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Original Article

Evaluation of the role of Neem leaf supplement in averting Alzheimer's Disease neuropathology and cognitive deficit in experimental model of Wistar Rats

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ABSTRACT

Background: Alzheimer's disease (AD) therapeutics interventions has a lot of limitation and the progression of the disease is alarming globally. There is need to discovery new and natural products such as neem plant with potential antioxidant property and document their therapeutic mechanism of neuroprotection through which natural products averts memory impairments by evaluating behavioral changes and neuroarchitecural changes in the PFC and hippocampus.
Methods: The twenty (20) healthy adult male Wistar rats used, were grouped as (n=5) groups A-D viz group A: control, B; AD-model C; oral 200mg/kg neem leaf supplement, and D: 200mg/kg neem leaf supplement treated AD model. Neuro-behavioral changes in memory was studied using Y-maze and open field test for object recognition. The brain samples were carefully removed for fixation in 10% formol calcium ready for tissue processing and staining using Haematoxylin and Eosin (H and E) stain, histochemical stain for Nissil bodies using Cresyl Fast Violent (CFV) stain and immuno-histochemical stain using Glial Fibrillary Acidic Protein (GFAP) for astrocytes. Data analysis was done using ANOVA and test for statistical significance set at $p<0.05$.
Results: Results show that neem leaf supplement reversed the declined spontaneous alternation behaviour, familiar object recognition time, recognition index while increasing novel object recognition and discriminatory index at $p<0.05$ in the AD model treated with neem leaf when compared with the AD model. These behavioral changes in neem leaf supplement correlates with histomorphological changes in the PFC and CA1 of the hippocampus having presence of neurons with neurites, lack chromatolysis and reduced proliferation of reactive astrocytes a neuroinflammatory response to neurodegeneration perturbing neural circuit as seen in the AD model.
Conclusion: Neem leaf supplement improves memory by inhibiting progressive proliferation of astrocytes, while boosting immune response for tissue repair that protect against loss of neurons and improve neuron function and connection in the prefrontal cortex and CA1 hippocampal region for memory consolidation.

KEYWORDS: Neem leaf, memory, oxidative stress, neuroinflammation, antioxidant, astrocytes

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1. Introduction

Neem (*Azadirachta indica*) is a global medicinal plant because of its vast therapeutic benefits such as regulation of immune activity, attenuates oxidative stress as an antioxidant, fights against infectious agent such as malarial parasite, fungi, bacteria and antimutagenic properties among others [1-2]. Neem leaf extract has powerful antiseptic, antimicrobial, antiviral, anti-fungal, analgesic, antibacterial, anti-inflammation and anti- histamine properties [2]. The working memory-related to spatial tasks reside in the prefrontal cortex while the hippocampus is the area of the brain largely responsible for memory and the consolidation of memories [3-4].These areas of the brain are affected by neurotoxin such as lead, ethanol, tetrodoxin, aluminum, fluoride etc [5-7] due to these toxin's ability to potentiate the synthesis of reactive oxygen species (ROS), that perturbs neuron and synaptic activity leading to the development of cognitive disorders such as Alzheimer's disease (AD) [8-9] in animal model. A fundamental research tool in translational neuroscience is animal behavior evaluation that helps to explain the physiological mechanism underlying the pathophysiology of neurodegenerative disorders [10]. In order to better explain AD-type pathology in vivo and testing therapeutic strategies of natural products [11-12] animal model are adopted in these experimental trials.

Aluminium, one of the top three common metals, gain access into human body through contamination in drinking water, food, cooking utensils, body deodorants, and pharmaceutical drug, and they gain access into the brain by their ability to permeate the blood brain barrier [13] to get accumulated in sensitive areas such as hippocampus and frontal cortex thereby contributing to the development of the pathophysiology of cognitive and motor disorders such as Alzheimer's disease (AD) and Parkinson's disease (PD) [14-16]. The use of natural herbal therapy (medicinal plants) in the treatment of neurodegenerative disorders such as Alzheimer's disease [12] cannot be overemphasized, since their pharmacological properties are linked with their antioxidant, neuroprotective and cholinergic activities [17]. Antioxidants help to mob off reactive oxygen species (ROS) or free radicals before they attack biological cells [18]. Neem plant's significance in health care and cognitive function is related to its rich source of phytochemicals such as azadirachtin, others are nimbolinin, nimbin, nimbanene, nimbidin, nimbidol, sodium nimbinate, gedunin, salannin, quercetin, nimbandiol, nimbolide, ascorbic acid, n-hexacosanol, amino acid, 7-desacetyl-7-benzoylazadiradione, 7-desacetyl-7-benzoylgedunin, 17-hydroxyazadiradione, and nimbiol [19-20]. The fresh leaves of neem are rich source of antioxidant due to the phytochemical components such as quercetin, azadirachtin, nimbolinin, nimbin, nimbidin, nimbidol, salannin, polyphenolic and other flavonoid [21-22]. This research was conducted to filled a knowledge gap mentioned by Sandhir et al., [23] on the need for continued research aimed to explain the potential mechanism through which neem leaf averts neurodegeneration and cognitive impairments, hence this study is aimed to evaluate mechanism through which neem leaf supplement enable neuroprotection in aluminium induced memory impairment by evaluating behavioral changes in Y-maze test, open field test, neuro-architectural alterations within the PFC and CA1 hippocampus in addition to changes in neuroinflammation reaction of the astrocytes.

Methods

Experimental Animals of Study:

Twenty (20) healthy adult male Wistar rats used in this study were obtained from the National Veterinary Research Institute (NVRI), Jos, Plateau State, Nigeria. All experimental investigations in this study was done in compliance with the guidelines to humane animal care standard outline according to the "Guidelines for the Care and Use of Laboratory Animals in research" [24] in the Animal House Department of Anatomy, Bingham University Karu, Nasarawa State, Nigeria.

Care for the experimental Animals: The experimental Wistar rats weighing between 120-150g were housed in well aerated metal cages whose floor was covered with fine wood shavings. They were kept in standard laboratory condition; maintained at an ambient temperature and a dark- light cycle (12:12 hours light and

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dark cycle respectively). They acclimatized into the new habitat for a period of two weeks while they were feed rat pellets [Vital UAC feeds, Nasarawa State Nigeria] and water ad libitum. The rat cages were regularly cleaned and wood shavings changed regularly after two days

Drugs of Study:

Drug material for AD induced neurotoxicity (AD animal model): Aluminum Chloride:

Experimental animal model in studies of Alzheimer's disease have shown that aluminium can be used to create pathological characterization of the disorder in animal models [10], it has been detected in the post mortem brains of people with dementia and AD [25] hence aluminum can exhibit neuro-oxidative tissue damage [26]. Aluminium chloride ($AlCl_3$) (98%; anhydrous) was obtained from Department of Chemistry, Bingham University, Karu Nigeria. The LD50 for $AlCl_3$ given orally is 200–1000mg/kg experimental studies using rats and mice [5]. This study used an oral dose of 200 mg/kg [7, 26,27]. There are evidence of neuronal cell loss and necrosis in 200 mg/kg, a characteristics of AD pathogenesis [7] and it is the non-lethal dose to model AD in animal study. $AlCl_3$ dissolved in distilled water was given at a daily dose of 200mg/kg bw orally.

