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ORIGINAL RESEARCH

Protective effects of *Nigella sativa* on Pre-fronto-cortical functions in mice Attention Deficit and Hyperactivity Disorder (ADHD) model

Folarin R*, Adefoluke S, Ogunwale T, Osinowo O, Ogunledun A, Ibrahim I

Neurophytotherapy Unit, Department of Anatomy, OlabisiOnabanjo University, Sagamu, Nigeria

***Correspondence: Dr. R Folarin, Neurophytotherapy Unit, Department of Anatomy, OlabisiOnabanjo University, Sagamu, Nigeria. Email: royhaan.folarin@oouagoiwoye.edu.ng; ORCID – https://orcid.org/0000-0003-1558-6158.**

Abstract

Background: Attention Deficit and Hyperactivity Disorder (ADHD) is a neurodevelopmental disorder associated with pre-fronto-cortical (PFC) cognitive dysfunctions. Ethanol is a psychoactive agent and its foetal exposure is employed in the modelling of ADHD.
Objective: To determine the curative roles of *Nigella sativa* in the PFC functions of mice induced with ADHD-like symptoms.
Method: Twelve BALB/c mice pups each from four groups of dams were studied. Normal saline was administered to the control (CTRL) dams, ethanol to the ETH dams, *Nigella Sativa* to the NS dams, and *Nigella sativa* followed with ethanol to the NSE dams. Novel object recognition test was used to assess recognition memory in the pups 15 days after weaning. Histological illustration of PFC was conducted using haematoxylin and eosin (H&E) stain.
Results: ETH mice exhibited the least recognition memory while *Nigella sativa* prevented this deficit in NSE mice by eliciting much higher recognition memory. This indicates the neuroprotective role of *Nigella sativa* despite prenatal exposure. NS pups also had the highest weight gain, as well as the glutamate and Glutathione, peroxidase (GPX) levels, while raising these levels in the ETH-exposed mice which had the lowest levels, indicating a neurochemical potentiation. The results of the histological analysis showed the protective roles of *Nigella sativa* on the PFC neuronal densities.
Conclusion: This study suggests the protective effects of *Nigella sativa* on the prefronto-cortical functions in mice ADHD model following maternal exposure to ethanol.

Keywords: Attention Deficit and Hyperactivity Disorder, Mice, Cognition, Dopamine, Pre-frontal Cortex, Glutamate, Nigella sativa.

Introduction

Attention Deficit and Hyperactivity Disorder (ADHD) is a neurodevelopmental disorder. [1,2] It is characterized by difficulty in paying attention, excessive activity, and difficulty in controlling behaviours which are not appropriate for age. [3]However, patients with ADHD may sustain attention for tasks they find interesting or rewarding, a symptomatic phenomenon referred to as hyper focus. [3, 4] Despite being the most commonly studied and

diagnosed mental disorder in children and adolescents, the exact cause of ADHD is unknown in the majority of cases. It affects about 5to 7% of children when diagnosed using the criteria of the Fourth Edition of the Diagnostic and Statistical Manual of Mental Disorders, (DSM-IV), [4] and 1 to2% when diagnosed with the criteria ofthe10th revision of the International Statistical Classification of Diseases and Related Health Problems (ICD-10). [5] As of 2015, ADHD is estimated to affect about 51.1 million people globally. The rates are similar between countries and depend mostly on how the condition is diagnosed.

Personality expression, decision making, social behaviour moderation, and the ability to plan complex cognitive behaviour are all traits for which the involvement of the Prefrontal cortex has been chiefly implicated, which all risk compromise in individuals suffering from ADHD. [6]To model this disorder in rodents for better understanding, preventive/therapeutic measures, ethanol, the most commonly consumed psychoactive drugs by humans and a central nervous system depressant, has been proven useful when administered to rodents during gestational days 6 to 16. Such prenatal ethanol exposure has been related to neurological abnormalities like Attention-Deficit/Hyperactivity Disorder (ADHD)-like behaviours in the offspring, as well as growth retardation and morphological abnormalities. [7]

Nigella sativa, (black caraway, also known as black cumin, *Nigella*, and kalonji, amongst other names), [8-10] is an annual flowering plant in the family Ranunculaceae, native to south and south-west Asia, and with widely published therapeutic potentials. As data is limited on the exact impact *Nigella sativa* oil may confer on the pre-fronto-cortical functions of ADHD-like mice model, this study sought to fill this gap in knowledge, as the search for natural alternatives to orthodox neuro-therapeutic drugs continues.

