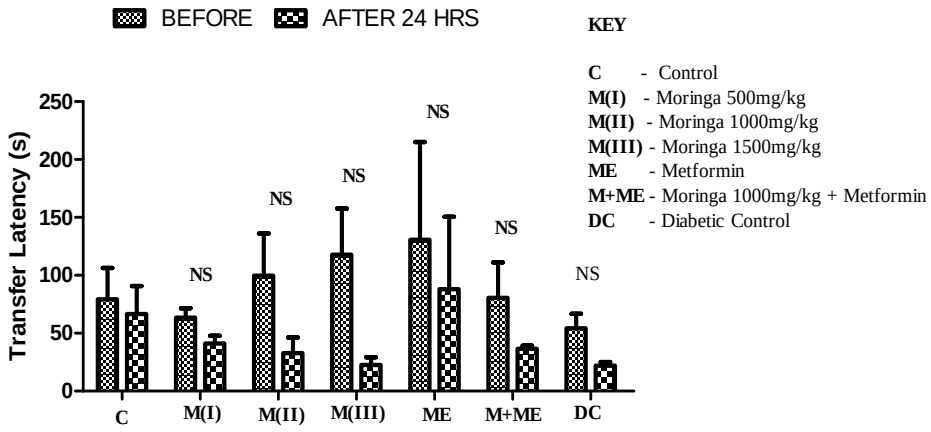

Figure 1: Comparison of the transfer latency time of animals. It shows the transfer latency for the animals in the different groups.

Discrimination ratio in rats

This comparison showed a negative (-ve) discrimination ratio for animals treated with moringa (1500mg/kg) and the untreated group while the control, animals treated with 500, 1000 mg/kg moringa, Metformin, and Metformin + Moringa (1000mg/kg) showed a positive discrimination ratios.

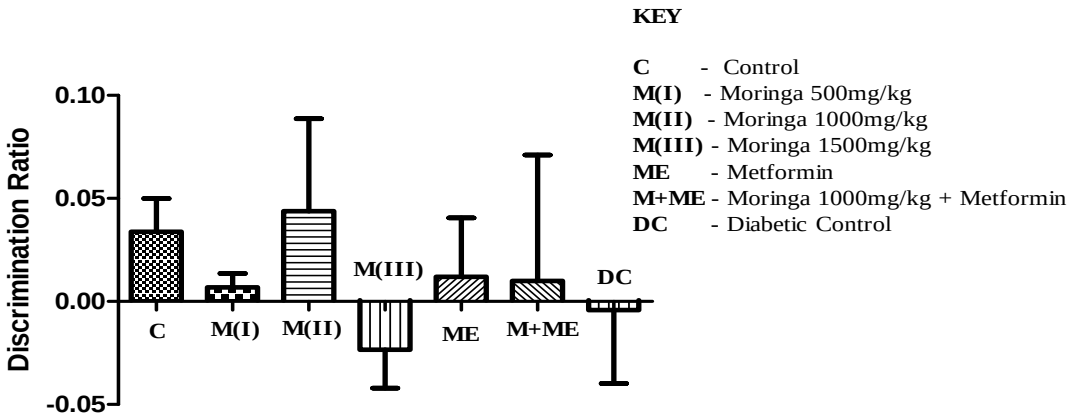

Figure 2: Comparison of the discrimination ratio in animals. There is a negative (-ve) discrimination ratio value for animals treated with moringa (1500mg/kg) and the diabetic control.

Percentage (%) of Alternation

The comparison showed no significant difference between the treated groups and the control group.