Neem leaf herbal supplement Capsule: The neem leaf herbal dietary supplement capsule (Nature's Way Brands LLC, Green Bay, USA) was procured from HealthPlus Pharmacy, FCT Abuja, Nigeria. The drug dose used in this research was at 200mg/kg body weight of each rat. The neem herbal capsule is taken daily in humans at a dose of 475mg/kg body weight in which two capsules are taken daily as recommended by the manufacture; Nature's Way Brand LLC, Green Bay, USA.

Experimental Animal Grouping and Protocol

Table 1: Experimental Animal Grouping and Experimental Procedure.

Groups (n=5)	Treatments	Duration (Days)
A- Control	Received Water and Rats Pellets *ad libitum*	14 days
B- $AlCl_3$ treated (AD-neurodegeneration model)	Received Water and Rats Pellets *ad libitum* + oral 200mg/kg of $AlCl_3$	7 days
C- Neem leaf supplement	Received Water and Rats Pellets *ad libitum* + oral 200mg/kg of neem leaf	7days
D- Neurodegenerative Repair Model ($AlCl_3$ treated + Neem leaf supplement)	Received Water and Rats Pellets *ad libitum* + oral 200mg/kg of $AlCl_3$ for 7 days before oral 200mg/kg of neem leaf for 7 days.	14 days

Behavioral testing: The behavioral testing and handling of experimental animals were done in accordance with the guidelines according to the "Methods and welfare considerations in behavioral research with animals" (National Institutes of Mental Health [28], and "Guidelines for the care and use of mammals in neuroscience and behavioral research" [29].

Y-maze Test for Working Memory: The Y-maze test is a cognitive test that uses spontaneous alternation to measure spatial working memory, short term memory, cognitive and motor activity. Y-maze test was carried out in a Y-maze composed of three arms spaced equally, each having an angle of 120°, 41cm long and 15cm high. The floor of each arm is made of Perspex is 5cm wide. The Y maze arms being labeled A, B, and C. Each rats were placed in one arm, allowed free movement till the tail completely enters another

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arm. The numbers of arm entries were taken and spontaneous alternation is defined as entry into all three arms consecutively (A-B-C). The rats began their trial at one end of the arm and were allowed to move freely to explore the Y-maze for duration of 5mins and the sequence of arm entry were recorded manually. The percentage of alternation is calculated as: {the number of correct 3 choices divided by the number of total arm entry minus 2) x 100}. Each animal was tested for 5 minutes and the apparatus was sanitized and dried [30]. The Y-maze test has a chance level of 67%, that shows good memory and below 22% shows impaired memory [31] for example if an animal makes the following arm entries; ACBCABCACABCA, the total number of arm entries made is 13 of which 3 are correct choices and it would calculate as:

$$\% \text{ alternation} = \frac{\text{Number of correct 3 arm choices}}{\text{Total number of arm entry} - 2} \times 100$$

Object Recognition test for Recognition Memory:

The novel object recognition (NOR) task is a commonly applied test paradigm for the measure of working memory, attention, anxiety, and preference for novelty in rodents in order to test the effects of pharmacological agents and brain damage. Experimental animal's performance indices in this test are discrimination index, preference index, and recognition index. The object recognition index is measure by the difference in the exploration time of novel and familiar objects while the recognition index is measure by the interval between time spent with novel object and time spent with sample object and the time allowed for rats to explore the sample in a first trial. The preference for a novel object means that presentation of the familiar object exists in the animals' memory [32] and this test demonstrates the role of prefrontal cortex and hippocampus [32] to recall memory. The NOR exploits the natural behavior of the animal and measures short-term and long-term memory as well as recognition memory. Chance level for object recognition is 50%, a score below this indicates impaired memory and a score above shows healthy memory. The chance level for both discriminatory index (DI) and recognition index (RI) is zero (0), which shows no preference for novel or familiar objects respectively, an increase or decrease of +1 or -1 indicates a preference for either novel or familiar object respectively [32].

Object exploration [%] for the sample object (OE)

$$OE = \frac{\text{sample object interaction}}{\text{Sample object interaction + novel object interaction}} \times 100$$

$$\text{Discrimination index (DI)} = \frac{\text{time novel object} - \text{time with familiar object}}{\text{Time with novel object + time with familiar object}}$$

Recognition index (RI) =

$$RI = \frac{\text{\% of familiar exploration during pretest - \% of familiar exploration during test}}{\text{\% of familiar exploration during pretest [32].}}$$

Euthanasia of the experimental Animals: The final body weights of the animals were taken 24 hours after the last dose of Neem leaf extract using an analytic weighing scale (Manufactured by P.M Hana Ltd, Hong Kong, China). The animals were cervical dislocated then decapitated [33].

Brain tissue collection and preservation: The cranium of the rats was dissected using some bone forceps to expose the brain within the cranium. The wet weight was rapidly taken using a sensitive pocket scale (Camry Electronic pocket scale: Model; EHA25 China), then fixed in labeled sample bottles containing 10% formolcalcium ready for histological tissue processing [34]. The coronal sections of the prefrontal cortex

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and hippocampus were excised using landmark described by Paxinos and Watson Brain Stereotaxis Brain Mapping guide (-2.80mm from bregma for the hippocampus coronal section and 1.70mm from bregma for the prefrontal cortex coronal section).

Prefrontal and hippocampal cortices histological tissue processing: Excised tissues were processed using an automated tissue processor (Leica TP1020; Leica Microsystems, Germany). It was set to processed the tissue according to Bancroft and Gamble [34]. The processed tissues were embedded in paraffin wax and sectioned using a Rotatory Microtome (Leica RM 2125; Leica Microsystems, Germany) set at 5µm tissue thickness.

Prefrontal-hippocampal CA1 horn staining for histological, histochemical and immunohistochemical assay: Haematoxylin and Eosin (H and E) stain was caried out to evaluate the general histoarchitecture of the PFC and CA hippocampal cortex (33), the Cresyl fast violet stain was done to demonstrate the Nissl substance that stains of ribosomal RNA and the immunohistochemical demonstration for astrocytes was done using GFAP antibody protein GFAP [30,34].

