Investigating Potential Ameliorative Effects of *Moringa Oleifera* Leaf Ethanolic Extract on the Visual Cortex Retinopathy Wistar Rats Models

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**ABSTRACT:** It is important to study possible relationships between retinopathy and visual cortex integrity. The human retina is a very delicate component of the human eye. Due to the retina’s vital role in visual perception, damage to it can cause permanent blindness. Conditions such as retinopathy can compromise the integrity of its vascularity thereby preventing visual signals being sent to the brain hence leading to vision impairment or permanent loss of vision. The visual cortex, located in the occipital lobe of the brain, is the center for visual signals, sent from the retina through the optic nerve and the associating visual pathway components, translation into its original meaning. Lesions to the visual cortex automatically translate into vision integrity compromise. Retinopathic conditions basically occur due to oxidative reactions and *Moringa oleifera* leaves phytochemicals have been proven to contain potent anti-oxidants. Hence, this investigation studied the visual cortex structural and functional integrity in established condition of retinopathy using a 50mg/kg body weight dosage of sodium iodate (NaIO₃). Four Groups of animals labeled A, B, C and D to serve as control [A], retinopathic group [B], treated group with lower dose of moringa extract [C] and treated group with higher dose of moringa extract [D] were studied respectively. Experiment lasted 11 days. Results showed retinopathy was induced, but without extensive damage to the visual cortex structural integrity. However, astrocytic reactions suggested mild assault to the visual cortex as well as elevated levels of the glutamate and GABA in the brain tissues. *Moringa* extract as used, however did not show significant evidence to ameliorate the observed changes associated with retinopathy.

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I. Introduction

The eye is the organ of sight for humans and mammals. Sight provided ability for interaction with the environment and the perception of light happens exclusively within the eye. Special nerve cells, called photoreceptors, absorb light rays with a spectrum of 400-700 nm (Solomon. *et al.*, 2007) and transform it into an electrical potential stimulus which then is transferred along the optic nerve to visual cortex located within the occipital lobe of the human brain. The retina of the eye is responsible for photoreception. Through the visual pathway, perceptions are transported to the visual cortex for processing and higher mental manipulations and responses. In trying to account for the multiplicity of visual areas, two main principles have been suggested namely: Hierarchical processing and Functional specialization. Hierarchical processing proposes that visual perception is achieved through a gradual process in which information is first represented in a localized and simple form and, through a sequence of processes, is transformed into more abstract, holistic, and even multimodal representations (DeYoe. *et al.*, 1988). Functional specialization, proposes that specialized neural pathways exist that process information about different aspects of the visual scene. In particular the visual system may consist of parallel hierarchical sequences, or processing streams, that are specialized for a particular functional task. The dorsal stream, also referred to as the “where” (Mishkin *et al.*, 1983) or “action”(Goodale *et al.*, 1991) stream, has been associated with spatial localization (or visually guided action) and the ventral “what” stream (Mishkin *et al.*, 1983), which are involved in object and form recognition and are the best-known of the processing streams.

*Moringa oleifera* (MO), also known as drumstick or horse radish tree) is a highly nutritious plant and consumed in many tropical and subtropical countries. The green pods are edible as well as the fresh leaves are due to their high nutritional composition (Mbikay. *et al.*, 2012). According to (Dalla. *et al.*, 1993) its leaves, pods, and flowers are packed with nutrients important to both humans and animals. It grows fast and reaches up to 12 m tall. The wood is light, but provides a fairly good fuel for cooking. It has density of 0.5-0.7 and yield approximately 4,600kcal/kg (Fuglie. *et al.*, 1993). The seeds are usually in water purification (Muyibi. *et al.*, 2017).
The leaf is a rich source of β-carotene, protein, minerals and flavonoids (zeatin, rutin, quercetin, beta-sitosterol, caffeoylquinic acid and kaempferol). It has been found to possess hypoglycemic (Gupta et al., 2012), anti-inflammatory (Muangnoi et al., 2012), anti-oxidant (Gupta et al., 2012) and hypolipidemic (Adisakwattana and Chanathong, 2011) properties. Mahajan et al. (2009) evaluated anti-inflammatory activity from the n-butanol extract of the seeds of Moringa oleifera against ovalbumin-induced airway inflammation in guinea pigs (Mahajan et al., 2009). The free radical scavenging effect of Moringa oleifera leaf extract was comparable with that of the reference antioxidants (Sreelatha et al., 2009).