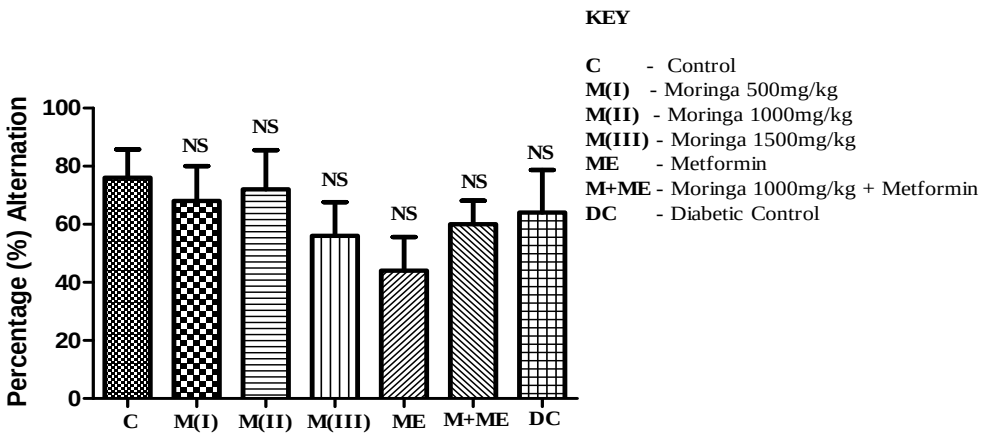

Figure 4: Comparison of the percentage (%) of alternation for the animals. It shows the alternation on simple alternation test (T-Maze) for animals exposed to different treatment protocol.

Immunolabelling Observation of the Prefrontal cortex (GFAP)

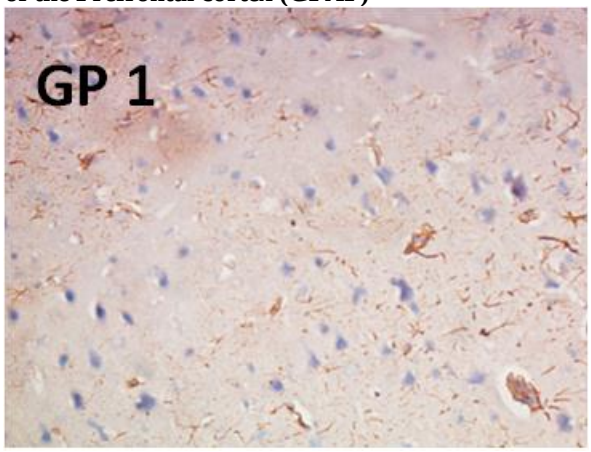

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Figure 5: Immunohistochemical section of the prefrontal cortex of group 1 rats administered with normal saline. Section of the prefrontal cortex of group 1 rats administered with normal saline showing normal distribution of the GFAP protein.

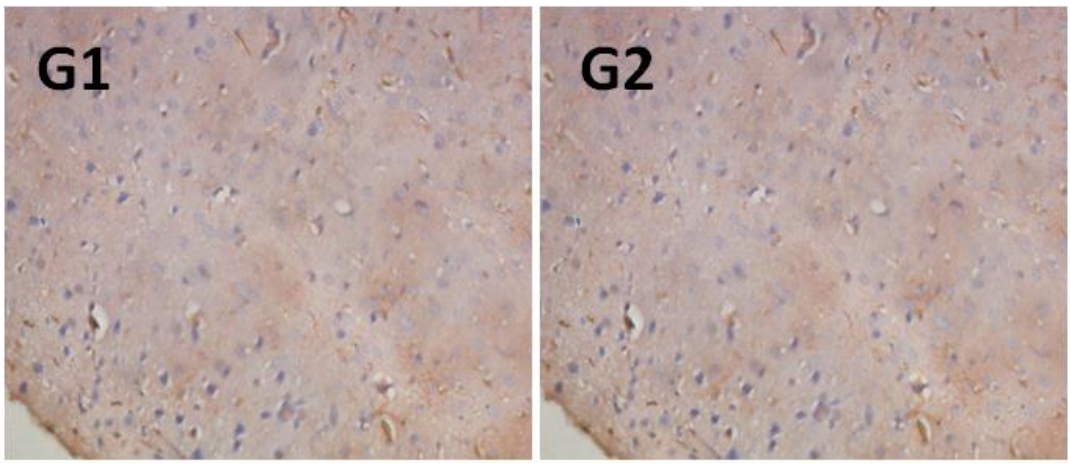

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Figure 6: Immunohistochemical section of the prefrontal cortex of group 2 animals administered with 500mg/kg of moringa leaf ethanolic extract. Section of the prefrontal cortex of group 2 animals administered with 500mg/kg of moringa leaf ethanolic extract showing normal expression of GFAP when compared with control.