Haematoxylin and Eosin (H and E) staining procedure: The PFC and CA1 horn was stained according to Bancroft and Gamble, [34] Sections were dewaxed in xylene for 10 minutes; then hydrate in descending grades of alcohol (absolute I and II, 100%, 90% and 70% respectively) for 2 mins; rinsed in water for one minute; stained in Mayer's Hematoxylin for 20 mins, rinsed in water for few minutes; differentiate in 1% acid alcohol. Rinse sections in slow running tap water for 10mins; then counter stained with eosin for 20mins; then rinsed in distilled water for 60 seconds, then dehydrate in ascending grades of alcohol 70%, 90%, 100% and absolute I and II and the slides cleared in xylene for 30 seconds. Then cover slipped using distyrene plasticizer and xylene (DPX) mountant and air dried.

Cresyl Fast Violet (CFV) for Nissl Bodies staining procedure: To stain the intracytoplasmic clumps or granules of RNA in the PFC and CA1 horn of the hippocampus as described by Bancroft and Gamble,(36) as follows- sectioned deparaffinized in xylene and hydrate in descending grades of alcohol (absolute I and II, 100%, 90% and 70% respectively) for 2 mins; then rinsed in water for one minute; sectioned were then dipped in cresyl fast violet solution for 5 minutes; then rinsed in two changes of distilled water; then alcohol for 30 secs each (85% alcohol; absolute alcohol for 30secs) then cleared in xylene for 1min and placed in balsam-xylene solution for 2mins then differentiated in two changes of absolute alcohol for 10-30secs each and check microscopically [30,34].

Glial Fibrillary Acid Protein (GFAP) staining procedure: GFAP is a major protein of the glial filaments in differentiated astrocytes. PFC and CA of the hippocampal tissue sections were treated with 0.01ml citrate buffer (PH 6.0) for 10mins to reveal the antigen as follows: sections were incubated in 0.3% hydrogen peroxide for 30 mins, thereafter blocked using 5% horse serum for 1-2hrs, slide sections were incubated with the primary antibody (1:500 monoclonal mouse anti-GFAP) at 4°C for 18-20hrs and rinsed in distilled water; incubate with biotinylated secondary antibodies (ABC kit, 1:200) and then with avidin-biotin complex; then developed with 0.05% diaminobenzene (DAB) and counterstained with Mayer's haematoxylin and rinse in distilled water; dehydrate in ascending grades of alcohol, 70%, 90%, 100% and absolute alcohol I and II , dry slide on hot plate, clear in xylene and mount with distyrene plasticizer and xylene (DPX) [30,34].

Statistical analysis: Data set were analyzed using GraphPad Prism 7 (GraphPad software, Inc., LA Jolla, CA). Student's t-test was done for paired comparisons and one-way ANOVAs were used for all multiple comparisons followed by the post hoc Tukey test where the significance was at p-values was set at $P<0.05$ indicated by asterisks ($*p < 0.05$). Data were expressed as mean ± standard deviation (SD).

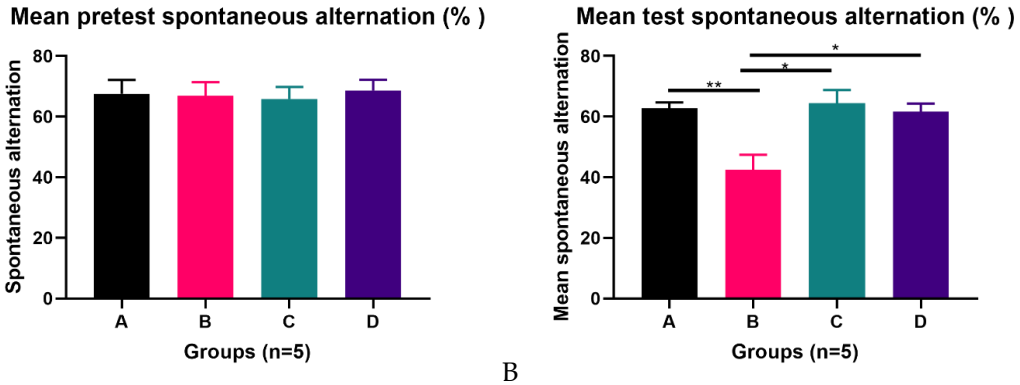
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Photomicrography of histological, histochemical and immunohistochemical results: Sections were visualized with LEICA digital bright field microscope and images captured using a X40 objective lens with an attached MV 500 Cameroscope ™ (5.1 MP) digital camera with phototube of X10.

Results

Neem leaf improves spatial memory induced by aluminium induced AD model: The spontaneous alternation behaviour during experimental testing for the neem leaf extract shows a significance difference when comparing between the control (A) and $AlCl_3$ treated (B), i.e: $AlCl_3$ treated had a significance decrease in spontaneous alternation behaviour when compared with the control (**A vs B: $p<0.0077$) as seen in Fig 1B. The $AlCl_3$ treated (B) had a significance reduction in spontaneous alternation behaviour when compared with the neem leaf treated group (C) and $AlCl_3$ induced AD treated with the neem leaf (D) groups using Tukey's multiple comparisons test (*B vs D; $P<0.0111$; *B vs D $p<0.0130$). but there was no significance when comparing the control groups (A) versus C and D ($p<0.8132$; 0.8139) and the neem leaf treated group (C) vs $AlCl_3$ induced neem leaf treated group (D) at $p<0.05$.

