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Potential Neuro-therapeutic Effect of Flaxseed Oil on the Striatum of Rotenone Mice Model of Parkinson' Diseases

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Authors' contributions

This work was carried out in collaboration between all authors. Author PDS designed the study, wrote the protocol and the final draft of the manuscript. Authors OOO and OJO managed the literature searches. Authors OOO and HBA wrote the first draft of the manuscript. Authors BRB, DJT and OOO managed the laboratory animals. Authors OFS, DJT, PDS, HBA and OOA performed the laboratory analyses. Authors OFS, BRB and OJO performed the statistical analysis. All authors read and approved the final manuscript.

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ABSTRACT

Aim: This study investigated the potential neuro-therapeutic effect of Flaxseed oil on the striatum of Rotenone mice model of Parkinson' diseases'
Study Design: Fifty-six adult male and female mice (*Mus musculus)* weighing between 23.9-26.3 grams were used for this study. The mice were randomly placed into four groups of fourteen mice each made up of equal number of male and female: A (Control; mice pellets), B (Rotenone 3 mg/kg, IP), C (Rotenone + Flaxseed oil 0.3 ml orally), and D (0.3 ml Flaxseed + Rotenone).
Place and Duration of the Study: Department of Anatomy, Olabisi Onabanjo University. Between May and September, 2016.

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Methodology: The brain were excised, weighed and appropriate sections taken and processed histology and stained with H&E and Nissl stains and immuno-cytochemically with GFAP.
Results: The results showed significant ($P<0.05$) reduction in the neuronal cell count, body and relative brain to body weight, which were increased by flaxseed oil treatments. Rotenone induced neural and striatal bundle degeneration which were ameliorated by flaxseed oil treatment.
Conclusion: In conclusion, the reduction in weight and neuronal derangements associated with rotenone induced Parkinson's disease in this study were reduced or alleviated as a consequence of the treatment with flaxseed oil, and hence flaxseed oil could be considered as a potential therapeutic candidate in the management of Parkinson's disease.

Keywords: Parkinson's disease; flaxseed oil; striatum; rotenone.

1. INTRODUCTION

The striatum (caudate and putamen) is the major input structure of the basal ganglia complex and is an essential part of neural networks involved in motor and non-motor function [1]. Striatal function is severely impaired in Parkinson's disease (PD), which depletes the neuro-modulatory influence of ventral midbrain dopamine-producing neurons on these circuits and disrupts the balance of multiple corticostriatal circuits.

Degeneration of dopaminergic neurons that project to the striatum is a hallmark of Parkinson's disease pathology [2]. PD manifests progressively in worsening motor and non-motor (cognitive and behavioral) dysfunction, which may in part reflect anatomical changes at the level of the putamen (motor) and caudate (oculomotor, cognitive and behavioral). Despite the established linked between PD and striatum, the morphological changes in both the striatal neurons and glia have been largely ignored as a possible cause of some of PD dysfunction. Previous magnetic resonance imaging (MRI) studies have variously reported decreased or non-significant volume differences for these striatal structures using manual or semi-automated tracing methods [3-9].

A growing body of evidence suggests that nutrition may play an important role in PD. Epidemiological and biochemical studies have recently identified promising components in certain food groups that may elicit neuroprotection in PD [10,11]. However, inclusion or exclusion of other food groups may trigger or exacerbate neurodegeneration.

Flaxseed oil comes from the seeds of the flax plant (*Linum usitatissimum,* L.). Flaxseed oil contains both omega-3 and omega-6 fatty acids, which are needed for health. Flaxseed oil contains the essential fatty acid alpha-linolenic acid (ALA), which the body converts into eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA), which is the omega-3 fatty acids found in fish oil [12]. Omega 3 and omega 6 fatty acids are essential fatty acids, that is, they cannot be synthesised and are essential components of the human diet for health. While both omega 3 and 6 fatty acids have positive structural properties when incorporated into bodily cell membranes, omega 3s are essentially anti-inflammatory [13,14].

In this study, we investigated the relevance of flaxseed oil, as a potential neuro-therapeutic candidate targeting striatal neurones and astrocytes in line with the growing body of evidence that suggests nutrition may play an important role in PD.