Age-related macular degeneration (AMD) or (ARMD), accounts for about half of the cases of vision impairment in developed countries. As the disease only affects elderly people over the age of 50, the prevalence of AMD is increasing with the demographic shift towards an ageing society (Jager et al., 2008). The macula is the region of the eye affected. It is a circular area at the center of the retina, responsible for high-resolution vision. Sufferers of AMD lose their ability to read and identify minute details hence, the disease has a huge impact on the daily activities of affected people (Jager et al., 2008). The first changes in the retina do not impact the visual acuity and are only visible as small yellow spots called drusen and hyper- or hypo-pigmented changes of the macula during an ophthalmological examination (Chakravarthy et al., 2006). Drusen are focal deposits of debris between retinal pigment epithelium (RPE) and Bruch’s membrane and their growth and extension are major indicators for a progress of AMD, but their exact formation pathway is not well established (Jager et al., 2008). The multi-factorial role of oxidative stress in retinal aging and the pathology of AMD has made treatment approaches complex.

Sodium iodate can be used to induce retina damage or retinopathy, especially in experimental animals. Sodium iodate is a white crystalline salt (Budavari, 1996). It is also known as an Organic salt, iodic acid, and sodium salt (Nikitakis et al., 1991): Registry of Toxic Effects of Chemical Substances [RTECS], 1992). Salts acts as a carrier of iodine, which helps to populate areas that are deficient of iodine. Sodium iodate is achieved by adding 20 to 80 mg of iodine per kilogram of sodium salt. NaIO₃ is a powerful oxidizer which is a fire hazard, when it comes in contact with flammable or combustible materials, it triggers fire (Sax, 1975). The toxic effect of sodium iodate (NaIO₃) on the retinal pigment epithelium (RPE) is well known (Noell, 1953, 1954; Grignolo et al., 1966). Earlier studies on the effect of NaIO₃ on the RPE used the electroretinogram (ERG) and concentrated on the c-wave, which arises in part from the RPE (Textorius & Weilinder, 1981; Nao-I et al., 1986) to assess the functional changes of the RPE. Studies have been conducted on the effects of NaIO₃ on the retina using the b-wave of the ERG in mice (Adachi-Usami et al., 1992; Hosoda et al., 1993). In those studies, it was discovered that there was an increase in the b-wave amplitude and a shift of the intensity-response curve towards higher stimulus intensities 24 hours after the intravenous injection of iodate, but 96 hours after injection the waves were greatly reduced. Histopathologic examination of the retinas at 24 hours showed severe damage in the outer layer of the retina and in the RPE. In fluorescein angiographic studies, there was a breakdown of the RPE barrier within 24 hours after the intravenous injection of iodate, but between 1 and 2 weeks after the injection, no leakage of fluorescein could be seen (Ringvold et al., 1981). The destruction of the RPE with intravenous injection of NaIO₃ caused choriocapillar atrophy, and after 1 week there was no fluorescein leakage (Korte et al., 1984).

The aim of this research was to investigate the ameliorative effects of ethanolic extract of Moringa oleifera leaf against the harmful effects of sodium iodate-induced retinopathy on the visual cortex of adult male Wistar rats. Increased oxidative stress and inflammatory mediators are implicated in the development of diabetic retinopathy, in adult male Wistar rats, and its development can be prevented or combated by the use of antioxidants. Powerful antioxidants, present within Moringa oleifera phyto-chemicals hence may not only aid in curbing retinopathic conditions but also present a relatively available and affordable alternative for individuals suffering from various types of retinopathy Age-related Macular Retinopathy (AMD).

