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RESEARCH PAPER

MORINGA OLEIFERA LEAF POWDER AMELIORATE NEURONAL DEGENERATION IN THE ENTORHINAL CORTEX OF ADOLESCENT MALE ALCOHOLIC RATS

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ABSTRACT

Moringa oleifera is an important medicinal plant that has shown great promise in CNS conditions. However, the potential protective effect of *M. oleifera* in the entorhinal cortex (EC) of adolescent rats with Alcohol Use Disorder (AUD) is still unknown. The effect of *M. oleifera* was therefore assessed in an adolescent model of AUD.Two groups of rats were oro-gastrically fed thrice daily with 5 g/kg ethanol (25% w/v), and 5 g/kg ethanol (25% w/v) plus *M. oleifera* (10 mg/kg body weight) respectively in diluted nutritionally complete diet (50%v/v). A control group was fed a nutritionally complete diet (50%v/v) made isocaloric with glucose. Cytoarchitectural study of the entorhinal cortex was examined with H&E. The extent of damage was assessed biochemically by determination of tissue levels of lipid peroxidation and protein oxidation. After 4 days of binge alcohol treatment, histologic and biochemical indices of degeneration in EC were significantly reduced by *M. oleifera* supplementation compared with the non-supplemented rats. In conclusion, *M. oleifera* attenuates alcohol-induced entorhinal degeneration in the rats by alleviating oxidative stress and reducing the expression of degenerative changes in the EC**.**

Key words: Alcoholism, Medicinal plant, Nutraceuticals, Neuroprotection

INTRODUCTION

Moringa oleifera is perhaps one of the most researched medicinal plants. It has shown great potential in several models of neurodegenerative conditions (Fahey, 2005). It was used as an antidepressant (Kaur *et al*., 2015), anti-inflammatory (Fard *et al*., 2015), anticataleptic (Joy *et al*., 2012) and as a cerebroprotective (focal ischemic stroke) agent (Kirisattayakul *et al*., 2013). In addition, leaves extract of *M. oleifera* exhibited antioxidant activity (Ijeomah *et al*., 2012) and ameliorated oxidative stress in a rat model of Alzheimer's disease (Ganguly and Guha, 2008; Ganguly *et al*., 2010). Stohs and Hartman (2015) have previously provided a comprehensive review of the safety and efficacy of *Moringa oleifera* in various disease conditions.

M. oleifera leaves is rich in essential minerals, vitamins and flavonoids (Manguro and Lemmen, 2007; Amaglo *et al*., 2010). The LD50 of the alcoholic extract of *M. oleifera leaves* was reported to be more than 2800 mg/kg (Okechukwu *et al*., 2013). Therefore, the leaves extract intake is safe at dose ≤ 1000mg kg-1 BW (Asare *et al*., 2012). However, the health benefits of *M. oleifera leaves* that are arguably hyped by scientific studies were mostly conducted with extracts. On the contrary products of *M. oleifera leaves* are available in the un-extracted form in powder or capsule formulations.

Consequently, the objective of this study was to determine if dried *eaves of M. oleifera leaves* has neuroprotective potentials in adolescent male rats exposed to binge alcohol consumption. Alcohol use disorder (AUD) is a direct sequel of binge alcohol consumption. There is currently no satisfactory clinical remedy for AUD, which is especially critical for the adolescents, where the consequences of AUD are more devastating than in the adults (Evrard *et al*., 2006; Welch *et al*., 2013). The current thought is that multimodal neuroprotective agents have high therapeutic potentials in the management of CNS conditions. Medicinal botanicals are naturally multimodal in action and hence they achieve some therapeutic synergy that is not classical with conventional medications. A search of this great potential in medicinal plants can anticipate some level of success.

