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RESEARCH ARTICLE

β-sitosterol Protects against Aluminium Chloride-mediated Neurotoxicity

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Abstract: ***Objective*:** The objective of this study is to investigate the neuroprotective effects of β-sitosterol using the $AlCl_3$ model of Alzheimer's Disease.

***Methods*:** $AlCl_3$ model was used to study cognition decline and behavioral impairments in C57BL/6 mice. Animals were randomly assigned into 4 groups with the following treatments: Group 1 received normal saline for 21 days, Group 2 received $AlCl_3$ (10 mg/kg) for 14 days; Group 3 received $AlCl_3$(10 mg/kg) for 14 days + β-sitosterol (25mg/kg) for 21 days; while Group 4 was administered β-sitosterol (25mg/kg) for 21 days. On day 22, we performed the behavioral studies using a Y maze, passive avoidance test, and novel object recognition test for all groups. Then the mice were sacrificed. The corticohippocampal region of the brain was isolated for acetylcholinesterase (AChE), acetylcholine (ACh), and GSH estimation. We conducted histopathological studies using Congo red staining to measure β -amyloid deposition in the cortex and hippocampal region for all animal groups.

***Results*:** $AlCl_3$ successfully induced cognitive decline in mice following a 14-day induction period, as shown by significantly decreased ($p < 0.001$) in step-through latency, % alterations, and preference index values. These animals also exhibited a substantial decrease in ACh ($p < 0.001$) and GSH ($p < 0.001$) and a rise in AChE ($p < 0.001$) compared to the control group. Mice administered with $AlCl_3$ and β-sitosterol showed significantly higher step-through latency time, % alteration time, and % preference index ($p < 0.001$) and higher levels of ACh, GSH, and lower levels of AChE in comparison to the $AlCl_3$ model. $AlCl_3$-administered animals also showed higher β-amyloid deposition, which got significantly reduced in the β-sitosterol treated group.

***Conclusion*:** $AlCl_3$ was effectively employed to induce a cognitive deficit in mice, resulting in neurochemical changes and cognitive decline. β -sitosterol treatment mitigated $AlCl_3$-mediated cognitive impairment.

Keywords: β-sitosterol, cognitive deficit, Alzheimer's disease, acetylcholinesterase, oxidative stress, AlCl3.

1. INTRODUCTION

Cognitive impairment is a predominant outcome of various neurological disorders. Exposure to toxins, depression, metabolic diseases, concussions, and aging [1] may all impair learning and memory [2, 3]. The hallmarks of Alzheimer's disease (AD) include gradual memory loss, cognitive dysfunction, and mental confusion. β -amyloid(Aβ) plaques and neuronal cell death are the two main contributors to the neurodegenerative process and the key hallmarks of AD [4]. β-sitosterol, a predominant component of the human diet, has been shown to have immune boosting, hypolipidemic and cardioprotective functions. Its role in neurological disorders is being explored [3, 5, 6]. β-sitosterol (SIT) is a counterpart of cholesterol not synthesized by mammalian tissue [7-6] and are primarily obtained by animals through their diet. Soybeans, rice bran, wheat germ, maize oils, vegetable oils and products manufactured from them as well as avocado oil (76 mg/100 g) are rich sources of SIT [8-10]. Taking 1.5–2.4 g/day of phytosterols and/or sterols is recommended by the European Food Safety Authority (EFSA) to decrease blood cholesterol [11]. Additionally, the US FDA has approved the role of foods containing phytosterol esters in reducing the risk of heart disease when ingested along with a low-saturated-fat and low-cholesterol diet [12].

Epidemiological investigations have indicated that aluminum (Al) is an environmental neurotoxicant. It is associated with neurodegenerative diseases [13]. Additionally, exposure to Al is known to cause neurobehavioral abnormalities in mice [14, 15]. Al is involved in amyloid deposition and eventual AD progression. According to research, excessive Al can lead to neurotoxicity from amyloid deposition. Previous *in vivo* studies have demonstrated that Al-induced cen-

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tral nervous system (CNS) injury can affect CNS neuropathology, neurobehavioral alterations, neurochemistry, and neurophysiology [16]. $AlCl_3$ may also be involved in the malfunctioning of cerebral microcirculation [4, 17]. The current study evaluated the effectiveness of β-sitosterol as a neuroprotective agent using the $AlCl_3$ model of AD. *In silico* study revealed that β-sitosterol interacts with AChE, while *in vivo* study showed that β-sitosterol ameliorated $AlCl_3$ mediated cognitive impairment in mice.