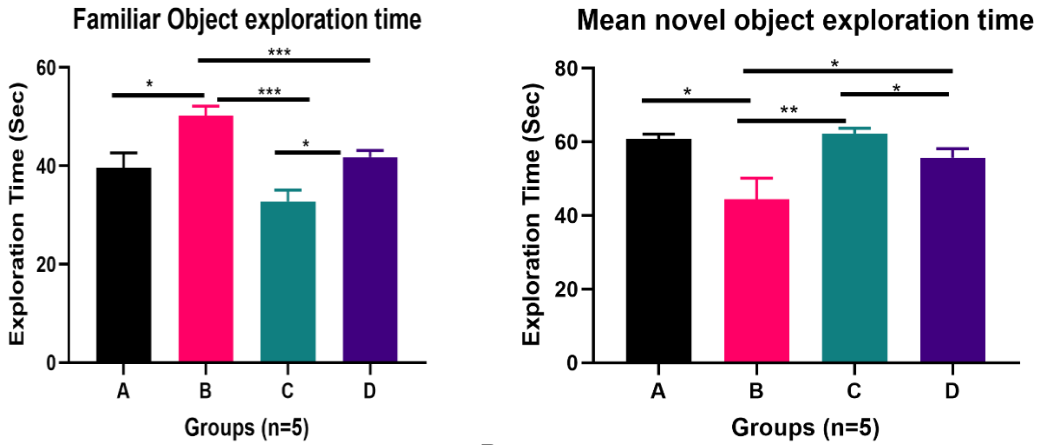


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Figure 1: Graphical representation of the mean pretest and test spontaneous alternation behaviour in percentage in experimental animals used in this study. Data analyzed using one-way ANOVA and expressed as Mean ± Standard Deviation (Mean ± SD). Statistical Significance is taken at $P<0.05$ (*) using Tukey Post hoc test. Legend: A- Control; B: $AlCl_3$ treated; C- neem leaf treated, and D: $AlCl_3$ +Neem leaf treatment. There was no significance difference @$p<0.05$ in the pretest spontaneous alternation of the experimental animals using Tukey multiple comparisons test as shown in Fig. 1A. However the spontaneous alternation behaviour during the neem leaf extract administration test shows a significance difference when testing between the control (A) and $AlCl_3$ treated (B), i.e: $AlCl_3$ treated had a significance decrease in spontaneous alternation behaviour when compared with the control (**A vs B:$p<0.0077$) as seen in Fig 1B. $AlCl_3$ treated (B) had a significance reduction in spontaneous alternation behaviour when compared with the neem leaf treated group (C) and $AlCl_3$ induced the neem leaf treatment (D) groups using Tukey's multiple comparisons test (*B vs D; $P<0.0111$; *B vs D $p<0.0130$). but there was no significance when comparing the control groups (A) versus C and D ($p<0.8132$; 0.8139) and the neem leaf treated group (C) vs $AlCl_3$ induced neem leaf treatment group(D) at $p<0.05$.

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A B

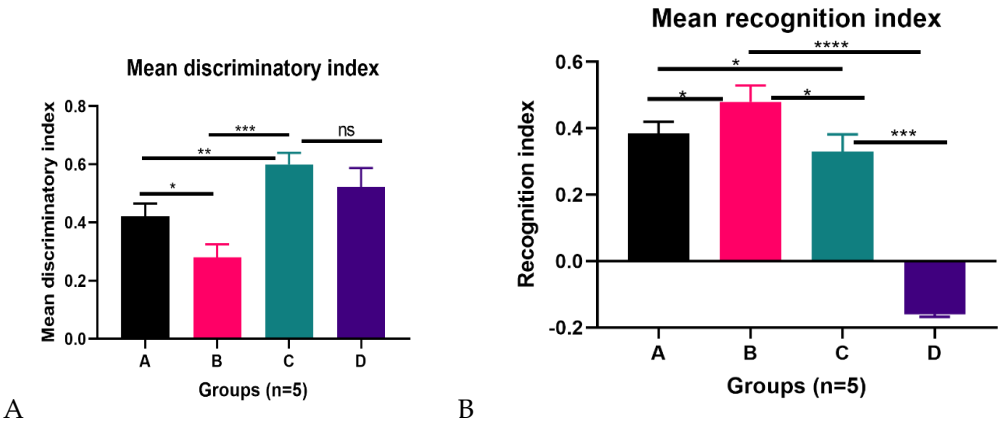
Figure 2: Graphical representation of mean familiar and novel object exploration time in experimental animals used in this study. Data analyzed using one-way ANOVA and expressed as Mean ± Standard Deviation (Mean ± SD). Statistical Significance is taken at P<0.05 (*) using Tukey *Post hoc* test. Legend: A-Control; B: $AlCl_3$ treated; C- neem leaf treated, and D: $AlCl_3$ +neem leaf treatment. In the novel object exploration time, the control (A) had an increased time spent exploring as compared with the $AlCl_3$ treated (B) group (*A vs B: p<0.0209) and $AlCl_3$ +neem leaf treatment (D) (*A vs D: p<0.0454). In the $AlCl_3$ treated (B) group it was noted that they have a lowered novel object exploration as compared with neem treated groups C and D (**B vs C p<0.0088; *B vs D p<0.0474) as seen in Fig 2A. The neem leaf treated group (C) had an increase novel object exploration time (*C vs. D p<0.0181)) using Tukey's multiple comparisons test. The familiar object exploration time in the control (A) group increased when compared with $AlCl_3$ treated (B) (*A vs B p< 0.0249). The control (A) group had no significant difference when compared with neem leaf treated (C) and $AlCl_3$ + neem leaf treated (D) groups. Novel object exploration time in $AlCl_3$ treated (B) decreased as compared with neem leaf treated (C) and $AlCl_3$ +neem leaf treated (D) groups. (***B vs C P<0.0001; ** B vs D p<0.0051) as seen in Fig 2B. The neem leaf treated (C) group had a decline in novel object exploration time when compared with the $AlCl_3$ + neem leaf treated (D) group (*C vs D p<0.0138). The $AlCl_3$ treated (B) had an increase in familiar object exploration as compared with novel object exploration. The neem leaf treated group (C) had a decline in familiar object exploration time as compared with novel object exploration time. The neem leaf treated $AlCl_3$ induced neurodegeneration model (D) had an increase in novel object exploration when compared with the familiar object exploration time.

Neem leaf improves recognition memory in open field test: In the novel object exploration time, the control (A) had an increased time spent exploring as compared with the $AlCl_3$ treated (B) group (*A vs B: p<0.0209) and $AlCl_3$ + neem leaf treatment (D) (*A vs D: p<0.0454). In the $AlCl_3$ treated (B) group it was noted that they had a lowered novel object exploration as compared with neem treated groups C and D (**B vs C p<0.0088; *B vs D p<0.0474) as seen in Fig 4A. The neem leaf treated group (C) had an increase novel object exploration time as compared with group D (*C vs. D p<0.0181) using Tukey's multiple comparison test. The familiar object exploration time in the control (A) group increased when compared with $AlCl_3$ treated (B) (*A vs B p< 0.0249). The control (A) group had no significant difference when compared with neem leaf treated (C) and $AlCl_3$ + neem leaf treated (D) groups. Novel object exploration time in $AlCl_3$ treated (B) decreased as compared with neem treated (C) and $AlCl_3$ + neem leaf treated (D) groups. (***B vs C P<0.0001; ** B vs D p<0.0051) as seen in Fig 4B. The neem leaf treated (C) group had a decline in novel object exploration time when compared with the $AlCl_3$ +neem leaf treated (D) group (*C vs D p<0.0138). The $AlCl_3$ treated (B) had an increase in familiar object exploration as compared with novel object exploration. The neem leaf treated group (C) had a decline in familiar object exploration time as

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compared with novel object exploration time. The neem leaf treated $AlCl_3$ induced neurodegeneration model (D) for AD had an increase in novel object exploration when compared with the familiar object exploration time.