II. Materials And Methods

The M.oleifera leaves were obtained from the Nigerian Natural Medicine Development Agency Federal Ministry of Science and Technology in powder form. The weight of sample collected was 11.9kg and the weight of dry sample was 2.96kg. The moisture content of wet sample was 75.15%. Sodium Iodate was obtained from Sigma Aldrich and stored in a cool and moisture free environ so as to prevent decomposition by heat or dissolution by water. The animals were first allowed to acclimatize for approximately 1 week in a well ventilated room before commencement of the bench work. The animals were kept in aerated plastic cages with constant supply of Standard Rat chow and water.

The 32 adult male Wistar rats (Rattus norvegicus) weighing between 100-160g were purchased from the Babcock University animal house. The rats allowed after which they were divided into four groups, i.e. A, B, C and D. The rats were kept in transparent plastic holdings (Cages) designed to hold containers meant for feed and water. The rats were divided into four groups as shown in the table below:
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Table 1: Table showing the grouping and treatment of experimental animals

<table>
<thead>
<tr>
<th>GROUPS</th>
<th>TREATMENT</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Control: distilled water only along with standard feed for 11 days.</td>
</tr>
<tr>
<td>B</td>
<td>Sodium iodate (50mg/kg body weight) (Zhou et al., 2014) was administered once on day 1; along with standard feed and distilled water for 11 days.</td>
</tr>
<tr>
<td>C</td>
<td>Sodium iodate (50mg/kg body weight) (Zhou et al., 2014) was administered once on day 1 and Ethanolic extract of M.oleifera leaves (500mg/kg body weight) (Owolabi et al., 2014) for 10 days along with standard feed and distilled water for 11 days.</td>
</tr>
<tr>
<td>D</td>
<td>Sodium iodate (50mg/kg body weight) (Zhou et al., 2014) administered on day 1 and Ethanolic extract of M.oleifera leaves (200mg/kg body weight) (Owolabi et al., 2014) for 10 days along with standard feed and distilled water for 11 days.</td>
</tr>
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Relative Organ Weight (ROW) was calculated by dividing the organ of interest, weight by the body weight of the animal from which the tissue was excised. This was necessary in order to evaluate organ mass in relation to the body mass of the animals. Thus,

\[
\text{(organ of interest)ROW} = \frac{\text{organ weight}}{\text{body weight}}
\]

The experimental subjects were euthanized through cervical dislocation 24 hours after the final administration, after which, the fur around the neck and brain was removed using a scalpel. Thereafter, the animal was decapitated; skull was opened enhancing the removal of the brain which was subsequently weighed. After weighing, a section of the pre frontal cortex was sectioned into sucrose solution in specimen bottle and stored in a freezing chamber to enhance preservation while the rest of the brain was immersed in 19% formal saline solution contained within a sample bottle.

Tests: Homogenisation

The pre-frontal cortices of the rat specimen already distributed into specimen bottles were extracted from the sucrose solution, placed in a clean and dry ceramic mortar and pounded with the use of a pestle in 1ml of distilled water. The cortices were homogenized one after the other according to their categorization and the resulting homogenates were obtained. The homogenates were collected individually into clean and dry centrifuge bottles, and then placed in a centrifuge for 20 minutes at 70r.p. Centrifugation was continued even after 20 minutes if some homogenate were not centrifuged properly after which decantation was carried out in order to separate the supernatant from tissue precipitate.

Tissue Processing for Histological Procedure

Histology of the visual cortex of the various groups was demonstrated using Haematoxylin and Eosin staining procedure. The excised required tissue was fixed in 10% formal saline in order to prevent decomposition. The tissue sample was dehydrated by passing them through increasing concentrated alcohol solutions ending in 100%. The alcohol content of the tissue was then removed by passing through toluene; a process known as clearing. Afterwards, the tissue sample was placed in melted paraffin until they were completely infiltrated in the substance and allowed to further harden with the excess shaved off in order to prepare for microtome sectioning. After sectioning, the paraffin was removed with xylene, hydrated in alcohol solutions of varying concentrations. The slides were then placed in haematoxylin stain for approximately 12 minutes, rinsed with distilled water and then further immersed in eosin for approximately 2minutes, hydrated with alcohol of varying concentrations, left to dry and then covered using a cover slip.