MATERIALS AND METHODS

Animals: Rats were procured from the animal unit of the Department of Pharmacology, Faculty of Basic Medical Sciences, Niger Delta University, Wilberforce Island, Bayelsa State, Nigeria. The rats were accommodated in the experimental room of the Department of Pharmacology; Faculty of Basic Medical Sciences, Niger Delta University, Wilberforce Island, Bayelsa State, Nigeria. They were maintained under light/dark cycle (12/12) hours, at a (25 ± 5) °C. Rats were allowed free access to rat chow and water, except during alcohol treatment when rat chow was withdrawn.

Animal Grouping: Eighteen adolescent male *Sprague Dawley rat*s (80 –120 g) were randomly divided into 3 groups: group A (control), group B (ethanol exposed only), and group C (ethanol exposed plus *M. oleifera leaves*).

***Moringa oleifera* leaf capsule:** Gift capsules of *M. oleifera* leaf powder (200 mg per capsule) were received from the Department of Pharmacognosy & Herbal Medicine, Niger Delta University, Nigeria.

Ethical Consideration: All procedures in this study are in accordance with the Guidelines for the Care and Use of Laboratory Animals (NRC, 2010).

Binge alcohol treatment: The protocol for alcohol-induced neurodegeneration has been previously described (Obernier *et al*., 2002). Briefly, groups, B and C rats were gavaged with 5 g/kg ethanol (25% w/v in a nutritionally complete diet (50% v/v) (Vanilla Ensure). In addition to the ethanol treatment, group C rats received *M. oleifera* (10 mg/kg body weight) as a supplement. The control rats were gavaged with 5 g/kg of a nutritionally complete diet (50% v/v) (Vanilla Ensure). All treatments were repeated every 8 hours for 4 days. After the first ethanol dose of 5 g/kg in day-1, subsequent doses were based on the intoxication state of the rat as assessed with a six-point behavioral scale: 0-normal rat (5 g/kg), 1-hypoactive (4 g/kg), 2-ataxia (3 g/kg), 3-delayed righting reflex and ataxia with dragging abdomen (2 g/kg), 4-loss of righting reflex (1 g/kg) and 5-loss of eye blink reflex (0 g/kg) (Knapp and Crews, 1999).

Sample collection: Rats were anesthetized with a mixture of ketamine (75 mg/kg) and diazepam (2.5 mg/kg) (ip) and humanely decapitated. Heads with brains in-situ in respective cranial cavities were completely immersed in 10% formalin saline for 48 hours. Post-fixed brains were exposed and excised from the cranial cavities. The entorhinal cortex was isolated for histological study.

Tissue processing: The brain tissue samples were subjected to routine tissue processing techniques and embedded in paraffin wax overnight, serially sectioned at 5cm thickness with a rotary microtome, mounted on a glass slide and stained with Haematoxylin and Eosin (Kumar and Kiernan, 2010).

Semi-quantitative evaluation of histological sections: randomly selected sections (n = 5 per group) were evaluated by light microscopy for tissue damage and neurodegeneration (shrinkage of the neuron, hyperchromasia, and nuclear pyknosis) (Kuo *et al*., 2011). A semi-quantitative neurodegeneration scale based on the 2nd layer of the entorhinal section was adapted and scored thus: 1 point for normal appearance; 2 points for a few degenerated neurons among normal neurons; 3 points for a lot of degenerated neurons with scattered normal neurons; and 4 points for complete degeneration with no residual normal neurons. Each of the two regions of the entorhinal cortex was scored. "Degeneration score" was designated as the average of these scores per section. Scoring was done on a 4 mm by 4 mm grid graticulate at 400X magnification. Grid was orientated to enclose the 2nd layer randomly selected sections. The scores from all the sections from each group were averaged to give a final score for each group. Values were expressed as mean ± SEM.

Photomicrography: The microscopic image was captured by a full HD microscope camera attached to the triocular of a compound microscope. Digital photomicrographs were transmitted directly into an attached computer (Microsoft windows 10 enterprise) by the microscope camera. Representative images were labeled and saved for histological analysis.