2. MATERIALS AND METHODS

2.1 Experimental Animals

Fifty-six adult male and female mice (*Mus musculus)* weighing between 23.9-26.3 g were used for this study. The animals were housed in clean plastic cages, well ventilated environment with temperature ranging between 24-28°C in 12 hours light and 12 hours dark cycle. The animals were given standard mice pellets and water *ad libitum*, and were allowed to acclimatize for two weeks before commencing the experimental protocols. Rotenone was bought from Abcam, while flaxseed oil was purchased from Organo Shoppe, Lagos, Nigeria. The institutional committee on Animal Care and Use in Research, Education and Testing (ACURET) approval was obtained and the animal experiments were conducted according to the NIH Guide on Laboratory Animals for Biomedical Research (NIH, 1978) and ethical guidelines for investigation of experimental pain in conscious animals [15].

2.2 Experimental Design

Following the two weeks of acclimatization, the animals were randomly divided into four (4) groups of fourteen (14) animals each made up of equal number of male and female mice as follows:

- Group A: (Control Group) Mice were given dry food pellet and clean water *ad libitum.*
- Group B: (Negative Control Group) Mice were given 3 mg/kg/day of Rotenone per body weight subcutaneously for 5 consecutive days
- Group C: (Post-treated) Mice were given 3 mg/kg/day of Rotenone per body weight subcutaneously for 5 consecutive days followed by a fourteen (14) days oral treatment with (0.3 mil/mouse) flax seed oil.
- Group D: (Pre-treated) Mice were given (0.3 mil/mouse) flax seed oil for fourteen (14) days consecutively followed by five (5) days administration of 3 mg/kg/day of Rotenone subcutaneously.

2.3 Tissue Sample Preparation

At the end of the experimental period the mice were euthanized by administering 10 g/kg body weight of Pentobarbital. The mice brains were carefully dissected out, weighed and fixed in 10% formal-saline for routine histological and immunocytochemical procedures.

2.3.1 Haematoxylin and eosin routine staining

Tissue sections were rinsed in distilled water for 5 minutes, then stained in haematoxylin for 15 minutes, rinsed in running tap water and differentiated in 0.3% acetic acid and rinsed in tap water before staining with eosin for 2 minutes. Sections were then dehydrated in 70% for 1 minute, 95% ethanol for 1 minute, 100% ethanol for 1 minute (2 changes) respectively and then taken to the oven overnight. Sections were subsequently cleared in xylene and then placed DPX mountant and cover slipped for light microscopy [16].

2.3.2 Methods: Cresyl fast violet for nissl substance

Tissue sections were de-waxed in xylene (2 or 3 changes of 3 min each), dehydrated in alcohol (100% x2), 3 min each, followed by staining in 0.1% Cresyl Violet for 15 min.The slides were quickly rinsed in tap water to remove excess stain, then washed in 70% ethanol, followed by dehydration through 2x3 min changes of absolute ethanol and finally cleared in xylene x2 and mounted in DPX [17].

2.4 Immunohistochemical Protocol

The paraffin embedded tissue was cut at 5 microns thick and allowed to heat on hot plate for 1 hour, then sections were taken to water, that is, through xylene, alcohols and finally water respectively. Antigen retrieval method was performed using citric acid solution pH 6.0 in a pressure cooker for 15 minutes. Sections were equilibrated by gently displacing hot citric acid with running tap water for 3 minutes. Blocking of peroxidises in tissue sections was done using peroxidise block for 15 minutes and then washed for 2 minutes with phosphate buffered saline (PBS) with tween 20. Blocking of protein was then performed with Novocastra® protein block for 15 minutes. Tissue sections was then washed for 2 minutes with PBS, then incubated with primary antibody e.g., Glial fibrillary acidic protein (GFAP) 1 in 100 dilution for 45 minutes, washed in PBS for 3 minutes and later added Secondary antibody for 15 minutes. Tissue section was then washed twice with PBS. Polymer was thereafter added and allowed for 15 minutes, washed twice with PBS and then added the diaminobenzidine (DAB) chromogen diluted 1 in 100 with the DAB substrate for 15 minutes, and then washed with water and counterstained for 2 minutes in Haematoxylin. Again the tissue section was washed, dehydrated, cleared and mounted in DPX mountant [18].

2.5 Photomicrography

Photomicrographs were taken using Omax led digital Microscope.

2.6 Statistical Analysis

Data were analysed using analysis of variance (ANOVA) by comparing values for different treatment groups with the values for individual controls. Results were expressed as mean ± SEM. The significant differences among values were analysed using Graph Pad version 7 at *P-value* = 0.05.