Neurotransmitter Assay

The neurotransmitter assayed for in this study is glutamate and gamma amino butyric acid (GABA)

Gamma Amino Butyric Acid (GABA)

The tissue samples were rinsed in cold phosphate buffer saline (PBS) (0.01 mol/ L, pH 7.0- 7.2) so as to eliminate excess blood. The tissues were shredded into tiny pieces and homogenized in 5- 10ml PBS with a glass homogenizer on ice. The resulting suspension was sonicated with an ultrasonic cell disrupter to further fragmentize the cell membranes. After that, the homogenates were centrifuged for 5 minutes at 500xg. The supernatant was removed and assayed.

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Glutamate Procedure

10 ml of the 0.1 M Glutamate Standard was diluted with 990 L of the Glutamate Assay Buffer to prepare a 1mM standard solution. 0, 2, 4, 6, 8 and 10 L and the 1m M standard solution was added into 96 well plate, generating the “blank” and 2, 4, 6, 8 and 10 m mole/well standards. Thereafter glutamate Assay buffer was added to each well to bring the volume to 50μL. Tissue or cells of size 1 x 10⁶ can be homogenized in 100μL of the Glutamate Assay Buffer. The samples were centrifuged at 13,000 centrifugal speed for about 10-15 minutes to remove insoluble material. The samples were later filled up to a final volume of 50μL with Glutamate Assay Buffer.

Immunohistochemistry: Glial Fibrillary Acidic Protein (GFAP)

The Sections were de-paraffinized thoroughly in three changes of xylene, 3 minutes each and hydrated through two changes each of 100% and 95% ethyl alcohols, 10 dips each. Then, it was washed well with distilled water. Then endogenous peroxide was quenched with 2 drops of freshly made 3% hydrogen peroxide for 5 minutes. The slides were washed gently in distilled water and rinsed in two changes of Tris buffered saline. The excess buffer was tapped off; Glial fibrillar acidic protein primary antibody was applied and incubated at room temperature for 30 minutes. The slides were rinsed in two changes of buffer. The excess buffer was tapped off; amplifier was applied to Incubate for 10 minutes. The slides were the rinsed in two changes of buffer. The excess buffer was tapped off; HRP polymer was applied. It was then incubated for 10 minutes. The slides were rinsed in two changes of buffer.

Cresyl Violet Staining

Cresyl Violet Acetate solution is used to stain Nissl substance in the cytoplasm of neurons in paraformaldehyde or formalin-fixed tissue. The neuropil will be stained a granular purple-blue. This stain is commonly used to identify the neuronal structure in brain and spinal cord tissue. The Cresyl Violet method uses basic aniline dye to stain RNA blue, and is used to highlight important structural features of neurons. The Nissl substance (rough endoplasmic reticulum) appears dark blue due to the staining of ribosomal RNA, giving the cytoplasm a mottled appearance. Individual granules of extra-nuclear RNA are named Nissl granules (ribosomes). DNA present in the nucleus stains a similar color.

Method (for paraffin embedded sections):

Sections were de-waxed in xylene (2 or 3 changes of 3 min each) and rehydrated in alcohol (100% x2), 3 min each. Sections were stained in 0.1% Cresyl Violet 4-15 min and quickly rinsed in tap water to remove excess stain. They were washed in 70% ethanol (the stain will be removed by this method). Also, they were immersed [sections] for 2 min in Differentiation solution - check staining on microscope and dehydrated through 2x3 min changes of absolute ethanol, cleared in xylene x2 and mount in DePeX and allowed dry in the fume hood.

Statistical Analysis

The data gotten from all the test was expressed as mean ± Standard Error of Mean (S.E.M). The statistical significance was evaluated by one way analysis of variance (ANOVA) using Graph Pad Prism (version 5). A value p <0.05 was considered statistically significant.

III. Results

Administration of sodium iodate (NaIO₃) along with treatment of the retinopathic condition, Acute Macular Degeneration (AMD) in particular showed forth, the following respective results on the area of study, Visual cortex of adult male Wistar Rats.