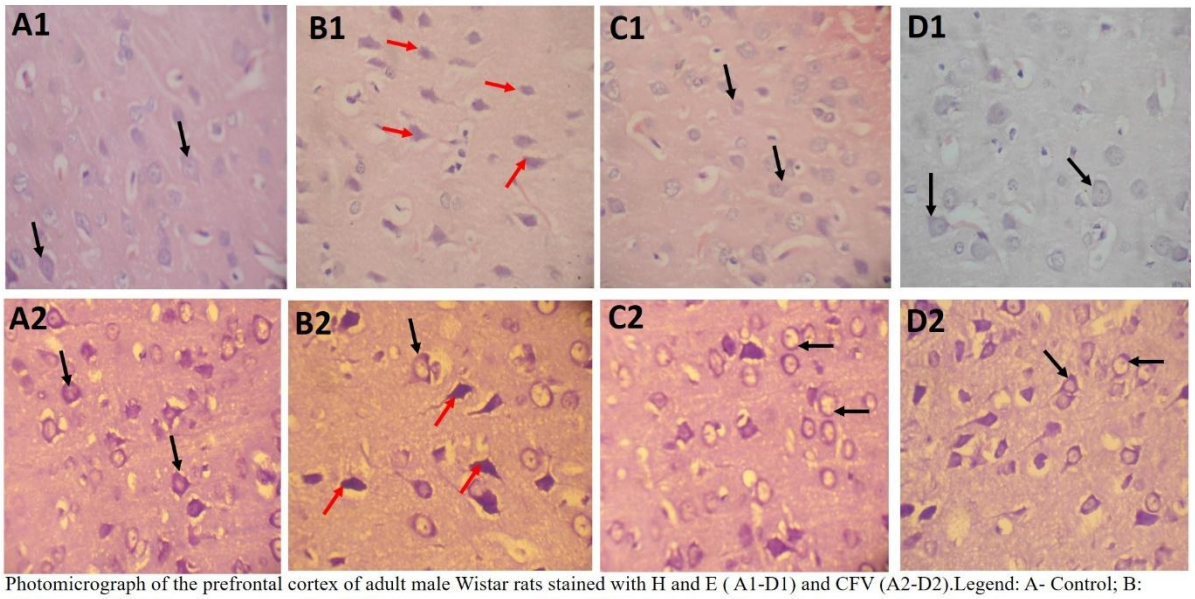
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Figure 3: Graphical representation of mean discriminatory and recognition indices of the experimental animals used in this study. Data analyzed using one-way ANOVA and expressed as Mean ± Standard Deviation (Mean ± SD). Statistical significance set at P<0.05 (*) using Tukey *Post hoc* test. Legend: A- Control; B: $AlCl_3$ treated; C- neem leaf treated, and D: $AlCl_3$ +neem leaf treatment. The discriminatory index of the control (A) increased as compared to the $AlCl_3$ treated (B) (*A vs B p<0.0224) and neem treated (C) (**A vs C p<0.0030). but there was no significant difference between the control and AlCl3 +neem treated (D) group as seen in Fig 5A. The discriminatory index for AlCl3 treated (B) declined when compared with neem leaf treated (C) and $AlCl_3$ +neem leaf treated (D) groups (***B vs C p<0.0010; **B vs D p< 0.0062). but there was no significant difference between neem treated (C) and $AlCl_3$ +neem leaf treated (D) group at p <0.05. The data from recognition index indicated that the control (A) group had a significant decline in recognition index when compared with $AlCl_3$ treated (B) (*A vs B p<0.0348), but had a slight increase in recognition index when compared with neem treated (C) (*A vs C p<0.0327) as shown in Fig 3B. Also, the control (A) had an increase in recognition index as compared with $AlCl_3$ +neem leaf treated (D) (***A vs D p< <0.0001). The $AlCl_3$ treated (B) treated when compared with neem leaf treated (C) and $AlCl_3$ +neem leaf treated (D) (* B vs C p<0.0192; ***B vs D p<<0.0001). The neem leaf treated (C) group had a significant increase as compared with the $AlCl_3$ +neem leaf treated (D) (***C vs D p<0.0001).

Effect of neem leaf and Aluminum on discriminatory and recognition index: The discriminatory index of the control (A) increased as compared to the $AlCl_3$ treated (B) (*A vs B p<0.0224) and neem leaf treated (C) (**A vs C p<0.0030). but there was no significant difference between the control and AlCl3 +Neem leaf treated (D) group as seen in Fig 3A. The discriminatory index for $AlCl_3$ treated (B) declined when compared with neem treated (C) and $AlCl_3$ +neem leaf treated (D) groups (***B vs C p<0.0010; **B vs D p< 0.0062). but there was no significant difference between the neem leaf treated (C) and $AlCl_3$ +neem leaf treated (D) group at p <0.05. The data from recognition index indicated that the control (A) group had a significant decline in recognition index when compared with AlCl3 treated (B) (*A vs B p<0.0348), but had a slight increase in recognition index when compared with the neem leaf treated (C) (*A vs C p<0.0327) as shown in Fig 3B. Also, the control (A) had an increase in recognition index as compared with $AlCl_3$ +neem leaf treated (D) (***A vs D p< <0.0001), also the AlCl3 treated (B) treated had an increase when compared with neem leaf treated (C) and $AlCl_3$ +neem leaf treated (D) (* B vs C p<0.0192; ***B vs D p<<0.0001). The neem leaf treated (C) group had a significant increase as compared with the $AlCl_3$ +neem leaf treated (D) (***C vs D p<0.0001).

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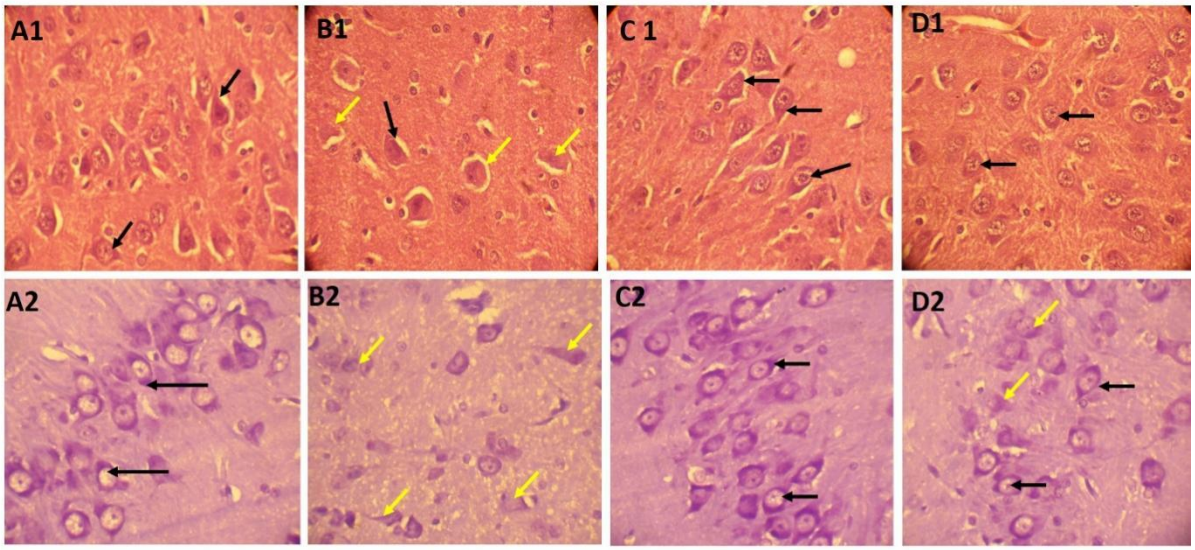


Photomicrograph of the prefrontal cortex of adult male Wistar rats stained with H and E (A1-D1) and CFV (A2-D2).Legend: A- Control; B: AlCl3 treated; C- Neem treated, and D: AlCl3 +Neem treatment. Mag. X400. Scale bar: 50µm. Red arrows: Necrotic or degenerated neurons; Black arrows: non-degenerated neuronal cell. A2, C2, and D2 have neurons positive for Nissl substances while B2 neurons characterized by chromatolysis.