2. MATERIALS AND METHODS

2.1. Experimental Mice

C57BL/6 mice weighing 20-25 grams 3-4 months old were placed for an acclimatization period for one week. The animals were housed in NIPER Kolkata Animal House for an experimental purpose in standard Individual Ventilated cage (IVC) cages. The mice were maintained under controlled room temperature (24-28^{o}C) and humidity (55-60% RH) with a 12-h light and dark cycle. All mice were provided with a commercially available normal pellet diet (NPD) and water *ad libitum*. The guidelines of the committee for the purpose of control and supervision of experiments on mice (CPCSEA), Govt. of India were followed, and prior permission was sought from the Institutional Animal Ethics Committee for conducting the study. Utmost care was taken to carry out all the experimental procedures ethically with minimum suffering to animals.

2.2. Experimental Design

The C57BL/6 mice were acclimatized for one week and then randomly divided into 4 groups comprising 6 animals/group. The doses of $AlCl_3$ (Sigma Aldrich) and β-sitosterol (TCI chemicals) were selected based on published literature [6, 18]. Mice with similar body weights and showing similar baseline behavioral changes were used for the study. Mice in groups 2 and 3 were administered with $AlCl_3$ (soluble in distilled water) orally. Mice in groups 3 and 4 were administered with β-sitosterol, which is soluble in 0.5% CMC (Carboxymethylcellulose) (Fig. **1**). From the 3rd week of administration, the mice were assessed using the Passive Avoidance Test (Ugo Basile), Novel Object Recognition Test (Orchid scientific), Y-Maze (Orchid scientific), and Smart Video Tracking system (Pen lab). The mice were euthanized for biochemical estimation using Amplex™ Acetylcholine/Acetylcholinesterase Assay Kit (Thermo Fisher), Glutathione Assay Kit (Abnova), and histopathological evaluation.

2.3. *In Silico* Study

Docking was carried out on Ubuntu 20.04 using Autodock Vina 1.1.2, an open-source application created at The Scripps Research Institute [19]. B-3D sitosterol's structure was retrieved in SDF format from the ChEMBL Database (curated database of bioactive molecules with drug-like properties) (https://www.ebi.ac.uk/chembl/). Before docking, the structure was altered by inserting hydrogens, partial charges, better bonds, bond angles, and incorrect dihedrals using the PRODRUG server (http://davapc1.bioch.dundee.ac.uk/cgi-bin/prodrg). The structure of β-sitosterol in PDBQT format (suitable format for protein IDs supported by autodoc vina) is then downloaded from the server. The Human AChE Dihydrotanshinone I complex (PDB ID: 4m0E) protein structure was retrieved from PDB RCSB (Research Collaboratory for Structural Bioinformatics Protein Data Bank). Bester *et al.* deposited the dimeric form [20]. With a resolution of 2.00, X-ray diffraction was used to determine the protein structure. The 3-D structure of the protein with its energy minimized was created using Autodock technologies. Polar Hydrogens and Kollman charges were added, heteroatoms and water molecules were subtracted, and the file format was changed to PDBQT. The grid size was set to 70 x 60 x 54, with a spacing of 0.375, and the x, y, and z coordinates were 13.175, 40.519, and 26.909 pixels. The docking was conducted based on grid box characteristics and information about the protein and ligand.

2.4. Behavioral Study

2.4.1. Y Maze

The Y-maze was used to assess spatial reference memory, while % alternations to different arms were calculated as a measure of memory. Following the normal Y-maze procedure, mice were allowed to explore all three arms of the labyrinth. Spontaneous alternation, a test of spatial working memory, is triggered by rodent's natural urge to explore previously unexplored locations. The standard protocol of the same was followed by Prieur *et al.* [21].

2.4.2. Novel Object Recognition

The Novel Object Recognition Test (NORT) was conducted as per the standard protocol followed by Ennaceur A [22]. We measured cognitive function by calculating the Preference Index (PI), the ratio of the time duration to explore a novel object to the time duration to explore both objects [23, 24].

2.4.3. Passive Avoidance Test

Passive avoidance behavior of rodents is defined as the conquest of the innate fondness for the dark compartment of the test apparatus following exposure to an unavoidable shock. The step-through avoidance test was performed in a two-chamber box with one illuminated and one dark chamber linked through an open gate using the standard protocol of the given studies and mentioned reference [25-27].

2.5. Biochemical Assays

2.5.1. Preparation of Brain Homogenates

The cerebral cortex and hippocampus from the brain samples stored at -80°C were removed, blood vessels removed, and quickly cut into small pieces on a cool plate to generate brain homogenate. The tissues were weighed and homogenized in cold phosphate-buffered saline (PBS) at a ratio of 1:9 (tissue weight (g): PBS (mL) volume) for the test. The homogenates were centrifuged at 5,000 g for 5 min and the supernatant was collected.