![Figure 1: Chart showing the relative organ weight of the experimental animals’ brains in Groups A-D. Values were reduced in the treated groups relative to the Group A [Control], albeit not statistically significant. [Group A: Control; Group B: administered sodium iodate; Group C: administered sodium iodate and high-dose moringa; Group D: administered sodium iodate and low dose moringa.]](image)
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**Figure 2**: Photomicrographs of the visual cortex of the Group A-D experimental animals [H&E, X160; X1600]. Visual cortex is demonstrated without extensive histo-architectural disruption. Certain neurons appear morphologically heterogeneous in the treated groups. [Legend: N- Neuron, G- Glia]

**Figure 3**: Photomicrographs of the visual cortex of the Groups A-D experimental animals [CFV, X640] demonstrating the Nissl bodies in the cortical neurons. Cortices are properly demonstrated for Nissl bodies without evidence of extensive cortical cytological disruption. *Legend: N- Neuron, CFV- Cresyl Fast Violet*
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Figure 4: Photomicrographs of the visual cortex of the Groups A-D experimental animals [GFAP, X640]. Visual cortex is demonstrated with moderate expression of GFAB in the cortex of all cortices. There are no sign of pathological [GFAP] astrocytic expressions. **Legend:** GEA- GFAP Expressing Astrocytes

Figure 5: Bar chart showing the levels of neurotransmitters glutamate and GABA across the experimental groups. Neurotransmitters were increased in the treated groups relative to the control. Where *: signifies that there is statistical significance when compared with Group A (p<0.05). Where ^: indicates that there is statistical significance when compared with Group C (p<0.05). ^*: indicates that there is statistical significance when compared with Group D (p<0.05).

IV. Discussion

Physical and morphological observations, histological results, immuno-histochemistry results, and biochemical results were obtained as the visual cortex of the various groups were studied and compared with one another. It is also important to note that the animals used in this research were treated with Sodium Iodate and Ethanolic extract of *M.oleifera* leaves. The purpose for treating the animals with sodium Iodate was to induce retinopathy. A research conducted by Zhou *et al.* 2014, tested for the effect of a single intravenous injection of sodium Iodate at different doses (20, 35, 50 and 70mg/kg). The extent of damage in retinal pigment epithelium in animals was mild at 20mg/kg and moderate with 35mg/kg and 50mg/kg and severe at 70mg/kg. The sole purpose for treating the animals with Ethanolic extract of *M.oleifera* leaves was to study for the ameliorative effect on the sodium Iodate induced retinopathy, particularly on the visual cortex. Researches done on sodium Iodate induced retinopathy as shown that retinal pigment epithelium is a critical site for pathology (Zulliger *et al.*, 1997).
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Relative Organ Weight
In this morphological result, there was significant difference in the relative organ weight of the animals across the group when compare to Group A. The result- bar chart- showed that Group A (Control) had the highest relative organ weight, followed by Group D (Sodium Iodate and low dose M.oleifera), then followed by Group C (Sodium Iodate and high dose M.oleifera) and lastly, Group B (Sodium Iodate) had the lowest relative organ weight.

Histological Observations
The visual cortex histology particularly in terms of its architectural organization and its cellular elements with respect to their characteristic morphologies and relative spatial distribution are being considered to evaluate the relative integrity of the visual cortex in the experimental animals. This is very important in determining the effects of the treatments on the structural and functional integrity of the cortex. Also, the functional integrity with respect to protein synthesis is being considered using the cresyl fast violet staining technique. Results on protein synthesizing condition of the tissues helps in determining the functional status of the tissue. The immunohistochemistry study technique is specifically important in studying the specific reactions of the astrocytes to the treatment as a measure of the effects of treatments on the tissue.