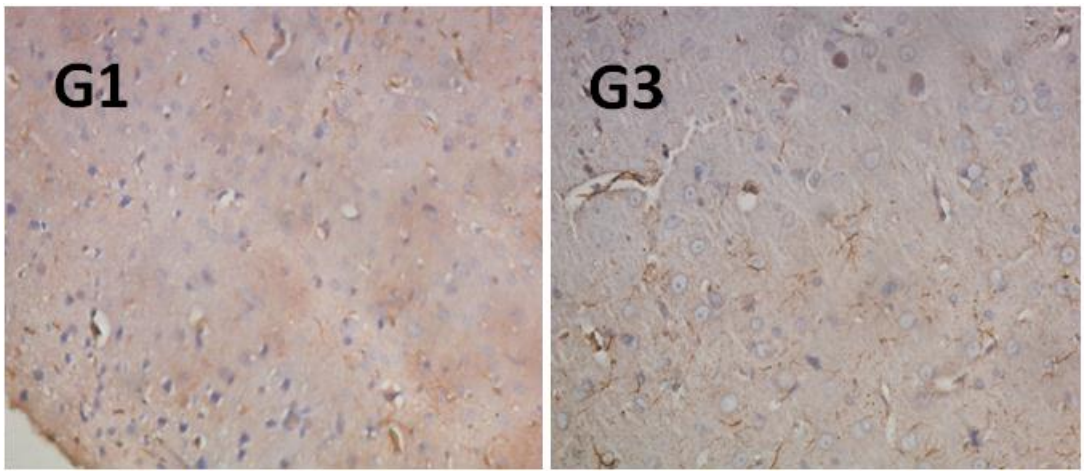

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Figure 7: Immunohistochemical section of the prefrontal cortex of group 3 animals administered with 1000mg/kg moringa leaf ethanolic extract. Section of the prefrontal cortex of group 3 animals administered with 1000mg/kg moringa leaf ethanolic extract showing normal expression of GFAP when compared with the control group.

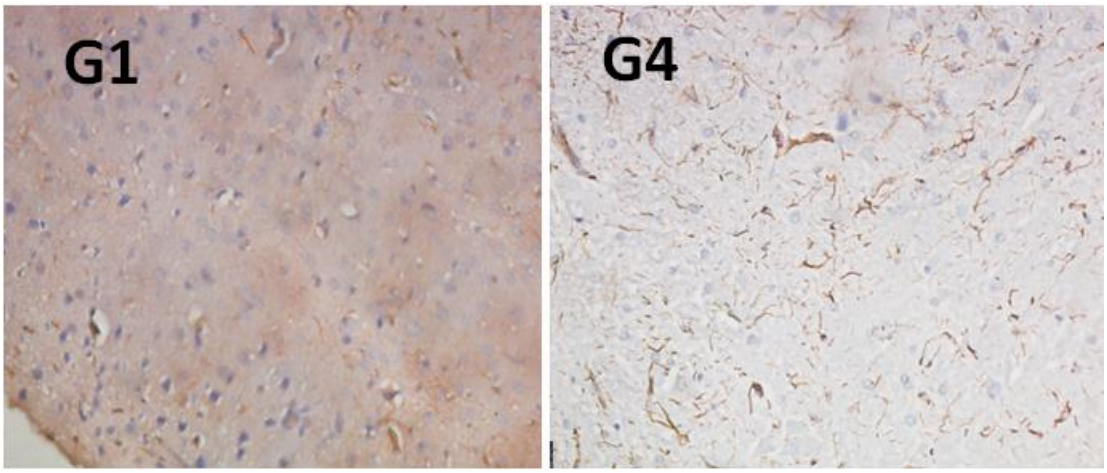

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Figure 4.28: Immunohistochemical section of the prefrontal cortex of group 4 animals administered with 1500mg/kg moringa leaf ethanolic extract. Section of the prefrontal cortex of group 4 animals administered with 1500mg/kg moringa leaf ethanolic extract showing normal expression of GFAP when compared with control.