3. RESULTS AND DISCUSSION

We have shown that the main pharmacologic features of flaxseed oil's effective neuroprotection include: 1) Prevention of

rotenone-induced loss of body in mice; 2) attenuation of rotenone-induced striatal neural degeneration; 3) Preservation/protection of striatal fibre bundles against rotenone-induced degeneration; 4) ameliorating pathological changes in astrocytes induced by rotenone.

Our results on the body weight showed significant interaction ($F_{(7, 32)}$ =44.53, P<0.0001); 2. Significant main effect of sex ($F_{(7, 32)}$ =1526, P<0.0001); 3. Significant main effect of treatment ($F_{(7, 32)}$ =63.24, P<0.0001) [Fig. 1]. Body weight is determined by many factors including genetic, epigenetic, metabolic, and environmental components. Under physiological conditions homeostatic behavioral adaptations protect against weight gain as well as weight loss [19]. However, regulation of body weight seems to be more effective in response to weight loss than to weight gain [20]. Rotenone induced significant loss in body weight in the mice model of Parkinson's disease.

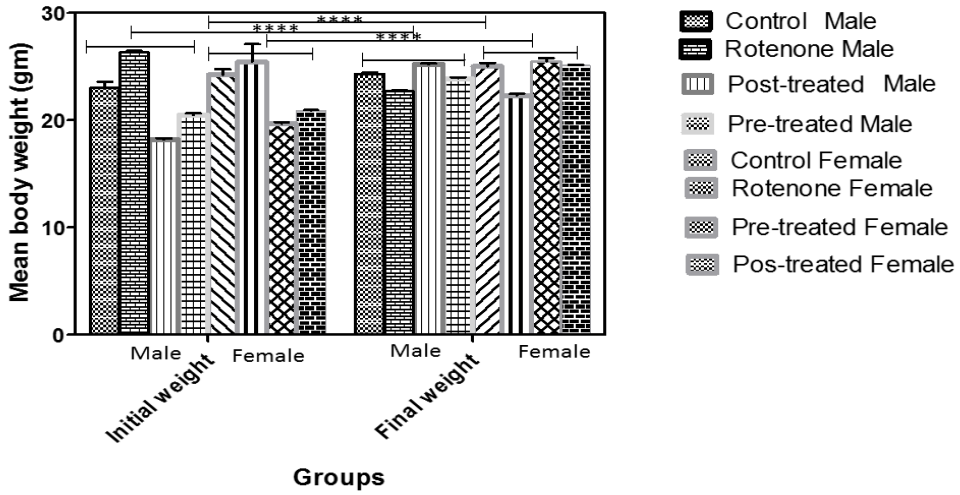

Fig. 1. Shows the mean ± SE initial and final body weight of male and female mice in control, rotenone, post-treated and pre-treated groups

The results of the two way ANOVA showed: 1.Significant interaction ($F_{(7, 32)}$ =44.53, P<0.0001); 2. Significant main effect of sex ($F_{(7, 32)}$ =1526, P<0.0001); 3. Significant main effect of treatment ($F_{(7, 32)}$ =63.24, P<0.0001)

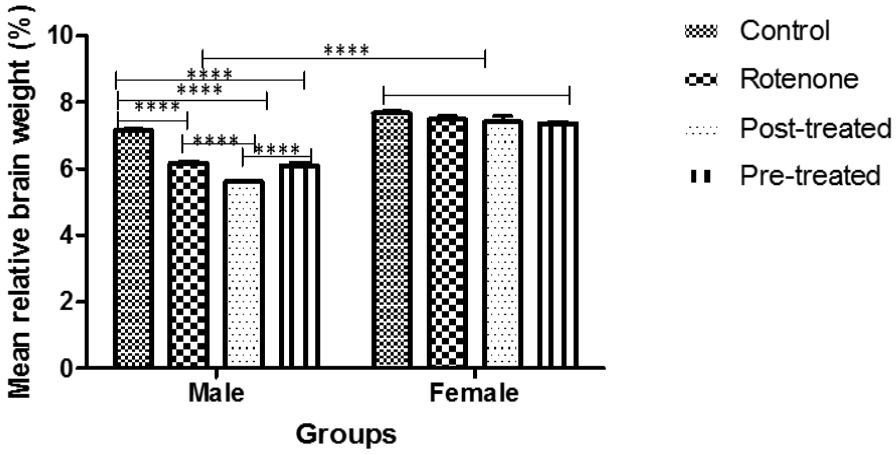

Fig. 2. Shows the mean ± SE of relative brain weight of male and female mice in control, rotenone, post-treated and pre-treated groups