Figure 4: Photomicrograph of the prefrontal cortex of adult male Wistar rats stained with H and E (A1-D1) and CFV (A2-D2) stains, Legend: A- Control; B: $AlCl_3$ treated; C- neem leaf treated, and D: $AlCl_3$ +neem leaf treatment. Mag. X400. Scale bar: 50µm. Red arrows: Necrotic or degenerated neurons; Black arrows: non-degenerated neuronal cell. The histological appearance of the prefrontal cortex of the control (A1) stained using H and E showed the presence of numerous pyramidal neurons with their neurite extension within the dense neuropil as compared with the $AlCl_3$ treated (B) for AD model of neurodegeneration which is characterized by palely stained neuropil, with pericellular spaces surrounding the homogenous degenerated pyramidal neurons, numerous pyknotic cells. However, neem leaf treated (C) showed the presence of numerous pyramidal neurons with their centrally located nucleus, axonal and dendritic outgrowths. The group D ($AlCl_3$ +Neem treatment), shows that neem leaf protects against aluminium induced oxidative tissue damages (seen in B1) as demonstrated in the presence of regenerating neurons with scanty neuropil and few necrotic neurons when compared with $AlCl_3$ treated (B). (see Fig 4 A1-D1) The CFV stained is used to demonstrate Nissl bodies a representation of ribosome required for protein or amino acid synthesis required in tissue injury repair and building block for neurotransmitters for neuron stimulation. The control (A) and neem treated (C) are positive for Nissl stain with absence of chromatolytic neurons (protein aggregation) as compared with $AlCl_3$ treated (B) characterized by a scanty neuropil with numerous necrotic neurons undergoing chromatolysis (see Fig 4 A2-D2). But the neem leaf treated $AlCl_3$ induced neurodegeneration has numerous neurons positive for Nissl granules and lack of chromatolytic neurons as seen in $AlCl_3$ treated (B).

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Photomicrograph of the hippocampus CA1 horn of adult male Wistar rats stained with H and E (A1-D1) and CFV (A2-D2) .Legend: A- Control; B: AlCl3 treated; C- Neem treated, and D: AlCl3 +Neem treatment. Mag. X400. Scale bar: 50µm. Yellow arrows: Necrotic or degenerated neurons; Black arrows: non-degenerated neuronal cell. A2, C2, and D2 have neurons positive for Nissl substances while B2 neurons characterized by chromatolysis.

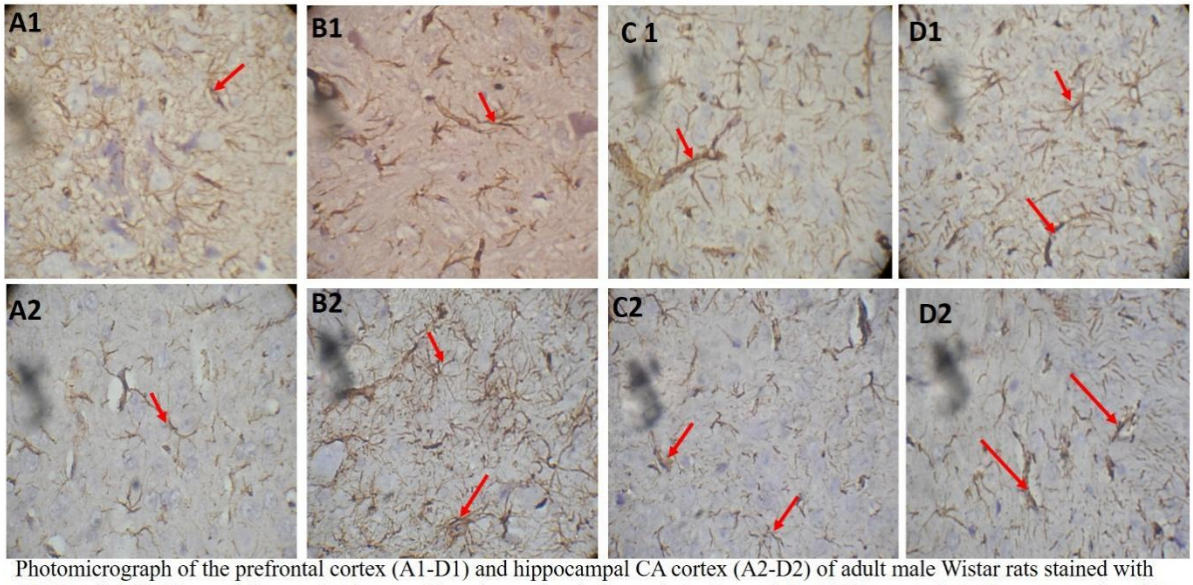
Figure 5: Photomicrograph of the hippocampus CA1 horn of adult male Wistar rats stained with H and E (A1-D1) and CFV (A2-D2). Legend: A- Control; B: $AlCl_3$ treated; C- neem leaf treated, and D: AlCl3 +neem leaf treatment. Mag. X400. Scale bar: 50µm. Yellow arrows: Necrotic or degenerated neurons; Black arrows: non-degenerated neuronal cell. The histomorphology of the hippocampus CA1 stained with H and E showed the presence of numerous pyramidal neurons with their neurite extension within the dense neuropil as compared with the $AlCl_3$ treated (B) for AD model characterized by few pyknotic neurons with homogenous cytoplasm. But the neem leaf supplement treated (C) showed the presence of numerous pyramidal neurons with their axonal and dendritic outgrowth (see Fig 5 A1-D1). The neem leaf supplement treated aluminum induced AD model (B) is characterized by numerous pyramidal numerous indicating neem leaf neuroprotective function as compared with $AlCl_3$ treated (B). In the CFV stained used to demonstrated Nissl granulation a representation of ribosome required for amino acid synthesis required in tissue injury repair and neurotransmitters synthesis required in neuron stimulation. The control (A) and neem leaf treated (C) are positive for Nissl stain with absence of chromatolytic neurons (protein aggregation) as compared with $AlCl_3$ treated (B) characterized by a scanty neuropil with few neurons undergoing chromatolysis (see Fig 5 A2-D2). But the neem leaf treated $AlCl_3$ induced neurodegeneration has numerous neurons positive for Nissl granules and lack of chromatolytic neurons as seen in $AlCl_3$ treated (B).