2.5.2. Determination of Acetylcholine Level

The primary neurotransmitter to be examined in cognitive decline is Ach [28]. The Amplex Red Acetylcho-

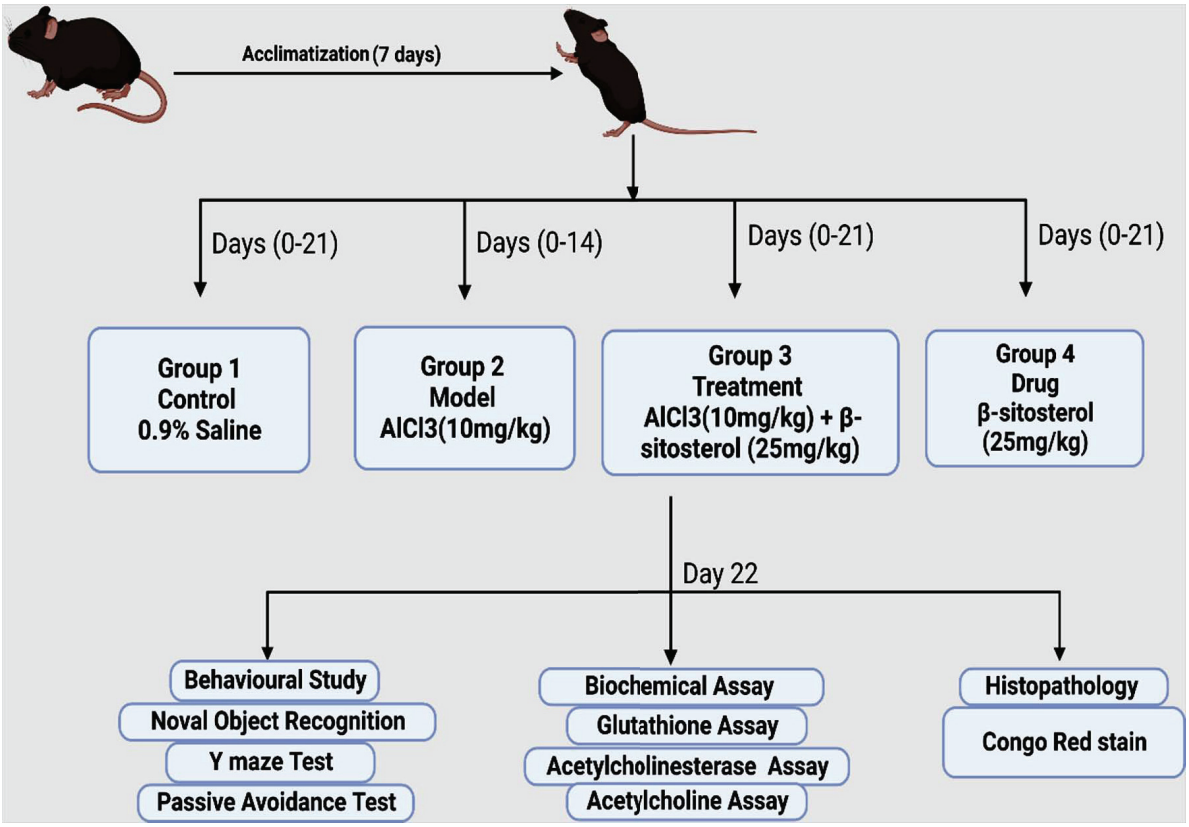

Fig. (1). Representation of experimental design. *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

line/Acetylcholinesterase Assay Kit (Invitrogen TM), which offers a sensitive method for detecting ACh and AChE in a fluorescence microplate reader, was used to measure ACh and AChE in the cortico-hippocampal homogenates based on manufacturer's instructions [29, 30].

2.5.3. Determination of Glutathione Level

Glutathione (GSH) is present in cellular systems as an antioxidant that neutralizes free radicals. GSH was estimated by Moron's method using the assay kit (Abnova). All samples were treated with the 5-sulfosalicylic acid dihydrate (SSA) solution. All of the SSA-treated centrifuged supernatants must have their SSA concentration brought down to 1% SSA by dilution with Assay Buffer. Brain homogenates were centrifuged at 5,000g, the supernatant was collected, and 0.6 mM DTNB(5,5′-dithiobis-(2-nitrobenzoic acid) was added to the supernatant. At the same time, absorbance was recorded at emission at 510 nm with excitation at 410 nm [31].