The results of the two way ANOVA showed: 1. Significant interaction (F (3, 16) =55.52, P<0.0001); 2. Significant main effect of sex (F (1, 16) =1199, P<0.0001); 3. Significant main effect of treatment (F (3, 16) =117.3, P<0.0001)

We further investigated the effects of the treatments on relative weight of the brain and neuronal cell count. Our findings showed significant effect of the treatments and sex on the parameters measured [Figs. 2 and 3]. The loss in relative weight of brain in the rotenone group and the significant gain in relative weight of brain in the Pre-treated in the male mice can be explained by the loss in body weight noted above [Fig. 1] and the significant loss in in neuronal cell count [Fig. 2] this result is collaborated by the neuronal degeneration seen in [Plate 1, B & Plate 2, B]. Currently, it is widely accepted that mitochondrial dysfunctions play an important role in pathogenesis of neurodegenerative diseases, such as Parkinson disease (PD) [21,22]. In this study, neuronal death is thought to be a consequence of the inhibition of mitochondrial complex I, which leads to a reduction in the energy supply and subsequent collapse of the mitochondrial membrane potential [23]. A recent study suggests that rotenone administration activates caspase-2 in mice neurons inducing the activation of downstream apoptotic effectors such as Bid, Bax, caspase 3 and 9, thus initiating apoptosis [24]. We further investigated the effect of flaxseed oil on relative brain weight and neuronal cell count. The results showed significant increase in the relative brain weight in the male pre-treated with flaxseed oil [Fig. 2] and significant increase in the neuronal cell count in the post and pre-treated mice [Fig. 3]. Flaxseed oil contains the essential fatty acid alpha-linolenic acid (ALA), which the body converts into eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) [12]. DHA is reported to play a neuroprotective role against oxidative stress in astrocytes [25], oligodendroglia cells [26], retinal ganglion cells [27], and human lymphocytes [28]. Our present study reported that exogenous intake of omega 3 in the form of flaxseed oil significantly protected against H2O2-induced oxidative injury, suggesting that omega 3 might be an effective supplement for the prevention of neurodegenerative diseases which are associated with oxidative stress. DHA has been reported to scavenge the intracellular radical productions induced by hydrogen peroxide (H2O2), superoxide anion (O2•−), and hydroxyl radical (•OH) [25]. Finally we looked at the role of astrocytes in rotenone induced Parkinson's disease and on the intervention with flaxseed oil. Our finding showed complete loss of astrocytes in the rotenone group [Plate 3, B]; during brain damage, astrocytic functions become transiently or permanently impaired, and the subsequent