Neem leaf prevents aluminum induced oxidative loss of neurons in the prefrontal and CA1 hippocampal neurons: The histological appearance of the prefrontal cortex of the control (A1) stained using H and E showed the presence of numerous pyramidal neurons with their neurites extension within the dense neuropil as compared with the $AlCl_3$ treated (B) for AD model of neurodegeneration which is characterized by palely stained neuropil, with pericellular spaces surrounding the homogenous degenerated pyramidal neurons, numerous pyknotic cells. However, the neem leaf treated (C) showed the presence of numerous pyramidal neurons with their centrally located nucleus and axonal and dendritic outgrowth. The group D ($AlCl_3$ + neem leaf treatment), shows that neem leaf protects against aluminum induced oxidative tissue damage (seen in B1) as demonstrated in the presence of regenerating neurons with scanty neuropil and few necrotic neurons when compared with $AlCl_3$ treated (B). (see Fig 5 A1-D1) The CFV stained is used to demonstrate Nissl /chromatophilic bodies which represents ribosome required for

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protein or amino acid synthesis required in tissue injury repair and building block for neurotransmitters. The control (A) and neem treated (C) are positive for Nissl stain with absence of chromatolytic neurons (protein aggregation) as compared with $AlCl_3$ treated (B) characterized by a scanty neuropil with numerous necrotic neurons undergoing chromatolysis (see Fig 5 A2-D2). But the neem leaf treated $AlCl_3$ induced neurodegeneration has numerous neurons positive for Nissl granules and lack of chromatolytic neurons as seen in $AlCl_3$ treated (B).



Photomicrograph of the prefrontal cortex (A1-D1) and hippocampal CA cortex (A2-D2) of adult male Wistar rats stained with GFAP (glial fibrillary acidic protein) immunohistochemcal stain Legend: A- Control; B: AlCl3 treated; C- Neem treated, and D: AlCl3 +Neem treatment. Mag. X400. Scale bar: 50µm. Red arrows: astrocytic processes. PFC and CA1 horn have more astrocytes proliferation (B1 and B2) as compared to the control and Neem treated groups with mild expression

Figure 6: Photomicrograph of the prefrontal cortex (A1-D1) and hippocampal CA cortex (A2-D2) of adult male Wistar rats stained with GFAP (glial fibrillary acidic protein) immunohistochemcal stain. Legend: A-Control; B: $AlCl_3$ treated; C- neem leaf treated, and D: $AlCl_3$ + neem leaf treatment. Mag. X400. Scale bar: 50µm. GFAP immunohistochemical is use as a biomarker for neuroinflammatory reaction indicated by proliferation of reactive astrocytes (gliosis). Red arrows: astrocytic processes. PFC and CA1 horn have more astrocytes proliferation (Fig8 B1 and B2) as compared to the control and the neem leaf treated groups with mild expression. The control and the neem leaf treated show mild expression of astrocytes indicated by the brownish pigmentation of intermediate filament of the astrocytic processes in the PFC and CA1 horn of the hippocampus as compared with the AlCl3 treated group to induced neurodegeneration a model for AD characterized by numerous reactive astrocytes expressed by the intensity of the brownish colouration of the astrocytic processes (Fig 6 B1 and B2). But the neem leaf treated AlC_{13} induced neurodegeneration group had a reduced proliferation of reactive astrocytes.

Neem leaf prevents aluminum induced necrosis and chromatolysis (loss of neurons and Nissl bodies) in the prefrontal and CA1 hippocampal neurons: The histomorphology of the hippocampus CA1 stained with H and E showed the presence of numerous pyramidal neurons with their neurite extension within the dense neuropil as compared with the $AlCl_3$ treated (B) for AD model characterized by few pyknotic neurons with homogenous cytoplasm. But the neem leaf supplement treated (C) showed the presence of numerous pyramidal neurons with their axonal and dendritic outgrowths (see Fig 6A1-D1). The neem leaf supplement treated aluminum induced AD model (B) is characterized by numerous pyramidal numerous demonstrating neem leaf neuroprotective functions as compared with $AlCl_3$ treated (B). In the CFV stained used to demonstrated Nissl granulation, the control (A) and the neem leaf treated (C) were positive for Nissl stain with absence of chromatolytic neurons (protein aggregation) as compared with $AlCl_3$ treated

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(B) characterized by a scanty neuropil with few neurons undergoing chromatolysis (see Fig. 6 A2-D2). But the neem leaf treated $AlCl_3$ induced neurodegeneration has numerous neurons positive for Nissl granules and lack of chromatolytic neurons as seen in $AlCl_3$ treated (B).

Neem leaf prevents continuous proliferation of aluminum induced reactive astrocytes a biomarker for neuroinflammation in the PFC and CA1 horn of the hippocampus: GFAP immunohistochemical is use as a biomarker for neuroinflammatory reaction indicated by proliferation of reactive astrocytes (gliosis). The control and the neem leaf treated show mild expression of astrocytes indicated by the brownish pigmentation of intermediate filament of the astrocytic processes in the PFC and CA1 horn of the hippocampus as compared with the $AlCl_3$ treated group to induced neurodegeneration a model for AD characterized by numerous reactive astrocytes expressed by the intensity of the brownish colouration of the astrocytic processes (Fig 6 B1 and B2). But the neem leaf treated $AlCl_3$ induced neurodegeneration group had a reduced proliferation of reactive astrocytes.

Discussion

Neem also referred to as *Azadirachta indica* has been massively applied in traditional or herbal medicine due to its extensive medicinal properties [23]. This study is carried out to explain the therapeutic mechanism of action of neem leaf supplement to avert the neuropathological responses as seen in aluminium induced AD model characterized by cognitive impairment, neuroinflammatory reaction in astrocytes and neuro histological changes in the PFC and CA1 of the hippocampus the region of the brain involved in learning and memory by evaluating behavioral changes in Y-maze, open field tests neuroarchitecural changes in the PFC and hippocampus and neuroinflammation reaction of the astrocytes. Evaluation of spontaneous alternation using the Y-maze behavioural test paradigm [30] to access spatial working memory and also short-term memory [32,35]. For the purpose of this research, two paradigms were employed; the spontaneous alternation behavior (SAB) and the reward paradigm. The reward paradigm employs the use of re-enforcements when the animal makes a correct entry and the SAB paradigm allows the animal to explore novel arms without re-enforcements.