2.6. Sample Collection for Histopathology

C57BL/6 mice were humanely euthanized at the end of the 3rd week through cervical dislocation and the brain samples were rapidly harvested and rinsed in cold saline to wash excess blood. The brain was kept in isolation using buffered formalin (pH 7.6) for fixation. The brain samples for the histopathological study were fixed in 10% formalin, while the remaining brain samples were stored at −80°C until assayed. Tissues were submerged in escalating alcohol concentrations (70%, 95%, and 100%) for two days to dehydrate the tissues. The tissues were then immersed in melted wax (between 60 and 70°C). Tissue was divided into 5-µm thin segments after the wax solidified. Xylene exchange was used to deparaffinized the wax (2 min each). We used 100% alcohol to dehydrate the tissue (5 min/3 min). 2 min in 95% alcohol, followed by further rehydration with distilled water (2 min). We further stained it with Congo red by submerging it in KOH solution after 5 min in Congo red solution. The section was immersed in hematoxylin and washed five times with tap water (3 min). With 70%, 95% (5 dips each), and 100% alcohol, the section's moisture was drawn out for 2 min before mounting with DPX (Dibutylphthalate Polystyrene Xylene). We cleaned the sections with xylene (2 min each). We viewed the sections under 40X magnifications and counted neurons with a Aβ-plaque in the cortex and hippocampal regions [32, 33]. We used 20 consecutive portions of each brain slice to calculate the number of plaques per mm^2.

3. STATISTICAL ANALYSIS

The results were analyzed using one-way ANOVA followed by Tukey's post hoc. $p < 0.05$ were considered significant, and results were presented as mean ± SEM. We used GraphPad Prism (version 5.0) software for data analysis.

4. RESULTS

4.1. *In Silico* Study of β-sitosterol with Acetylcholinesterase

In silico studies revealed that β-sitosterol binds to AChE [5]. The binding pockets of proteins with the appropriate orientation require the least amount of energy to bind, which

is -8.6 (in kcal/mol) [34]. The more stable the bonding and the less energy generated from the receptor-ligand contact, the better fit the molecule is in the protein's binding pocket and the more stable the β-sitosterol-protein relationship. The most popular AchE-docked conformations were analyzed. We found that the compound interacted over the binding cavity with the acidic amino acid residues Phe 297, Phe295, and Phe338 through an OH group by forming a hydrogen bond with carbonyl oxygen with a bond length of 3.8-4.6 and bond energy of -8.6 Kcal/mol. Away from the protein's active site, β-sitosterol joined the side chain of Phe295 with a hydrogen bond. Additionally, it demonstrated hydrophobic interactions with Trp286 and Tyr341. Additionally, Tyr124 and Arg296 participated in the creation of a water bridge with β-sitosterol (Fig. **2**) [34-36].

4.2. Behavioural Studies

4.2.1. Effects of β-sitosterol on Cognitive Functions

The Passive Avoidance Test (PAT) was used for the assessment of the fear-conditioned memory of mice. We found the step-down latency for the $AlCl_3$ administered mice to be higher than the control. Co-administration of β -sitosterol could partially reverse mediated memory impairment. $AlCl_3$-administered mice were less interested in the novel object, thus showing a low preference index than controls. This preference was reversed in $AlCl_3$-β-sitosterol-administered animals. The number of alterations was less in the $AlCl_3$ group, which changed upon $AlCl_3$- β-sitosterol co-administration. The step-down latency (A) in PAT reduction ($p < 0.001$) in the $AlCl_3$ model while the same got reversed by β-sitosterol ($p < 0.001$). % Alteration (B) in the Y maze also showed significant improvement ($p < 0.001$) in $AlCl_3$ and β -sitosterol administered animals when compared to $AlCl_3$ alone group. Preference index (PI) in novel object recognition decreased in the $AlCl_3$ group, which improved significantly ($p < 0.001$) after $AlCl_3$ + β -sitosterol co-administration (Figs. **3A-C**).

4.3. Effects of β -sitosterol on Acetylcholinesterase and Acetylcholine Level

$AlCl_3$ treatment led to a significant increase in AChE level ($p < 0.001$) compared to the control. This was significantly reduced in β-sitosterol and $AlCl_3$ co-treatment group ($p < 0.01$) (Fig. **4A**). $AlCl_3$ treatment showed a subsequent decrease in ACh levels ($p < 0.001$), which significantly increased ($p < 0.001$) in $AlCl_3$ and β-sitosterol co-treated animals (Fig. **4B**).

4.4 Effects of β-sitosterol on GSH Level

$AlCl_3$ treatment led to a significant decrease in GSH levels (Fig. **4C**) in cortico-hippocampal homogenates in comparison with control animals ($p < 0.001$). When $AlCl_3$ was co-administered with β -sitosterol, a significantly increased GSH ($p < 0.001$) level was observed.