ADHD remains a neuropsychiatric disorder of societal bane, for which the identification of a more effective and less detrimental treatment plan remains important and relevant. Therefore, this study investigated the possible prophylactic or therapeutic potentials of *Nigella sativa* oil in the treatment of BALB/c mice models of ADHD, with a focus on the pre-fronto-cortical functions and symptoms. The specific objectives of the study included the histological, neurochemical and neurobehavioural investigations of the aforementioned aim using through haematoxylin and eosin (H&E) photomicrography of the prefrontal cortex, spectrophotometric quantification of pre-fronto-corticalglutamate, dopamine and glutathione peroxidase (GPX); and short-term recognition memory assay using the novel object recognition respectively.

Methods

Acquisition of Research Materials

Absolute Ethanol manufactured by Sigma Aldrich - USA was procured from IBRA HADAD, Lagos. The male and female Bagg Albino (BALB/c) mice pups used for the research were in-bred within the Neurophytotherapy Research Laboratory of the Faculty of Basic Medical Sciences Animals Holding Facility, Ikenne, Ogun State, where they were maintained under controlled conditions of temperature, humidity and 12-hour diurnal cycles throughout the study. Ventilated transparent cages were constructed with sawdust beddings. Pelletised Grower's mash chicken feeds were procured from Animal Care® depot in Sagamu. Ethical permission for the study (OOU-AREC/18/VII/23-018) was obtained from the Anatomical Research Ethics Committee (AREC) of Olabisi Onabanjo University, in conformity with the International Animal Care and Use Committee (IACUC) ethical guidelines.

Experimental Design and Dosing

As summarized in Table I, the experimental pups were sorted into four (4) groups following their weaning periods, in agreement with the four regimens to which the respective dams were exposed, before or during their gestational periods.

Table I: Grouping of experimental animals, administration of substances, doses, duration, and routes of administration.

Pup Group tag	*Substance administered to dams*	*Dosage*	*Duration*	*Route of administration*
CTRL	Normal Saline Only	10ml/kg	Ad libitum	Orally
ETH	Ethanol only	7.59ml/kg	Gestational Day 6-16.	Orally
NS	*Nigella sativa* only	1ml/kg	10 Days Before Copulation	Orally
NSE	*Nigella sativa*	1ml/kg	10 days before Copulation	Orally
	Ethanol	7.59ml/kg	Gestational days 6 to 16.	

Measurement of body weight

The body weights of the animals were measured at the commencement and end of the study using a digital weighing scale (KERRO®, 2016; 0.1gaccuracy).

Novel Object Recognition (NOR) Test

The Novel Object Recognition (NOR) test was performed for each group of mice, 1 week after weaning. The test which comprised habituation, training/familiarization, and testing phases, was conducted following the SBFNL protocol. [11]

Sacrifice and assays

Following neurobehavioral assays, the animals designated for neurochemical assay were sacrificed by cervical dislocation and followed immediately by excision and homogenization of the pre-frontal cortex in 0.1 M phosphate-buffered saline (PBS). Other animals designated for histological analysis were euthanized, with the whole brain immersed in 10% formal saline and taken through the routine tissue processing for H&E staining.

Histological analyses were carried out through visual assessment of photomicrographs, while densitometry and size comparison was further conducted on the photomicrographs using Image J analytical software.