Biochemical assessment of entorhinal damage: Randomly selected rat's entorhinal cortex tissue samples from each of the groups were subjected to biochemical analyses. The extent of tissue damage in association with or without our treatment regime was assessed by determining the level of advanced oxidation protein products (AOPP) and malondialdehyde (MDA) in the sampled tissues. These are biomarkers of oxidative stress in proteins and lipids respectively. Brain of anaesthetized rats (n = 4 per group) were quickly removed from the cranial cavity and homogenized in 1:4 volume of 0.1M phosphate buffer (pH 7.4). The homogenates were centrifuged at 8000rpm for 10 min at 4°C to yield a clear supernatant fraction that was used for all the biochemical analysis.

Assay of AOPP levels: Spectrophotometric determination of AOPP levels was performed by Witko's method (2003). Samples of entorhinal cortex, two hundred microliters were diluted 1/5 in 20 mMPBS pH 7.4 and10 µl of 1.16 M potassium iodide was added to each tube, followed by 20 µl of 10% acetic acid. The absorbance of the reaction mixture was read immediately at 340 nm, against a blank, containing 1000 µL of 20 mM PBS, 10 µL of potassium iodide and 20 µL of acetic acid. Chloramine T solution (0-100 µmol/L) was used as calibrator. The chloramine T absorbance at 340 nm is linear within a range of 0-100 µmol/L, and AOPP concentrations were expressed as µmol/L of chloramine T equivalents.

Determination of malondialdehyde (MDA): The assay for brain lipid peroxidation was done by the method of Wright *et al*., (1981) with some modifications. The reaction mixture in a total volume of 3.0 ml containing 0.4 ml aliquot of brain homogenate was mixed with 1.6 ml 0.15 M Tris-KCl buffer pH 7.4to which 0.5 ml of TCA (10%), and 0.5 ml TBA (0.75%) were added. All the test tubes were placed in a boiling water bath for a period of 45 min. The tubes were shifted to ice bath and then centrifuged at 3000xg for 10 min. The amount of malondialdehyde (MDA) formed in each of the brain samples were assessed by measuring the optical density of the supernatant at 532 nm. The results were expressed as the µmol MDA formed/gram of tissue by using a molar extinction coefficient of 1.56×10^5 M^{-1} cm^{-1}.

Statistical analysis: The one-way analysis of variance and the Turkey's post-hoc analysis were used to assess for intergroup differences (GraphPadPrism-5, San Diego, USA). Values were expressed as mean ± SEM. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Histopathologic analysis: The six layer of the entorhinal cortex was very distinct in the control group. In the alcohol group, only the outer two layers were recognizable, the inner four layers were not clearly delineated. In the *M. oleifera* group, the six layers were fairly evident. In the three groups, Layer I was poorly cellular and Layer II contained cell islands of rounded cells, as expected. The nuclei of the control group show prominent nuclei (white arrows, Figure 1). However, this was not evident in the *M. oleifera* group. The alcohol group showed condensed nuclei (black arrow head, Figure 1). Layer III contained cells of various sizes and shapes, but the medium pyramidal cells were the predominant type. Layer IV was a cell sparse layer. Layer V had large pyramidal neurons. Layer VI was characterized by the presence of cells of various sizes and shapes.

Semi-quantitative analysis of neuronal damage: Semi-quantitative assessment of the neurons in the 2nd layer shows that the level of neurodegeneration in the entorhinal cortex was significantly higher ($p < 0.01$, Fig 2) in the alcohol fed rats compared with the control. However, *M. oleifera* supplementation to alcohol fed rats significantly reduced ($p < 0.05$) the extent of neuronal degeneration in entorhinal cortex (Figure 2).

Biochemical assessment of entorhinal cortex damage (MDA and AOPP): To biochemically assess the level of entorhinal cortex damage with or without *M. oleifera* supplement in EtOH exposed rats, the levels of lipid peroxidation (LP) in the entorhinal cortex were determined by the tissue MDA concentrations. Mean MDA was highest in the alcohol group and lowest in the control group (Figure 3). Tissue MDA level is directly proportional to the degree of tissue damage. The administration of *M. oleifera* to alcohol-fed rats lowered the mean MDA level compared with the rats that were fed alcohol without *M. oleifera* supplement (alcohol group). ANOVA result showed that mean MDA level of the alcohol group was significantly different from the control group ($p < 0.001$) and from the *M. oleifera* group ($p < 0.001$).