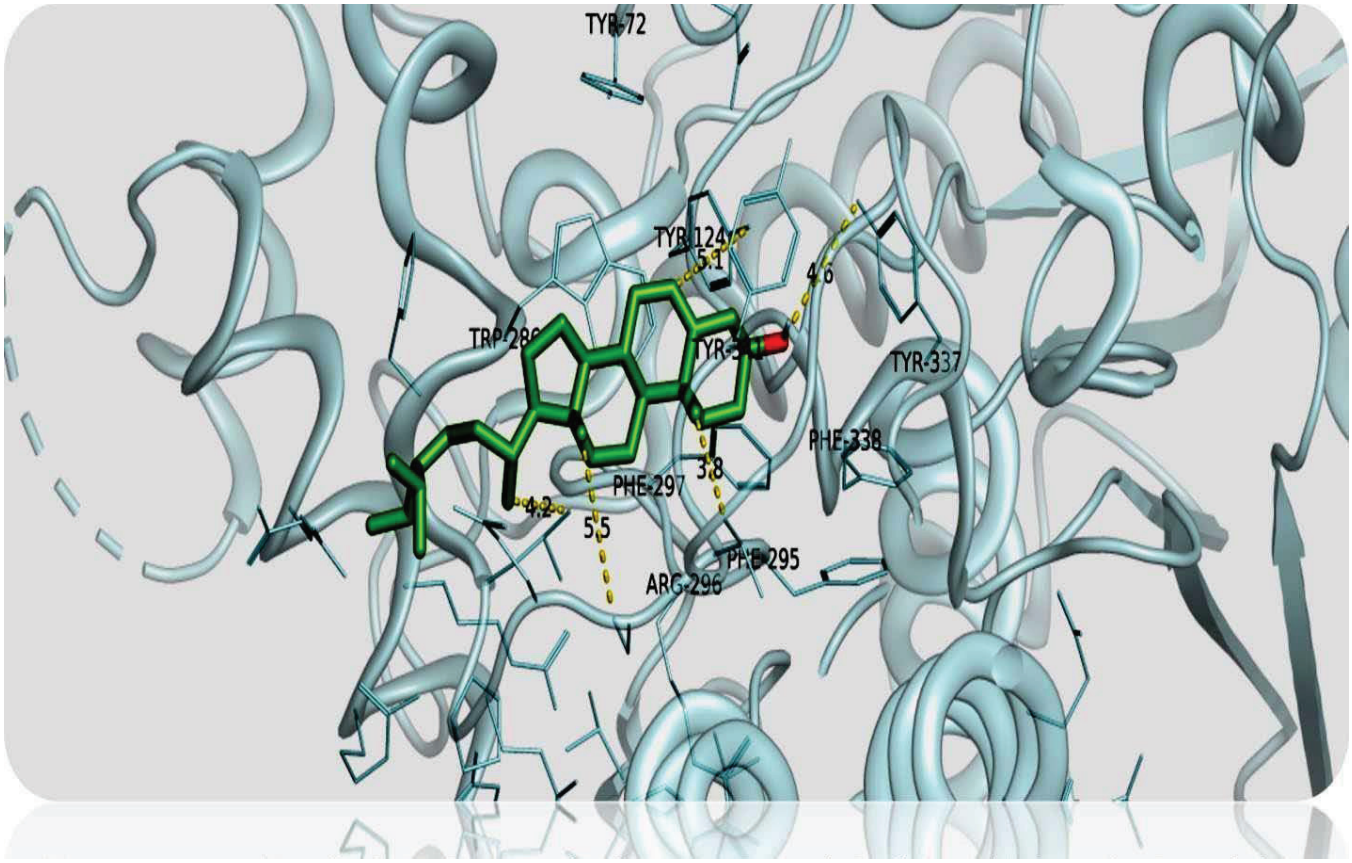

Fig. (2). Bound ligand in the pocket region of AChE protein showing hydrophobic and hydrogen bonding with the side chain residue of the amino acid is seen. Interactive amino acid Tyr, Phe with a bond length of 3.8- 4.6 Å and lowest energy of binding -8.6 (in kcal/mol). *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