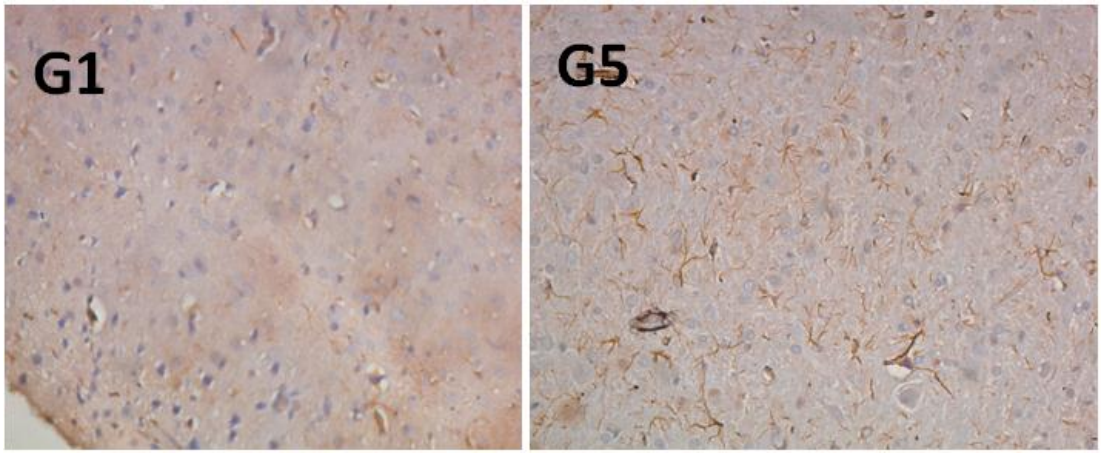

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Figure 4.29: Section of the prefrontal cortex of group 5 animals administered with metformin. Section of the prefrontal cortex of group 5 animals administered with metformin showing normal expression of GFAP when compared with control.

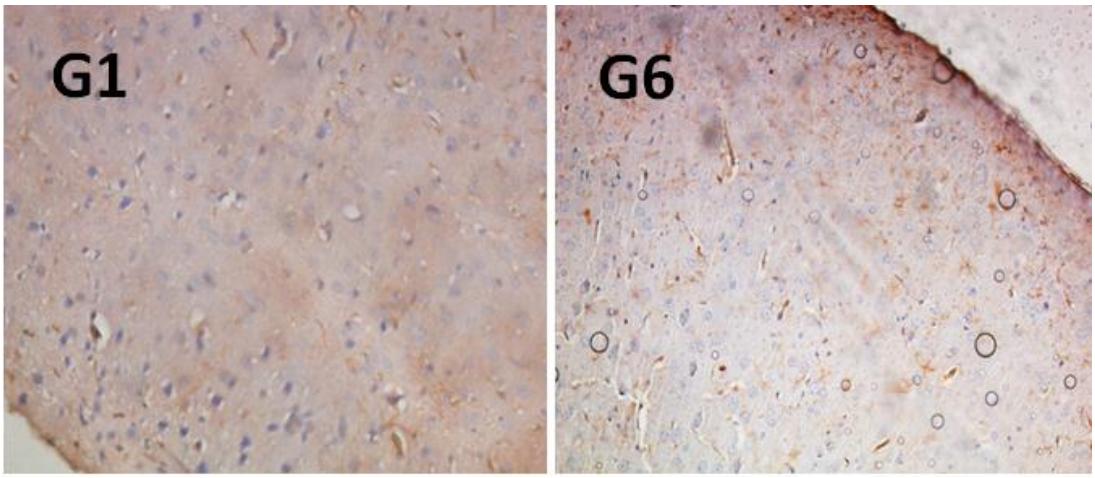

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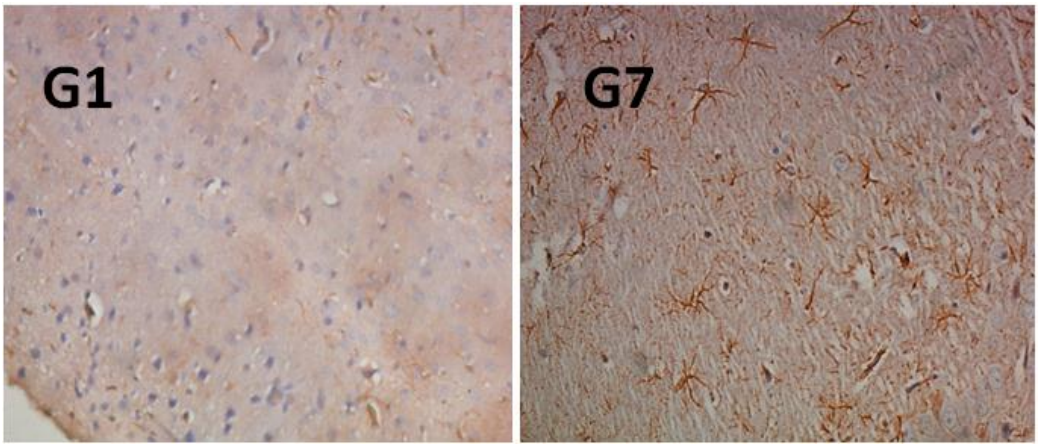
Figure 4.30: Immunohistochemical section of the prefrontal cortex of group 6 animals administered with 1000mg/kg moringa leaf ethanolic extract + metformin. Section of the prefrontal cortex of group 6 animals administered with 1000mg/kg moringa leaf ethanolic extract + metformin showing normal expression of GFAP when compared with control.