Relative Brain Weights

The respective weights of the excised whole brains were measured, and the relative brain weights (RBW) of the animals across the groups was calculated using the formula:

RBW = (Brain Weight/Body Weight) x 100

Neurochemical assay

The homogenized tissues were centrifuged at 5000 rpm for 5 minutes, and a liquid chromatography-tandem mass spectroscopy (LC-MS/MS) method with protein precipitation, was employed to assay the levels of dopamine (DA), Glutathione peroxidase (GPX) and Glutamate in the samples.

Statistical analysis

The data such as body weights, relative brain weights, novel object investigation time, discrimination index, glutamate, dopamine and GPX levels were expressed as mean (± standard error of the mean) and were analysed with the One-way Analysis of Variance (ANOVA) using the GraphPad Prism (version 5.0) software. Statistical significance was

assumed at $P < 0.05$. Bonferroni posthoc test was conducted to determine the locations of statistical significance in the differences between groups.

Results

Physical Observation

On physical observation, the dams to which ethanol was administered were hyperactive following the administration of the agent and similar behaviour was observed among the pups reporting the highest level of hyperactivity and impulsivity in the ethanol groups while there was observable normalcy in the activity and attention of the control and *Nigella sativa*-treated group. The pups in the NSE group showed a reduced level of inattentiveness and hyperactivity as compared to the disordered group.

Body Weight Changes

Figure 1 shows that the NS group showed the highest body weight increase, while the ETH group had the least weight increase at 80%. However, the NSE group gained more bodyweight than the ETH group. The differences recorded in this analysis were statistically significant($p = 0.034$).

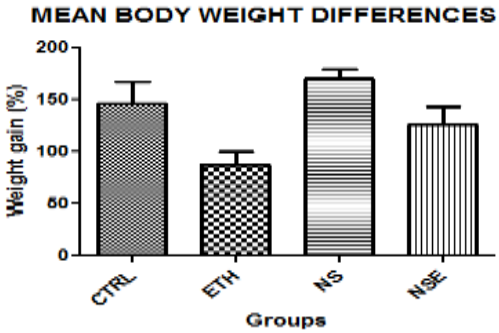

Figure 1: Percentage weight gain in all animal groups.

Relative Brain Weight

Figure 2 shows that the pups in the ETH group had the significantly highest relative brain weight when compared with all other groups. The NSE group recorded the least value of relative brain weight.

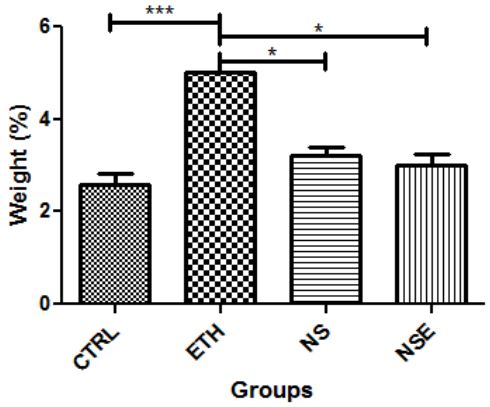

Figure 2: Relative Brain Weight across all Groups

Novel Object Recognition Test (NOR)

As displayed in Figure 3, the exploration time around the novel object was least in the ETH group while the NSE group spent a longer time investigating the novel object.

Total Investigation and Discrimination Index

As shown in Figure 4, the ETH group had a 47% total investigation Time. On the other hand, the NSE group had a total investigation time of 66.9%, which was higher than 50% indicating a greater investigation time of the novel object. The ETH group showed the least discrimination index, with a value of -0.1, while the pre-treated NSE group had a higher discrimination index value of 0.26.

Neurochemical assay

GPX analysis

Figure 5 shows the highest GPX value while the Control group had the lowest GPX value. However, a comparison of both ethanol-administered groups revealed a significantly higher GPX level in the pre-treated NSE mice than in the untreated ones.