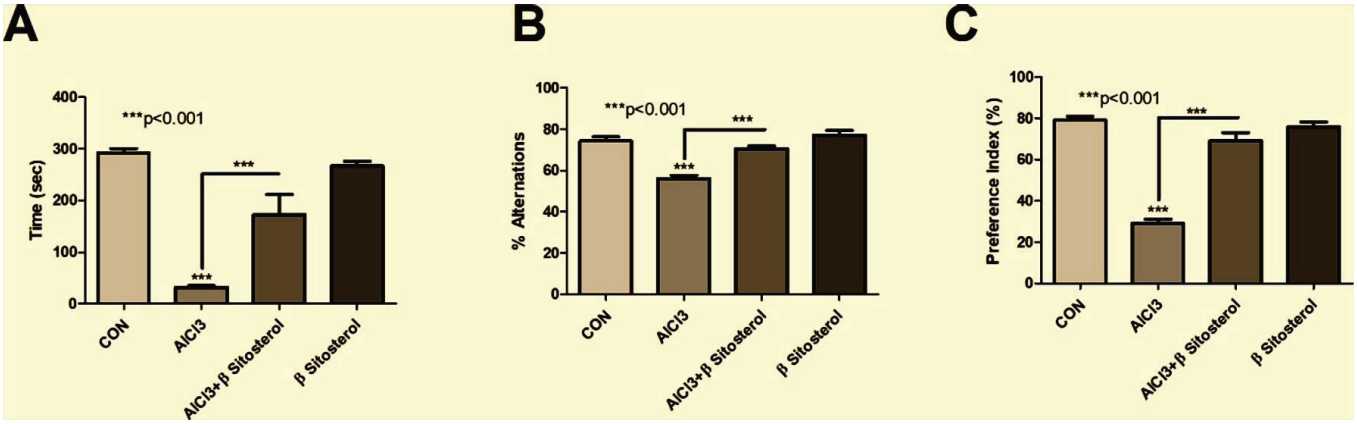

Fig. (3). Effect of β-sitosterol on learning and memory. The step through latency in PAT in (**A**) $AlCl_3$-administered mice show reduced step through latency but the same was reversed after β-sitosterol administration. The number of alterations (**B**) was less in the $AlCl_3$ Group which was reversed after administration of β-sitosterol. Preference index (PI) in novel object recognition test (**C**) decreased in the $AlCl_3$ Group which improved significantly ($p < 0.001$) after $AlCl_3$ + β-sitosterol administration. Data is presented with Mean ± SEM. *p* values < 0.05 was considered statistically significant. *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

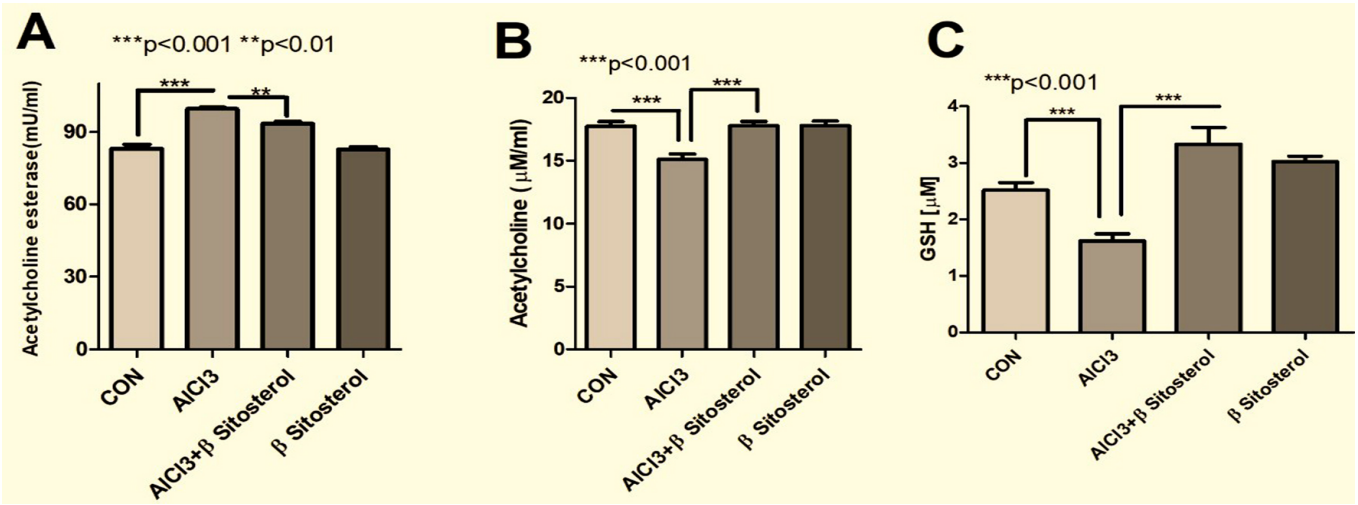

Fig. (4). Effects of β-sitosterol on **A**) AChE, **B**) ACh, (**C**) and GSH levels. Aluminium chloride treatment led to a significant increase in AChE level ($p < 0.001$) as compared to control which was significantly reduced in β sitosterol and $AlCl_3$ co-treated animals ($p < 0.01$). $AlCl_3$ treatment showed a decrease in ACh levels ($p < 0.001$) which significantly increased ($p < 0.001$) in $AlCl_3$-β sitosterol treated animals. $AlCl_3$-treated animal showed a significant decrease in GSH levels compared to control ($p < 0.001$). $AlCl_3$ and β-sitosterol co-administration led to a significantly increase in GSH ($p < 0.001$) level. Data is presented with Mean ± SEM. A *P* value less than 0.001 was considered statistically significant. *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