X400

Figure 4.31: Immunohistochemical section of the prefrontal cortex of group 7animals who were the diabetic control. Section of the prefrontal cortex of group 7 animals who were the diabetic control showing increased expression of GFAP when compared with control.

Immunolabelling Observation of the Hippocampus (GFAP)

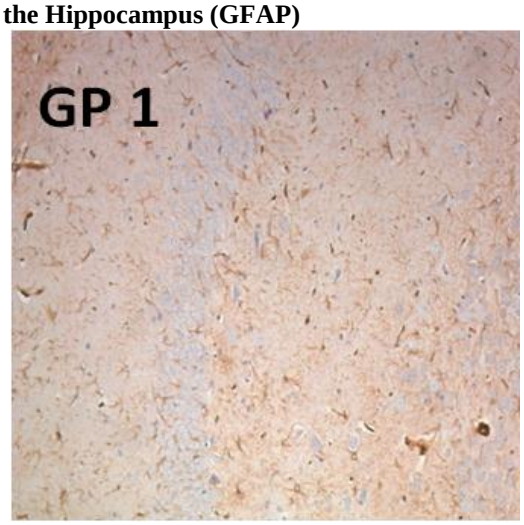


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Figure 4.32: Immunohistochemical section of the hippocampus of group 1 rats administered with normal saline. Section of the hippocampus of group 1 rats administered with normal saline Showing normal distribution of the GFAP protein.

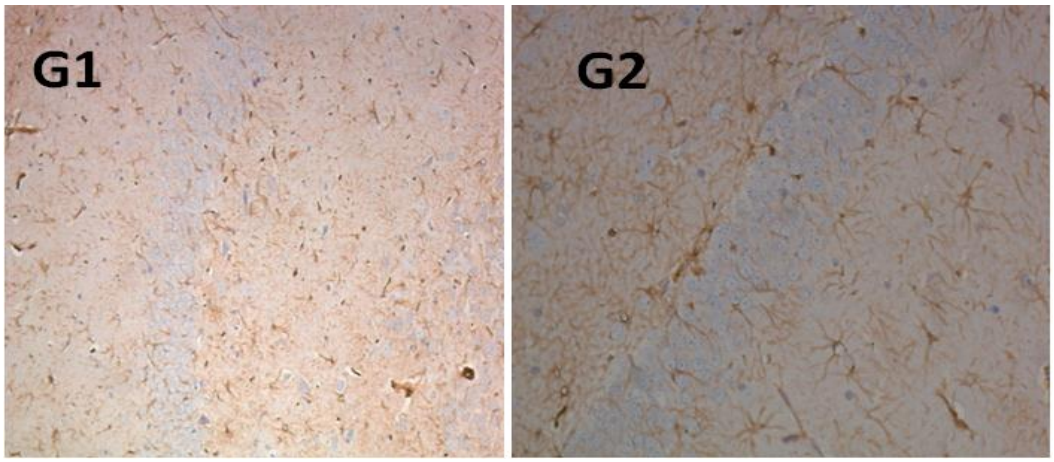

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Figure 4.33: Section of the hippocampus of group 2 animals administered with 500mg/kg moringa leaf ethanolic extract. Section of the Hippocampus of Group 2 animals administered with 500mg/kg moringa leaf ethanolic extract showing normal expression of GFAP when compared with control.