Photomicrographs of the visual cortex of the Control Group [A] experimental animals show that the visual cortex is properly demonstrated; so also the neurons and glia showing that the group can suitable serve as reference for the other experimental [treated] animals. Animals in Group B were treated with sodium iodate to induce retinopathy that can lead to total blindness due to compromised photoreceptors. The results on histological features in this group are being presented as photomicrographs. Photomicrographs of the visual cortex of the Group B experimental animals show that the visual cortex is generally demonstrated without extensive general histoarchitectural disruption. Slight morphological alterations in neurons and their spatial distribution are however observed. In addition, pyramidal cells are hardly observable. This shows that retinal disruption by the agent did not cause immediate or concurrent extensive disruption of the visual cortex tissue. However, cells of the visual cortex have signs of morphological alterations as a result. This therefore suggests that retina degeneration in the context of this research lead to slight morphological alterations of certain neurons. It is possible that such effects are as a result of the extended effects of the administered agent form retinal cells.

Photomicrographs of the visual cortex of the Group C experimental animals show that the visual cortex is properly demonstrated. Neuron and glia have normal morphologies relative to the control. This also indicates a positive role of the intervention against the administered agent. Photomicrographs of the visual cortex of the Group D experimental animals also show that the visual cortex is demonstrated without extensive histo-architectural disruption. However, certain neurons are morphologically heterogeneous and spatial organization appears affected resulting in less definitive cortical arrangement.

Cresyl Fast Violet
Nissl bodies of the neurons are being demonstrated using the cresyl fast violet staining technique. Photomicrographs of the visual cortex of the Groups A-D experimental animals demonstrating the Nissl bodies in the cortical neurons show that cortices are properly demonstrated for Nissl bodies without evidence of extensive cortical cytological disruption. Observable major aberrations are not also observed in the expression of Nissl bodies across the groups; again, this shows that the tissues are relatively normal except for the mild morphological aberrations that have been observed.

Neurotransmitter: Level of Gamma Amino Butyric Acid (GABA) and Glutamate
GABA is the major inhibitory neurotransmitter present in visual cortex cells (Kalloniatis and Tomisich, 1999), most especially in the amacrine cells, and also in horizontal cells. In this biochemical result, there was a significant difference in the level of GABA of the treated animals across the group, when compared to Group. Result as represented by the bar chart shows that Group D (Sodium Iodate and Low dose M.oleifera) has the highest level of GABA, followed by Group C (Sodium Iodate and high dose M.oleifera), and then Group B (Sodium Iodate), and Group A (control) had the lowest level of GABA. Glutamate on the other hand is the major excitatory neurotransmitter present within the visual cortex (Robert and Charles, 1981) and it could be noted, slight increases within each experimental groups occurred. Glutamate in excess levels can be very deleterious and could lead to a series of brain related diseases such as atrophied lateral disease (Lou Gherig’s disease) and Epilepsy due to excitotoxicity. Hence, this might explain the relative significant increase in GABA (depressant) levels so as to counter glutamate’s negative effect.
Level of GFAP

The glial acidic fibrillary protein technique is being employed to observe the nature of tissue response to the administered agents and intervention regimens. Photomicrographs of the visual cortex of the Groups A-D experimental animals show that the visual cortex is demonstrated with moderate expression of GFAP in the cortex of all cortices. There are no sign of pathological astrocytic expressions.

The human brain for instance has been reported to exhibit plasticity, in manners linkable to retinal function (Rosa et al., 2017). This fact is naturally expected to explain among others why retinopathy may cause visual cortex damage. Retinal distortions, changes or degeneration was linked to visual cortex alterations (Boucard, 2009), so also functional alterations in the visual cortex that accompany retinal degeneration (Chen et al., 2016); but there is not such evidence in the current investigation on retinopathy. Duration of the retinopathic condition might play a major role. Hence, in the current research, there was no significant evidence, connecting retinal degeneration to structural alterations in the visual cortex. Investigations may therefore also focus on factors that determine the extent, nature and timing of cortical changes that are found to be directly associated with retinal degeneration.

V. Conclusion

Then visual cortex was not extensively affected structurally when retinopathy was induced using sodium iodate. GABA levels were however locate in the visual cortex of experimental animals.

VI. Recommendation

This research was based on the administration of certain dosages of moringa according to reviews but more researches should be undertaken especially at higher doses to ascertain the efficacy and safety at such doses and the nature of interactions between Moringa oleifera ethanolic extract and sodium iodate with respect to the visual cortex integrity in the condition of retinopathy.

References


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