4.5. Beta-amyloid Plaques Deposition

Congo red staining showed that Aβ deposition in the cortico-hippocampal area of the mouse brain was higher in the $AlCl_3$ group than in the control group (Figs. **5A-D**). Aβ deposition was significantly reduced in animals on $AlCl_3$+ β-sitosterol as compared to $AlCl_3$ ($p < 0.001$). Regarding Aβ deposits, there was no discernible difference between the $AlCl_3$+β-sitosterol and the control group of animals.

5. DISCUSSION

AD is a neurodegenerative disorder characterized by Aβ-plaque deposits, neurofibrillary tangles, and neuronal death, ultimately leading to cognitive impairment [37]. The mice with cognition deficits have hippocampal Aβ plaque formation and impaired neuronal function, which is associated with memory loss. According to reports, β-sitosterol can cross the blood-brain barrier (BBB) [38, 39] and block the formation of Aβ. Previous research using the APP/PS1 (APP/PS1 are double transgenic mice that express mutant human presenilin and a mouse/human amyloid precursor protein modeling AD) mice model reveals that β-sitosterol improves memory and learning deficits and reduces the Aβ-deposition [5, 28]. We performed *in silico* studies, which showed strong binding between β-sitosterol and AChE. *In vivo*, $AlCl_3$-induced cognition deficit got reversed by β-sitosterol. The human cholinergic system plays a vital role in cognition. Cholinergic synapses play a critical role in retriev-