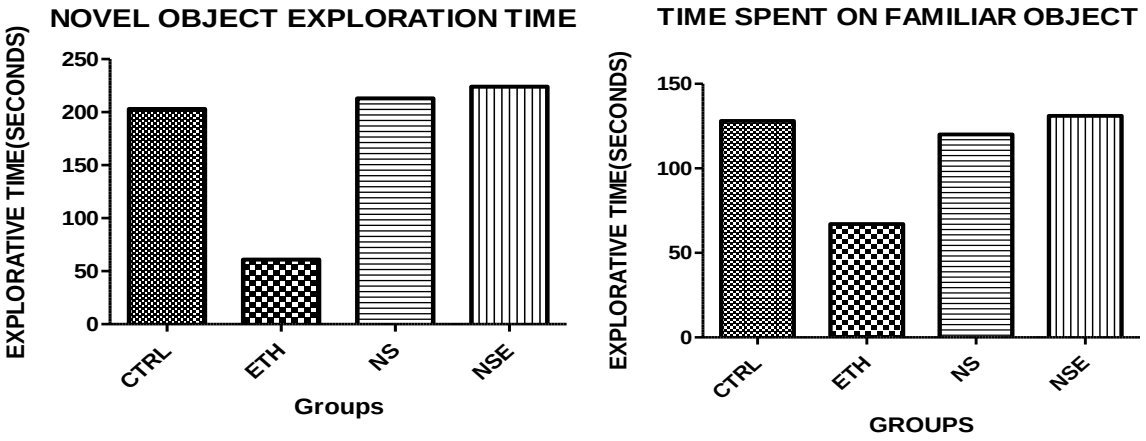

Figure 3: Exploration times around the novel and familiar objects

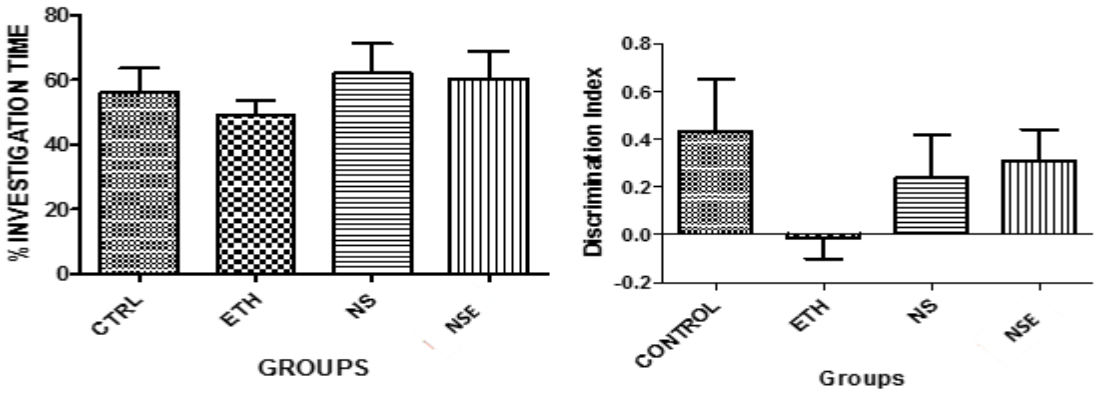

Figure 4: Percentage Investigation Time and Discrimination Index

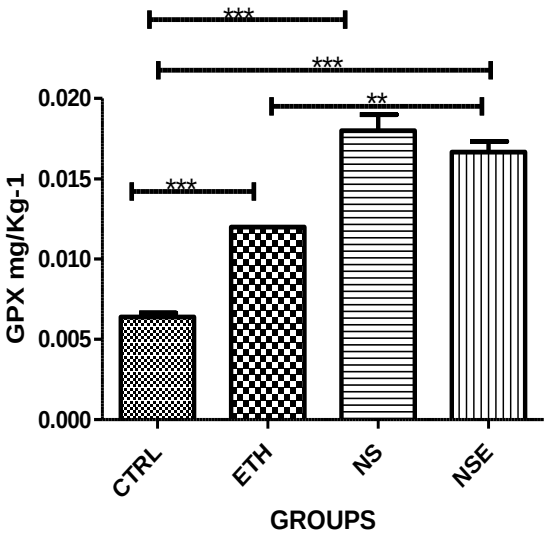

Figure 5: Level of GPX enzyme in all Groups. Number of asterisks (*) is an indication of the level of significance in direct proportion at p<0.05