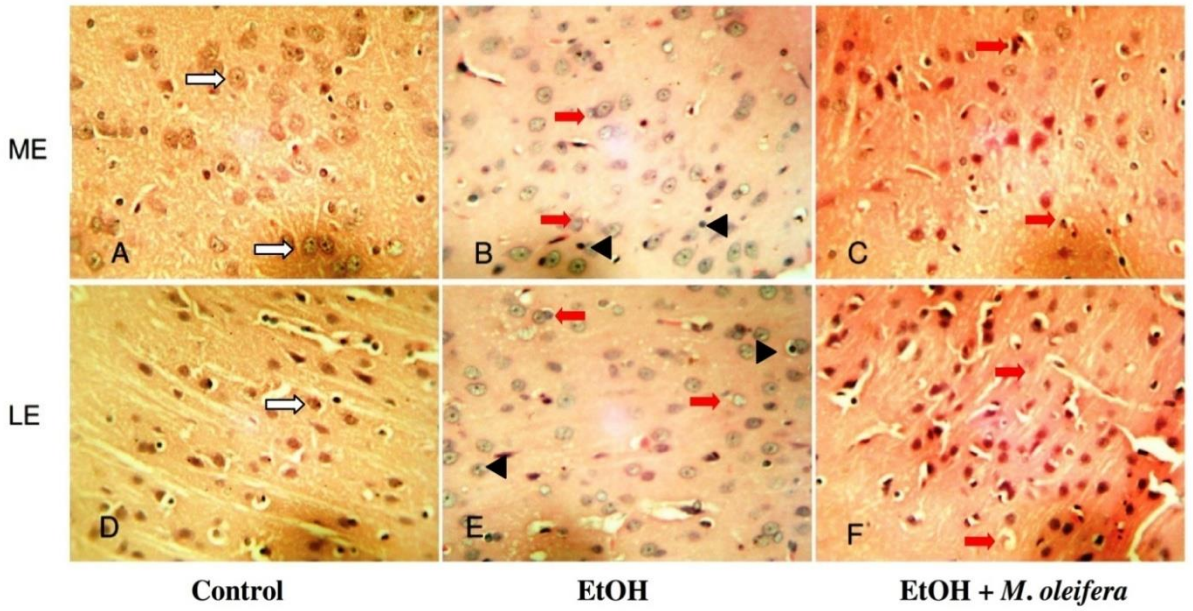

Figure 1: *M. oleifera* reduced the degree of neuronal degeneration in entorhinal cortex of alcoholic rats.

The morphology of neurons in the medial and lateral entorhinal cortices is shown (Figure 1). Histopathologic analysis revealed that the neurons in the ME and LE cortices were normal in the control group (Fig 1A and D respectively). The alcohol group exhibited moderate to severe degenerative changes (Fig 1B and E). *M. oleifera* fed rats showed mild to moderate degenerative changes in the ME and LE (Figs1C and F respectively). Red arrows and black arrow heads show degenerating neurons. H/ E Magnification X 400

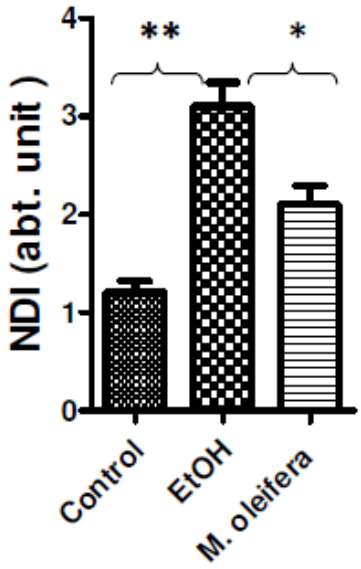

Figure 2: *M. oleifera* significantly reduced the level of neuronal degeneration in entorhinal cortex of alcoholic rats.