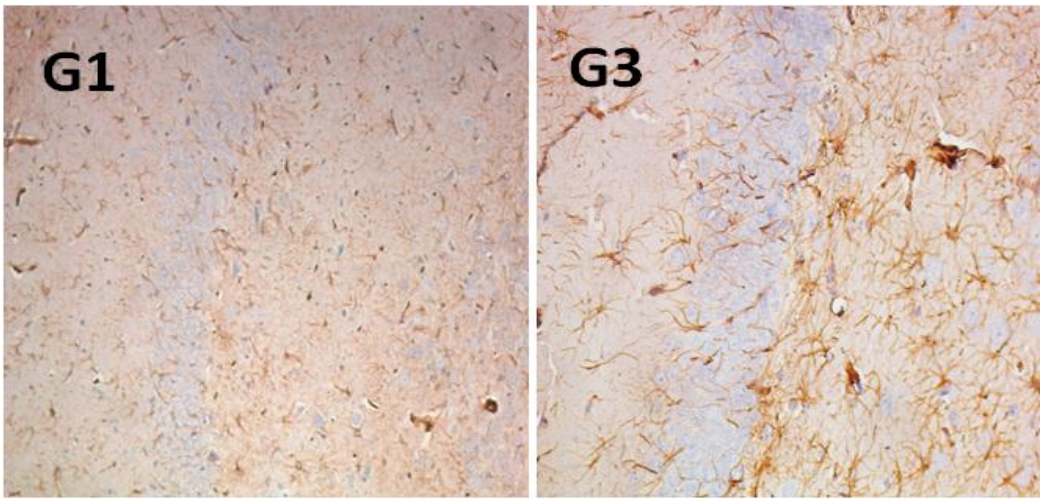

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Figure 4.34: Immunohistochemical section of the hippocampus of group 3 animals administered with 1000mg/kg moringa leaf ethanolic extract. Section of the Hippocampus of Group 3 animals administered with 1000mg/kg moringa leaf ethanolic extract showing mild increased expression of GFAP when compared with control.

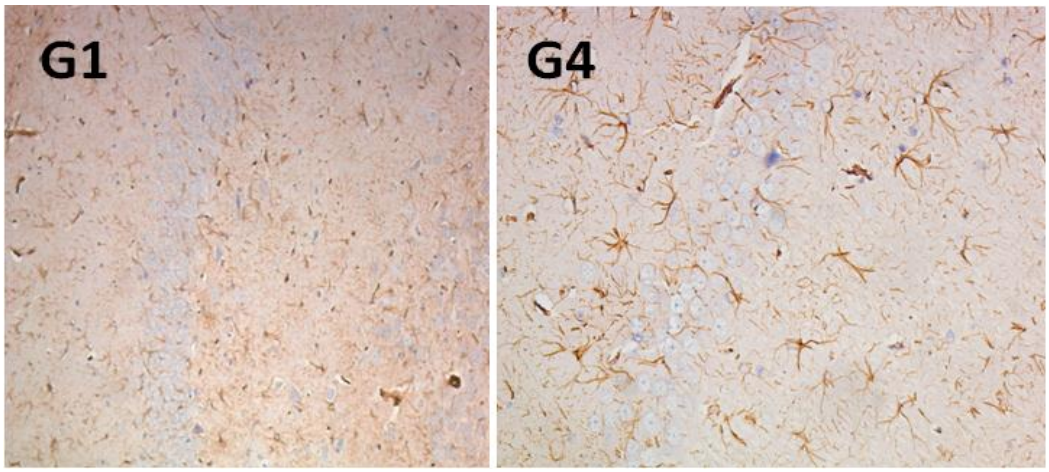

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Figure 4.35: Immunohistochemical section of the hippocampus of group 4 animals administered with 1500mg/kg moringa leaf ethanolic extract. Section of the hippocampus of group 4 animals administered with 1500mg/kg moringa leaf ethanolic extract showing mild increased expression of GFAP when compared with control.

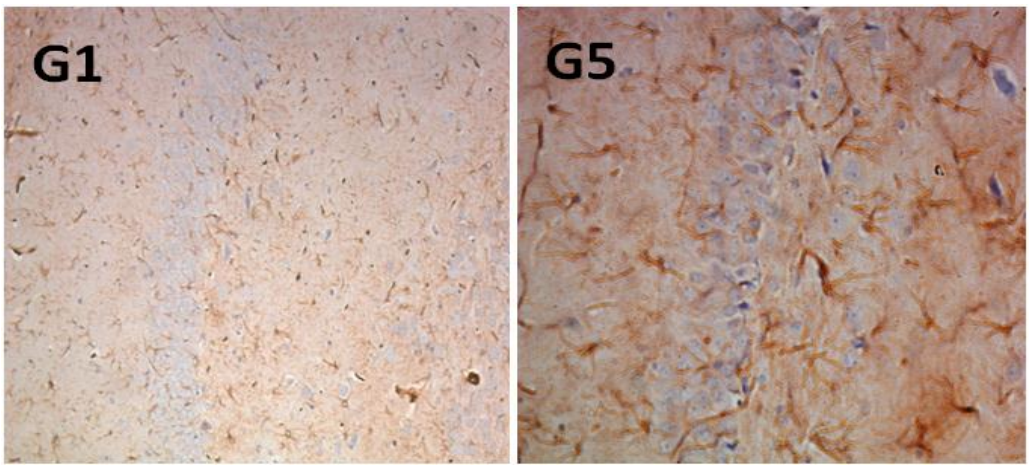

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Figure 4.36: Immunohistochemical section of the hippocampus of group 5 animals administered with metformin. Section of the Hippocampus of Group 5 animals administered with metformin showing moderate increased expression of GFAP when compared with control.