Glutamate and Dopamine analysis

As shown in Figure 6, the ETH group recorded the highest glutamate level from among the experimental groups (NS, ETH, and NSE), while the NS group had significantly lower levels. The glutamate level in the pre-treated NSE group was further significantly lower than the NS group.

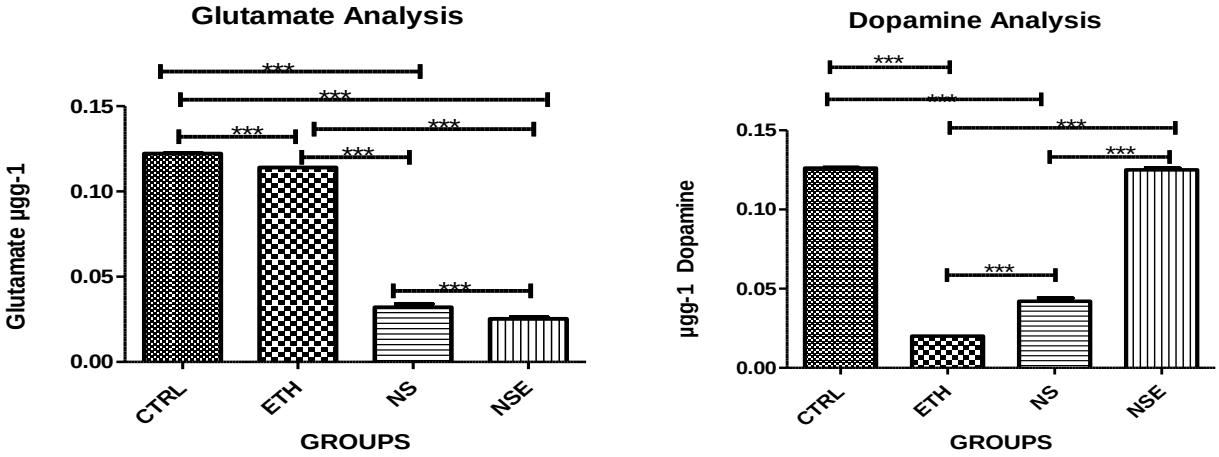

Figure 6: Glutamate and Dopamine Levels in the Prefrontal Cortex region across all Groups. Number of asterisks (*) is an indication of the level of significance in direct proportion at p<0.05

Histological assay

As illustrated in Plate 1, a much higher neuronal density was observed in the NSE group compared to the ETH group, even though the ETH group had a higher density than both the control group and the NS group.