There was no significant difference between the control and *M. oleifera* groups. Similarly AOPP level in the entorhinal cortex of the three groups was determined as the tissue chloramines-T equivalents. High tissue level of AOPP indicated high level of oxidative damage, while a low level indicated a low level of oxidative damage. Mean AOPP was highest in

the alcohol group and lowest in the control group (Figure 4). *M. oleifera* supplementation to alcohol exposed rats significantly reduced the mean level of AOPP ($p < 0.05$) compared to the alcohol group.

Semi-quantitative analysis of neuronal damage: Semi-quantitative assessment of the neurons in the 2nd layer shows that the level of neurodegeneration in the entorhinal cortex was significantly higher ($p < 0.01$, Fig 2) in the alcohol fed rats compared with the control. However, *M. oleifera* supplementation to alcohol fed rats significantly reduced ($p < 0.05$) the extent of neuronal degeneration in entorhinal cortex (Figure 2).

Biochemical assessment of entorhinal cortex damage (MDA and AOPP): To biochemically assess the level of entorhinal cortex damage with or without *M. oleifera* supplement in EtOH exposed rats, the levels of lipid peroxidation (LP) in the entorhinal cortex were determined by the tissue MDA concentrations. Mean MDA was highest in the alcohol group and lowest in the control group (Figure 3). Tissue MDA level is directly proportional to the degree of tissue damage. The administration of *M. oleifera* to alcohol-fed rats lowered the mean MDA level compared with the rats that were fed alcohol without *M. oleifera* supplement (alcohol group). ANOVA result showed that mean MDA level of the alcohol group was significantly different from the control group ($p < 0.001$) and from the *M. oleifera* group ($p < 0.001$). There was no significant difference between the control and *M. oleifera* groups. Similarly AOPP level in the entorhinal cortex of the three groups was determined as the tissue chloramines-T equivalents. High tissue level of AOPP indicated high level of oxidative damage, while a low level indicated a low level of oxidative damage. Mean AOPP was highest in the alcohol group and lowest in the control group (Figure 4). *M. oleifera* supplementation to alcohol exposed rats significantly reduced the mean level of AOPP ($p < 0.05$) compared to the alcohol group.

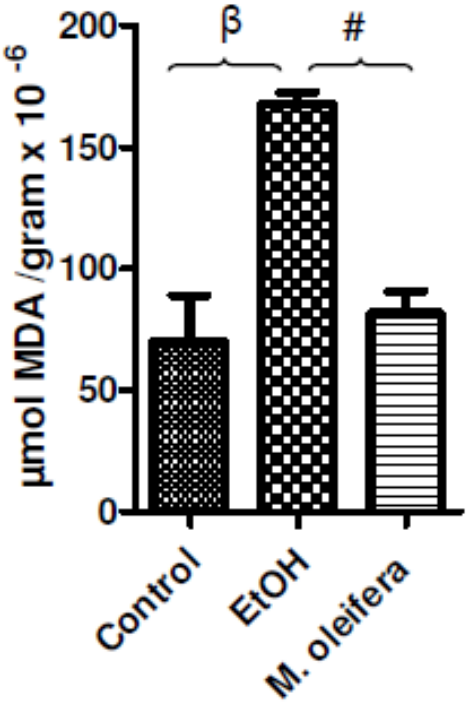

Figure 3: *M. oleifera*significantly reduced the level of lipid perooxidation in entorhinal cortex of alcoholic rats. β = p< 0.001; # = p< 0.01.