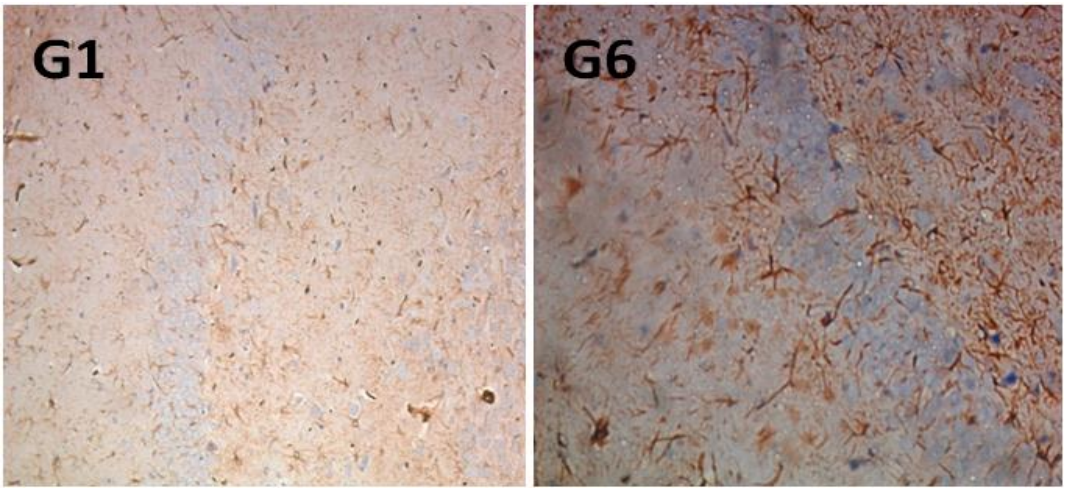

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Figure 4.37: Immunohistochemical section of the hippocampus of group 6 animals administered with 1000mg/kg moringa leaf ethanolic extract + metformin. Section of the hippocampus of group 6 animals administered with 1000mg/kg moringa leaf ethanolic extract + metformin showing mild increased expression of GFAP when compared with control.

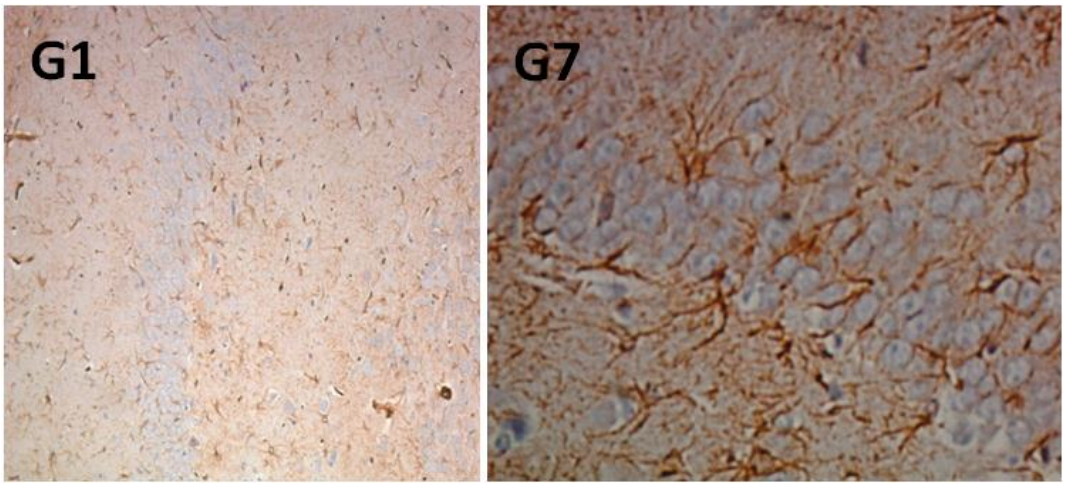

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Figure 4.38: Immunohistochemical section of the hippocampus of group 7 animals who were the diabetic control. Section of the hippocampus of group 7 animals that were the diabetic control showing severe increased expression of GFAP when compared with control.