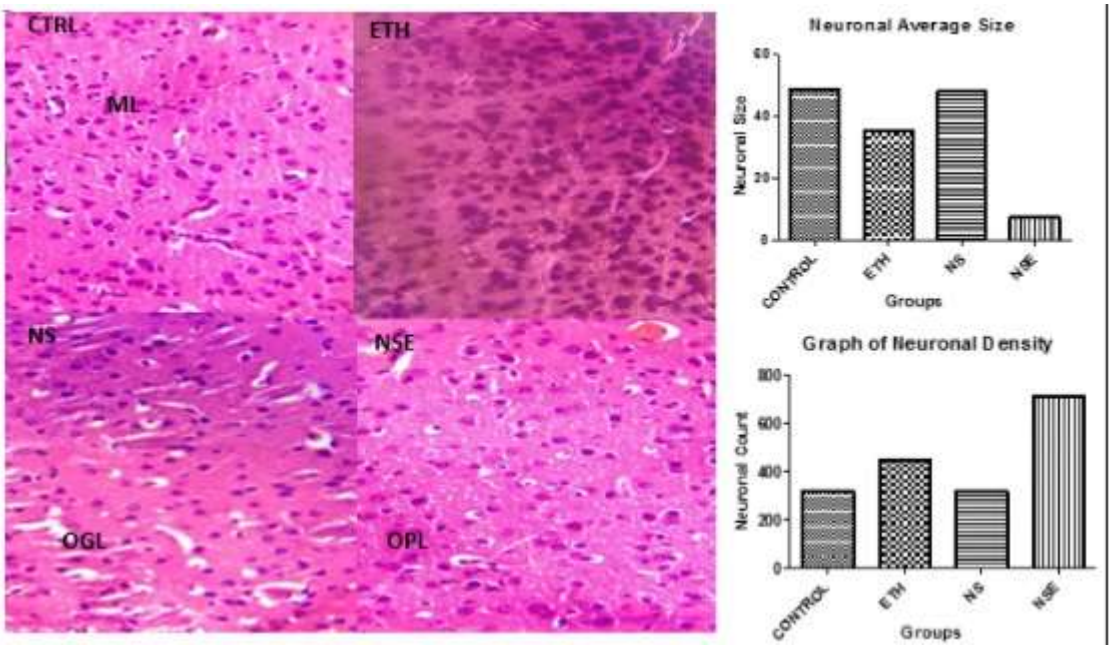

Plate 1: 400X Photomicrograph of Pre-frontal cortex in all groups. ML – Molecular layer; OGL – Outer Granular Layer; OPL – Outer Pyramidal Layer. Featured are neuronal sizes and density across groups.

Discussion

Novel object recognition test is a test for recognition memory. Cognition is a primary function of the pre-frontal cortex, as shown in Figure 3, the ETH-exposed pups had the least explorative time on the novel objects while the NSE-treated pups recorded the highest explorative time. This affirms the adverse effect of prenatal ethanol exposure on the cognition of pups while confirming the preventive roles played by *Nigella sativa* against this effect.

Also, the highest value of the discrimination index was recorded amongst the NSE group. This, in corroboration of the exploration time finding, further shows the effect of *Nigella sativa* in preventing the recognition memory caused by maternal exposure to ethanol. The earlier reported defect in recognition memory in the ETH group was re-affirmed in the present study. Therefore, this finding corroborates the earlier reports on the adverse effect of maternal ethanol exposure on recognition memory. [12]It also corroborates earlier reports on the neuro-pharmacological potentials of *Nigella sativa.* [13]

Furthermore, a positive discrimination index indicates a higher novel object recognition memory. The ETH group had a discrimination index of -0.1, indicating a lower recognition memory as a result of the prenatal ethanol exposure, while the NSE group had the highest discrimination index in corroboration of the prophylactic effect of maternal pre-gestational exposure to *Nigella sativa* on the NSE pups. The NS group further had a positive value of +0.4 indicating a higher novel object recognition memory by the NS group. This establishes the prophylactic role of *Nigella sativa* against the impairment of recognition memory conferred by prenatal ethanol exposure, as recorded in the untreated ETH-untreated group.

From the neurochemical data, glutathione peroxidase (GPX), which is an enzyme that prevents oxidative damage was highest in the NS group as compared with other test groups. This indicates the medicinal potentials of

*Nigella sativa*on GPX levels and explains the level of GPX recorded in the NSE group, which was not as high as the value for the NS groups but significantly higher than the untreated ETH group. GPX is known to play a protective role against oxidative stress. Glutamate is the most abundant of a group of endogenous amino acids in the mammalian central nervous system which presumably functions as excitatory neurotransmitters and under abnormal conditions, may behave as neurotoxins. As neurotransmitters, these compounds are thought to play an important role in the functions of learning and memory. As neurotoxins, they are believed to be involved in the pathogenesis of a variety of neurodegenerative disorders, in which cognition is impaired. Moreover, the brain structures which are considered anatomical substrata for learning and memory may be particularly vulnerable to the neurotoxic actions of these excitatory amino acids, especially in the elderly, who are also the segment of the population most susceptible to impairment of mnemonic function. In the present study, the role of excitatory amino acids in the processes of learning and memory and the pathogenesis and treatment of disorders thereof came to focus. The ETH group had the lowest GPX level, indicating the role of indirect ethanol exposure in oxidative stress.