When running the reward paradigm, the control group A, showed normal time in locating the correct arm with the food even after it was removed, however the aluminum induced memory impaired model took a longer time to locate the correct arm this effect was reversed in those treated with neem leaf this collaborates neem leaf reported antioxidant potential to averts neurodegeneration mechanism that perturbs normal neuronal activity and function mediated by ROS production thereby collaborating report made by Raghavendra et al., [36] that neem leaf supplement can improve reference memory, working memory and spatial learning. In the SAB paradigm, the neurodegenerative model, group B showed a higher percentage of alternation due to loss of spatial memory of the Y-maze which was averted by Neem leaf treatment. This report collaborates with finding reported by Wan et al, [37] that the neem leaf enhances memory and cognition demonstrated in the normal sequence and increased number of SAB percentage in Y-maze associated with its antioxidant potential and memory enhancing ability as reported by Raghavendra et al., [36].

The novel object recognition test is based on the innate ability of a rodent's exploratory behavior and is configured to measure working memory, attention, anxiety and preference for novelty in rodents. According to D'Isa et al., [32], the index for measuring the chance level in the novel object recognition is 50, a score below 50 is indicative of memory impairment and vice versa for both the familiar and novel object explored by the animal. In the novel object exploration time, the control (A) had an increased time spent exploring as compared with the $AlCl_3$ treated (B) group Hence, neem leaf supplement improves recognition memory in open field test. This support report made by Raghavendra et al., [36]. The $AlCl_3$ treated (B) had an increase in familiar object exploration as compared with novel object exploration. The neem leaf treated

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group (C) had a decline in familiar object exploration time as compared with novel object exploration time. Neem leaf treated $AlCl_3$ induced AD model (D) had an increase in novel object exploration when compared with the familiar object exploration time. In this study, neuro-pathological changes in the PFC and the hippocampus cortical neurons were examined to report structural changes and neuronal function in animal behaviour relative to cognition and memory. It was observed that the PFC and CA1 horn of the hippocampus treated with aluminum had neuropathological features characterized by palely stained neuropil, with pericellular spaces surrounding the homogenous degenerated pyramidal neurons and numerous pyknotic cell was ameliorated by Neem leaf treatment in the memory impaired model. The observation in the aluminum induced neurodegeneration and neuroinflammation supports report made by Akinrinade et al, [5] and Campbell et al, [14]. The neuroprotective and repair role of neem leaf against aluminum induced neurodegeneration correlates with report presented by Haynes et al, [38]. Nissl bodies represent aggregation of rough endoplasmic reticulum that are seen to be abundant in large neurons where they function in the production and dispersal of chemical substances such as proteins/peptides important for protein synthesis, an important cellular process for neurons and are found in the soma and dendrites of the neuron, when there is reduced aggregation of the rough endoplasmic reticulum, the neuronal body swells and displaces the nucleus toward the periphery of the cell [7]. The aggregation of this rough endoplasmic reticulum appears normal in control and neem leaf, this demonstrates role of the phytochemicals of Neem leaf to protect the integrity of the neurons via it anti-inflammatory and antioxidant potential [1,21] whereas the neurodegenerative model (B) had a chromatolytic neurons this effect was ameliorated in the neem leaf treated AD model group. The chromatolytic neurons is indicative of loss of Nissl granules and indicator of neurodegeneration [5,7].

Alzheimer's disease is often associated with elevated inflammation [27] which is associated with neuronal dysfunction resulting in learning and memory disorders [16,42]. In this present study aluminium induces neuroinflammation demonstrated by an increased expression of astrocytes in PFC and hippocampal cortex this biomarker for neuroinflammation has being linked to mitochondrial dysfunction and loss of neuron function hence, supporting report by Appanna et al., [40] that aluminum induces oxidative stress which is associated with neuroinflammatory response seen in astrogliosis as well as the accumulation of tau and apoptosis resulting in neuronal dysfunction [15] and this has being linked to impaired hippocampal long-term potentiation in rats [43] thereby changing the neuronal and synaptic ultrastructure in the hippocampus and PFC [44]. The neem leaf neuro-inflammatory and antioxidant properties enable it's to regulate normal brain function and prevent neurodegenerative mechanism [23]. GFAP immunohistochemical is use as a biomarker for neuroinflammatory reaction indicated by proliferation of reactive astrocytes (gliosis). In this study, neem leaf prevents continuous proliferation of aluminum induced reactive astrocytes a biomarker for neuroinflammation in PFC and CA1 horn of the hippocampus.Hence suggesting that aluminium induce neuroinflammation resulting in loss of neuronal cell and dendritic spine, and thereby leads to learning and memory deficits [5,7] as demonstrated in this present report while also demonstrating the neuroprotective role of neem leaf supplement in the experimental models of Alzheimer's disease [36]. The presence of reactive astrocytes in neurodegenerative model increased, this support reports made by [5,7] which implies that aluminum is an activator for oxidative stress and neuro-inflammation. However, the neem leaf treatment reversed this inflammatory reaction due to its anti-inflammatory property [5, 36]. Neem leaf helped to protect and possibly maintain the neurocognitive role of neurons in the PFC and CA hippocampus in retaining long-term memories [44]. According to Sandhir et al., [23] and Memudu et al [44] neem leaf supplement potentiates its role to avert neurodegeneration and memory deficit is mediated by its anti-oxidant and anti-inflammatory property to protect neuron cellular integrity, prevent distortion of neural circuit, modulate signaling pathways [24] for neuronal activity and synaptic connection.

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Conclusions

Neem leaf supplement improves memory by inhibiting progressive proliferation of astrocytes, while boosting immune response for neuron repair and protection against oxidative stress mediated loss of neurons which improves neuron function and connection in the prefrontal cortex and CA1 hippocampal region for memory and cognitive functions.

Abbreviations:
ROS: Reactive oxygen species
AD: Alzheimer's disease
PD: Parkinson's disease
PFC: prefrontal cortex
NVRI: National Veterinary Research Institute
LD50: Lethal Dose 50
$AlCl_3$: Aluminumm Chloride
NOR: novel object recognition (NOR)
OE: Object exploration
DI: Discrimination index
RI: Recognition index
H and E: Haematoxylin and Eosin
CFV: Cresyl Fast Violet
RNA: Ribonuclie Acid
GFAP:Glial Fibrillary Acid Protein

Declarations

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Author contributions:
Author contributions for CRediT roles are as follows**: Adejoke Elizabeth Memudu:** Conceptualization and design of the study; writing and proofreading final draft; Data curation; Methodology; Supervision; Validation; analysis and interpretation of data; **Grace Mchibuma Jibaniya:** Investigation; Methodology; Writing-original draft; acquisition of data, or analysis and interpretation of data.

Conflicts of interest:
The authors declare that they have no conflicts of interest.

Ethical approval:
Study procedures were compliant with the principles of the Declaration of Helsinki.

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