DISCUSSION

Over the years, astrocytes have been recognized as key player in the cognitive process, the main energy reservoir of the brain (Attwell and Gibb, 2005) and also key player in neuroinflammatory conditions. Astrocytes response to insult and injury by becoming reactive which is displayed notably by hypertrophic morphology, upregulation of the cytoskeletal protein, GFAP, proliferation, loss of non-overlapping domains, and ultimately glial scar formation. The functional roles of reactive astrocytes appear to be both neuroprotective and neurotoxic.

Moringa leaf extract which was used for this study has been reported to produce anti-diabetic and anti-amnesic effects (Ganguly and Guha, 2008; Jaiswal, 2009; Sholapur and Patil, 2013). This study sought to evaluate the efficacy of ethanol extract of moringa leaves in preventing hyperglycaemic-induced cognitive impairment. In this study, behavioural studies and immunohistochemical analysis (GFAP analysis) were adopted to determine the neuroprotective effect of *Moringa* leaf ethanolic extract.

From the result of this study, there was no significant difference between the treated groups and the control groups in the T-maze and Elevated plus maze memory test. However, in the novel object recognition test, animals treated with moringa (1500mg/kg) and the untreated groups recorded a negative discrimination ratio which signifies preference to the familiar object. This negative discrimination ratio in the untreated group and the group treated with moringa (1500mg/kg) points to the negative effect of hyperglycaemia on memory.

In the immunochemical assessment, the hippocampal section showed marked upregulation of the GFAP protein in the untreated animals, mild upregulation in animals treated with moringa 1000mg/kg, moringa 1500mg/kg and moringa 1000mg/kg + metformin 14.29mg/kg. There was moderate upregulation in animals treated with metformin 14.29mg/kg. The section of the prefrontal cortex showed a mild upregulation in the GFAP in the untreated group while other groups in the section showed normal expression of the GFAP as compared to the control group.

Marked expression of the GFAP in the untreated group (In this study) is indicative of neuronal insult and damage in the group (Umegaki *et al*., 2013). Previous studies have shown that reactive astrocytes are expressed as a hallmark feature of pathology which is correlated with the intensity of neuronal dysfunction and death (Escartin and Bonvento, 2008; Salmina, 2009; Serrano-Pozo *et al*., 2011).

Normal GFAP expression was observed in treated groups. This findings indicates that *M. oleifera* was able to protect against the hyperglycemic induced neuronal damage.

This neuroprotective effect may be attributed to the phytochemicals present in the moringa leaves. The phytochemicals include phenols, vitamin C and E which are known antioxidants. This studies agree with some earlier findings that moringa leaves protect the cognitive faculties of the brain via nootropics activity by providing substantial antioxidants like its phenols, vitamin C and E to combat oxidative stress" (Ganguly and Guha, 2008).

It was also noticed that the moringa leaf ethanol extract performed better than the standard drugs (Metformin) in some treated animals from immunolabelling observations. The negative (-ve) discrimination ratio noticed in animals treated with moringa 1500mg/kg tends to portray a fact that moringa leaves may exhibit some levels of toxicity to the brain tissue in high dose, though there is no strong supportive result from the other two behavioral study and immunolabelling results for same group of animals.

It is very striking to note that though the blood glucose of the untreated animals had returned to normal 2 weeks after induction of hyperglycaemia, the effect of the initial insult was not abated 4 weeks after restoration of normoglycaemia. This was evident by the abnormal changes in the brain of the untreated animals in immunohistochemistry and poor performance in novel object test.

CONCLUSION

This study shows that oral administration of *M. oleifera* leaf extract to a diabetic patient could prevent the neuronal damage triggered by diabetes mellitus. Also combination of metformin and moringa leaf extract does not result in a synergistic effect. Metformin appeared to have cancelled out or reduce neuroprotective effects of *M. oleifera*.

Competing interest

The author(s) declare that they have no competing interest.

Authors' contributions

1. *Davies Koofreh conceived and designed the work. Critically revised the manuscript to make it fit for publication.*
2. *Nsikak Udokang analyzed and interpreted the data.*
3. *Augustine Edet carried out the field work and paid for the analysis of samples.*
4. *Uduak Inwang was responsible for acquisition of data and also participated in analyzing and interpreting the data.*

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