Glutamate is an excitatory neurotransmitter that has been associated with the enhancement of cognitive functions, [14-16] though, still incompletely defined in terms of its concentrations during disease conditions. Its highest value in the ETH pups in the present study is, therefore, not understood, even though this may indicate abnormal excitation of neurons as a result of maternal ethanol administration. The NSE group which had the lowest glutamate level may indicate a protective effect of *Nigella sativa* on the glutamate level of the pups in the group.

Dopamine is also involved in cognitive functions, to such extent that, a low dopamine level is an associated symptom of ADHD. [17,18] The ETH group had the lowest dopamine level while the preventive group, the NSE group, had the highest dopamine level, in corroboration of the neuroprotective potentials of *Nigella sativa*. [10]

From the analysis of the mean body weight differences, the ETH group had the least bodyweight increase while the NS group had the highest body weight increase. This is in concordance with the previously recorded effect of maternal ethanol consumption on neuroanatomical and behavioural development in mice. [19].Alcohol consumption has also been explained to interfere with nutrients absorption, and thus malnutrition. Therefore, alcohol-induced compromise of maternal nutritional status is deleterious to the foetus, as foetal abnormalities like ADHD, Intrauterine Growth Restriction (IUGR) or Foetal Alcohol Spectrum Disorder (FASD) abound. [20] The NS group had the highest weight difference and the longest curve in the growth analysis. This indicates the medicinal advantages of *Nigella sativa* on diet and body weight, although this differs from the reports of Bano *et al*, which suggests that direct administration of *Nigella sativa* decreases body weight via the suppression of appetite. [21]This seems to identify a difference between the weight limiting effects exhibited by *Nigella sativa* upon direct exposure and its weight enhancing effects in pups of pre-gestationally exposed dams. In line with the bodyweight analysis from the present study as well as earlier reports, [19] maternal ethanol consumption is further corroborated as deleterious to cellular development and body weight/growth.

On brain to body weight ratio, depicted by relative brain weight, the ETH pups showed the highest relative brain weight while the pups in the NSE group had the least relative brain weight. While this may be

a complementary consequence of the reduced total body weight in a different proportion to that of the brains, the relative brain weight finding in the ETH group in the present study contradicts a previous report in which the total brain volume was reduced upon maternal exposure to alcohol. [22]

On neuronal density and size, the NSE group had the highest density when compared to the ETH group. While a higher neuronal density may be indicative of a higher level of neuroplasticity and neural communication between the cortical regions of the brain, this finding corroborates the suggested neuroprotective roles of *Nigella sativa.* It also suggests the occurrence of lower cortico-cortical sensations in the prefrontal cortices of the ETH pups. Reduced neuronal size may be indicative of a diseased state. However, the lower sizes recorded in the NSE pups may mean that *Nigella sativa* was unable to prevent the ethanol-induced neuronal size deficits in these pups as it did with the neuronal densities. Nevertheless, this study was limited by the use of basic H&E stain for histological illustration of the prefrontal cortex. The use of Nissl and dendritic markers, as well as antibodies for immunohistochemical analysis, are recommended. Female mice are also recommended for use towards validation of the findings reported among male mice in this study.

Conclusion

In line with the prophylactic potentials reported in this study, *Nigella sativa* is suggested as a neuro-protective agent with the potentials of protecting against ADHD and related neurological disorders. Therefore, there is a need for further translational studies.

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Authors' Contributions: FR conceived and designed the study, performed data analysis, and interpretation, and drafted the initial manuscript. II participated in the review of the draft. AS, OT, OO, OA, and II participated in data collection and analysis. All the authors approved the final version to be manuscript.
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