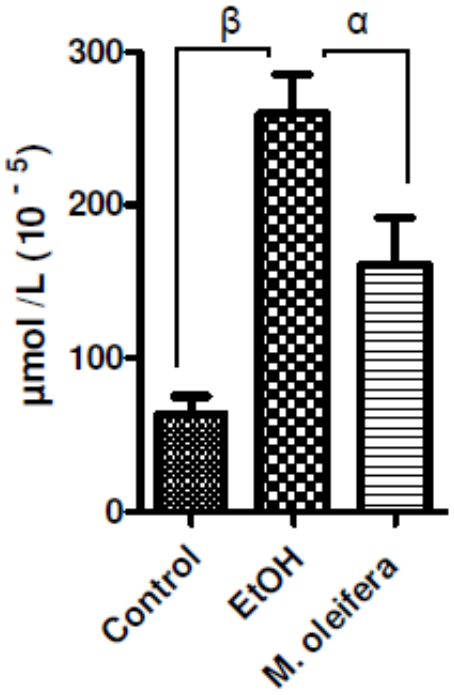

Figure 4: *M. oleifera* significantly reduced the level of protein oxidation in entorhinal cortex of alcoholic rats. α = p <0.05; β = p < 0.001.

DISCUSSION

The present study evaluated the neuroprotective potentials of dried *M. oleifera leaves* in an adolescent rat model of AUD. Our study demonstrated that *M. oleifera leaves* could protect the entorhinal cortex from degeneration during binge alcohol intoxication in the adolescent rats, which hitherto, has not been reported. Although, studies have reported that *M. oleifera* has neuroprotective element (Sutalangka *et al*., 2013; Giacoppo *et al*., 2017), but none has specifically reported the neuroprotective effect of *M. oleifera* in adolescent rats exposed to binge alcohol. Though, the present study agrees with the previous reports that *M. oleifera* is a neuroprotective agent, it, however, does not lay weight to this fact, but that *M. oleifera leaves*, in the dried unprocessed form (ie, as commonly consumed in developing countries) can protect the neurons from alcohol induced degeneration. The word of Hippocrates: "Let food be thy medicine and medicine be thy food", may be wholly true in this case, though there is very little agreement as to what Hippocrates really meant. In the current study, the histological profiles of control, alcohol and *M. oleifera* groups were compared, and it was observed that *M. oleifera* administered to alcohol-exposed rats significantly lowered the neurodegenerative changes in the EC, compared to the rats exposed to alcohol without *M. oleifera* administration (Figure 1, $p < 0.05$). Which suggested, though based on morphological characterization, that *M. oleifera* may have safeguarded the cells of the entorhinal cortex from alcohol-induced damage.

The CNS, compared to other human systems, relatively has a high lipid and oxygen content, making it very susceptible to oxidative damage (Adibhatla *et al*., 2010). To determine if the neuroprotective effect of *M. oleifera* is related to its antioxidative property, we estimated the levels of protein oxidation (AOPP) and lipid peroxidation (MDA) in samples of the EC. High levels of protein oxidation and lipid peroxidation is associated with tissue oxidative stress (Tonin *et al*., 2014; Shetty *et al*., 2015). The present study demonstrated that an unprocessed *M. oleifera* leaves have antioxidant activity in binge alcohol intoxication. *M. oleifera* significantly reduced the levels of AOPP and MDA in alcohol exposed rats. We, therefore, concluded that the protective effect of *M. oleifera leaves* in adolescent AUD rats is plausibly associated to its anti-lipid peroxidative properties. The antioxidant activity of *M. oleifera leaves* has been reported previously (Vongsak *et al*., 2013; Stohs and Hartman, 2015).

CONCLUSION

The results of this study suggest that powder from *M. oleifera leaves* attenuate alcohol-induced neurodegeneration by reducing degenerative changes and alleviating oxidative stress in the entorhinal cortex. Hence, *M. oleifera* leaves, as it is commonly used in powder formulation can be beneficial in neurodegenerative condition.

DISCLOSURE STATEMENT: The authors declare that they have no competing financial interests.

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AUTHORS' CONTRIBUTIONS

Oyinbo C.A and Robert, F.O were involved in the conception, design, animal experimentation, tissue analysis, interpretation of data, statistical analysis, and manuscript write-up. Johnbull T, Atoni A.D were involved in the design, animal experimentation, tissue analysis, interpretation of data, and manuscript write-up.