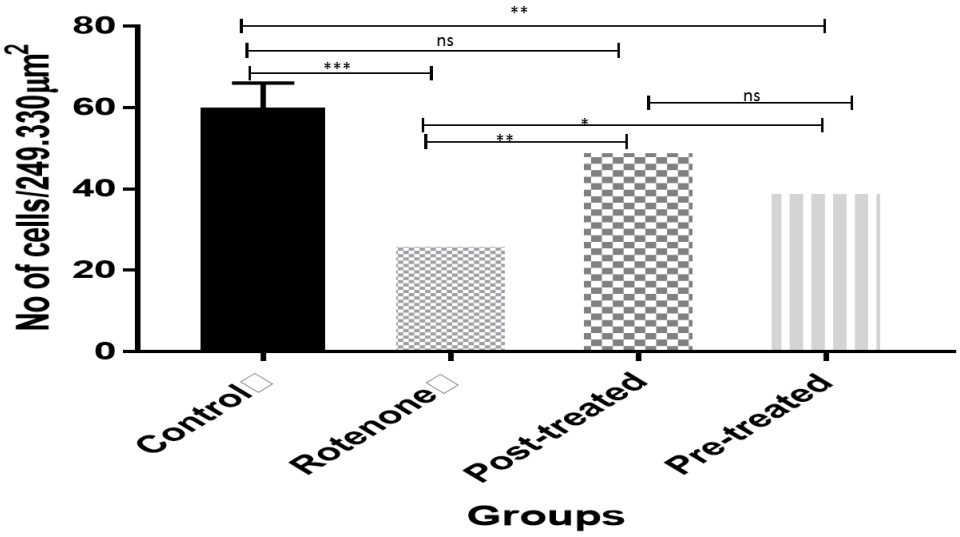

Fig. 3. Showing the mean number of cells per 249.330um^2 area of the striatum in experimental groups; Control (60 ±3.78), Rotenone (27 ±2.52), Post-treated (50 ± 3.24), Pre-treated (40 ± 3.67). The results of the one way ANOVA showed a significant effect of the treatments on the cell population ($F_{(3, 8)}$ =27.76,P=0.0001), while Tukey's multiple comparisons test at α < 0.05 showed significant decrease in the cell population between the Control (60 ±3.78) versus Rotenone(27 ±2.52), Control (60 ±3.78) versus Post-treated (50 ± 3.24), Control (60 ±3.78) versus Pre-treated (40 ± 3.67) and significant increase between Rotenone(27 ±2.52) versus Post-treated (50 ± 3.24), Rotenone(27 ±2.52) versus Pre-treted (40 ± 3.67)

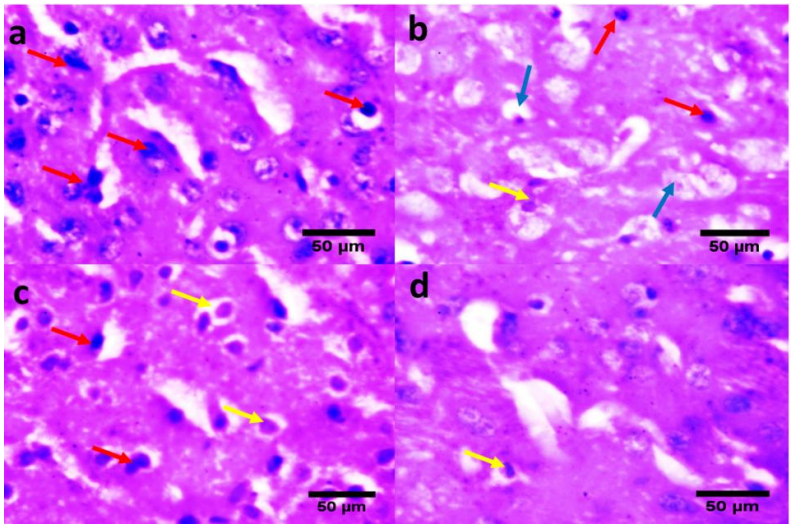

Plate 1. Showing the photomicrographs of mice striatum stained with H&E X1000: (a) Striatum of control group showing normal neuronal nuclei(red arrows); (b). Striatum of Rotenone treated mice few normal neurons (red arrows), degenerated neurons (yellow arrow) and vacuolated cytoplasm (blue arrow); c). Striatum of Rotenone exposed mice and post-treated with flaxseed oil showing some normal neurons (red arrows) and degenerating neurons (yellow arrow); (d) Striatum of Rotenone exposed mice and pre-treated with flaxseed oil showing some normal neurons (and degenerating neurons (yellow arrow)

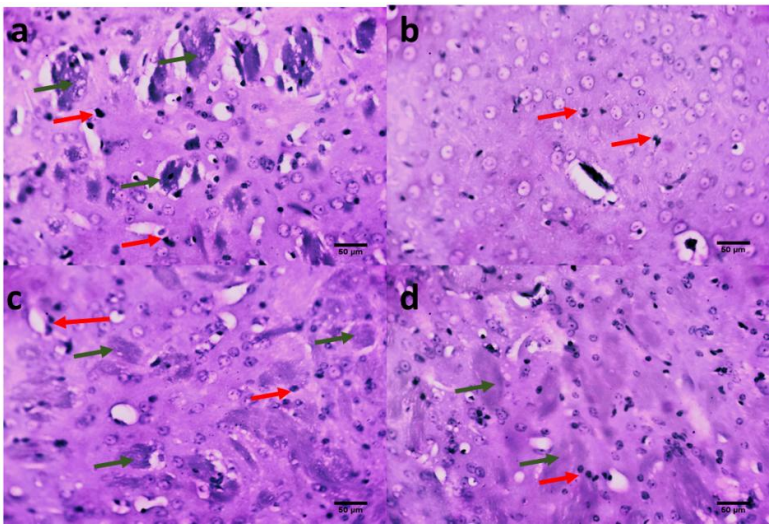

Plate 2. Showing the photomicrographs of mice striatum stained with Nissl X1000: (a) Striatum of control group showing normal neuronal nuclei (red arrows) and striatal fibre bundles (green arrow); (b). Striatum of Rotenone treated mice few normal neurons (red arrows) and degenerated striatal fibre bundles; (c) Striatum of Rotenone exposed mice and post-treated with flaxseed oil showing some normal neurons (red arrows) and fewer striatal fibre bundles (green arrow); (d) Striatum of Rotenone exposed mice and pre-treated with flaxseed oil showing some normal neurons (red arrow) and even fewer striatal fibre bundles (yellow arrow)

impact on neuronal cells may lead to pathological conditions and neurodegenerative diseases [29,30], because neurons are more susceptible to injury than astrocytes, as they have limited antioxidant capacity, and rely heavily on their metabolic coupling with astrocytes to combat

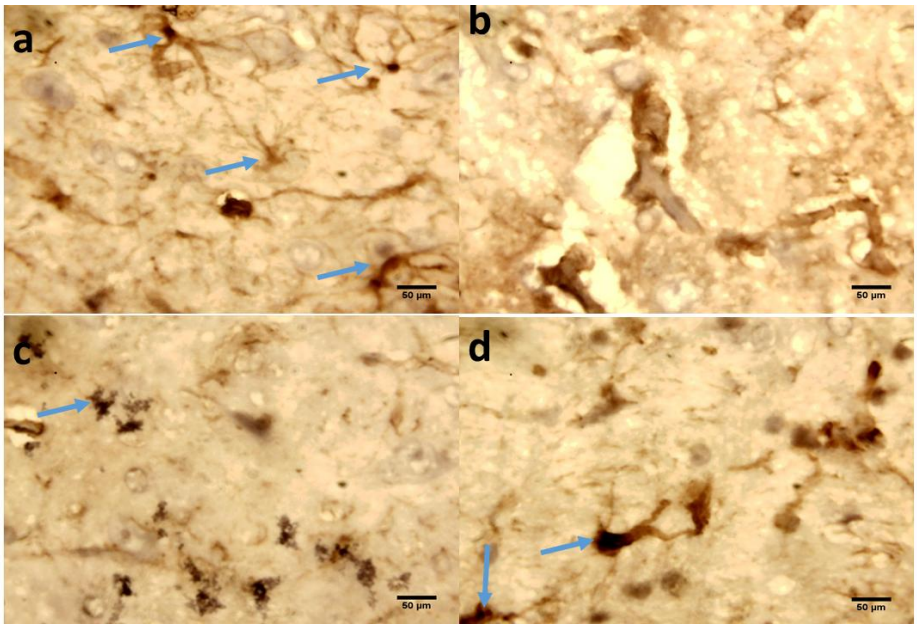

Plate 3. Showing the photomicrographs of mice striatum stained with GFAP X1000: (a) Striatum of control group showing normal neuronal astrocytes (blue arrows); (b). Striatum of Rotenone treated mice with no astrocytes in view; (c)Striatum of Rotenone exposed mice and post-treated with flaxseed oil showing some clumps of astrocytic processes (blue arrows); (d) Striatum of Rotenone exposed mice and pre-treated with flaxseed oil showing some astrocytes (blue arrows)

oxidative stress [29]. However, severe brain damage also results in astrocyte dysfunction, leading to increased neuronal death [31]. Our results also revealed that intake of flaxseed oil was able to preserve some astrocytes [Plates 3, C & D]. DHA modulation of astrocytes also demonstrates fine tuning of neuronal activity through inhibition of pro-inflammatory mediators and an important regulation of astrocytic activity [32]. In addition, astrocytes release antioxidant molecules like glutathione (GSH) and superoxide dismutases and supply neurons with neurotrophic factors, such as nerve growth factor (NGF), basic fibroblast growth factor (bFG), that constitute an important attempt to protect neurons during brain damaging processes, including PD [33,6,34,35].

4. CONCLUSION

In conclusion, the reduction in weight and neuronal derangements associated with rotenone induced Parkinson's disease in this study were reduced or alleviated as a consequence of the treatment with flaxseed oil, and hence flaxseed oil could be considered as a potential therapeutic candidate in the management of Parkinson's disease.

CONSENT

All authors declare that 'written informed consent was obtained from the patient (or other approved parties) for publication of this paper and accompanying images'.

ETHICAL APPROVAL

As per international standard or university standard, written approval of ethics committee has been collected and preserved by the authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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