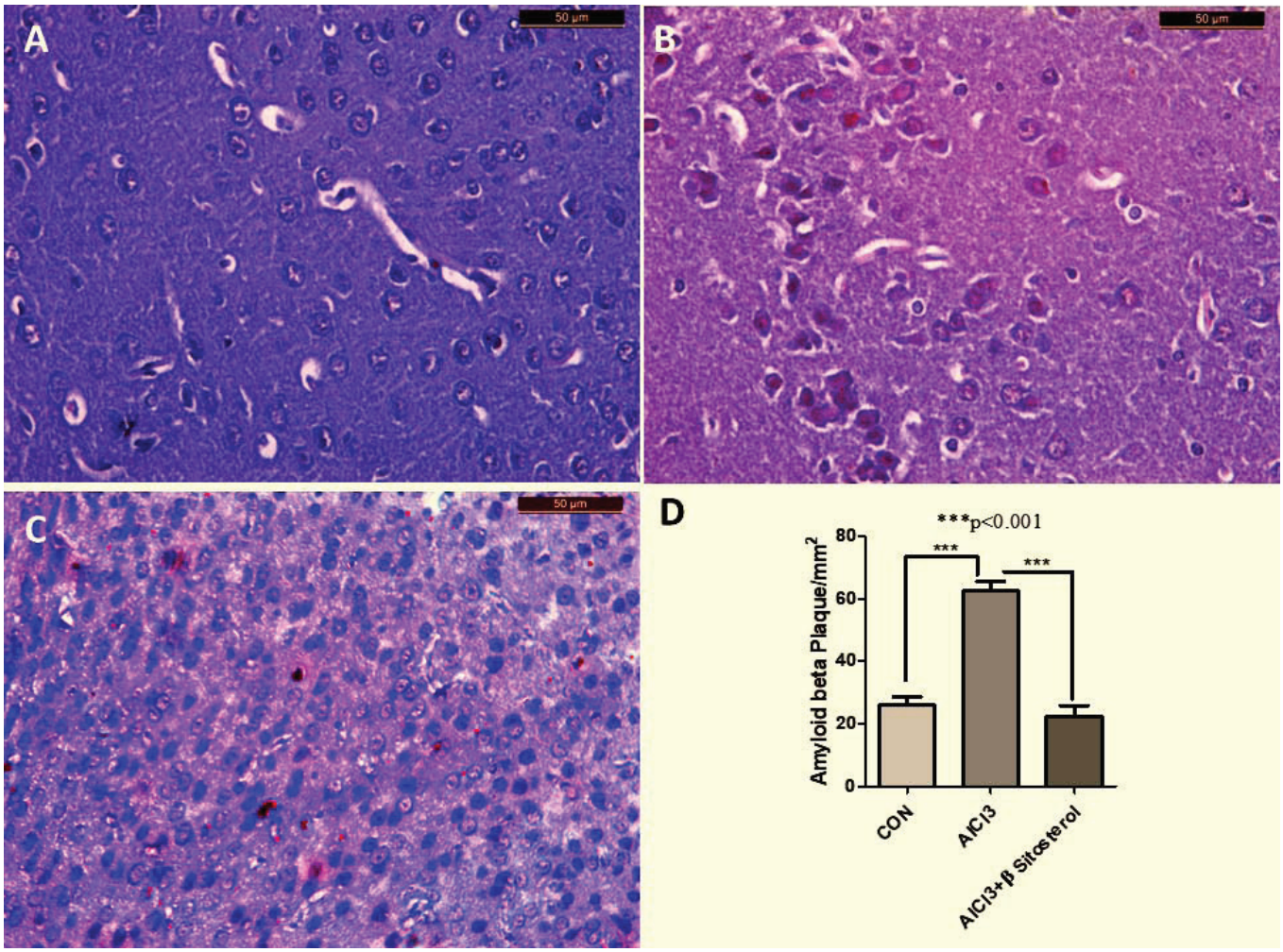

Fig. (5). (A-D) Representative images for Aβ plaques using Congo red staining in the cortico-hippocampal region shown in (**A**) Control Group, (**D**) $AlCl_3$ Group (**C**) $AlCl_3$+β-sitosterol Group. $AlCl_3$ treatment significantly increased Aβ plaques as compared to control ($p < 0.001$). β-sitosterol and $AlCl_3$ co-treated animals showed a significant decrease in Aβ plaque load as compared to $AlCl_3$ Group of animals ($p < 0.001$). Data are presented with Mean ± SEM. *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

ing and retrieving new information [28]. Cognitive impairment is linked to the degeneration of cholinergic neurons, which results in deficits in memory consolidation, acquisition, retention, and recovery [40, 41]. Increased cholinergic activity can thereby reverse cognitive impairment [42, 43]. Cholinesterase inhibitors have demonstrated promise as cognitive enhancers. Donepezil, rivastigmine, and tacrine have received clinical approval for disorders linked to cognitive loss [44]. A study performed using double transgenic mice (APP/PSEN1) has shown that β-sitosterol showed potent anticholinesterase activity. It effectively reaches the brain, inhibits cholinesterase metabolism-related enzymes, and acts as a free radical scavenger, followed by cognitive improvements [45]. In the present work, we show that β-sitosterol reduced AChE levels in the $AlCl_3$ model of AD. β-sitosterol treatment also improved fear conditioned, spatial, and visual recognition memory based on the behavioral studies performed using PAT, Y maze, and NORT, respectively.

Studies have suggested that β-sitosterol is a potent antioxidant [46] with anti-inflammatory properties [47]. The scavenging of reactive oxygen species (ROS) and the preservation of cellular redox balance are both crucial functions of GSH. The decline in the ratio of reduced to oxidized glutathione is thus a sensitive indicator of cellular oxidative stress [48, 49]. The glutathione assay revealed an increase in GSH levels in the treatment group in comparison with $AlCl_3$ model, indicating reduced brain oxidative stress. Effective treatment for AD and cognition decline prevents Aβ formation or promotes its degradation [50, 51]. The pathways that are linked to the Aβ degradation have been reported as BACE1(β-site APP cleaving enzyme-1) inhibition [5, 51] as well as *via* increasing the activity of β- and γ-secretase activities, which helps in reducing Aβ deposition [39]. Here we show that β-sitosterol treatment reduced Aβ deposition.

CONCLUSION

We conclude that β -sitosterol inhibited cortico-hippocampal AChE and increased ACh levels. It reduced oxidative stress and Aβ deposits in the cortex and hippocampus, eventually preventing $AlCl_3$-mediated cognitive impairment in the animals. However, further studies using β -sitosterol will help us understand the signaling processes responsible for the neuroprotective functions of β-sitosterol.

LIST OF ABBREVIATIONS

ROS = Reactive Oxygen Species

BBB = Blood-Brain Barrier

PAT = Passive Avoidance Test

AD = Alzheimer's Disease

Aβ = β-Amyloid

SIT = β-Sitosterol

EFSA = European Food Safety Authority

Al = Aluminum

CNS = Central Nervous System

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was conducted under the approval from the institutional animal Ethics Committee under CPCSEA Govt. of India (CPCSEA), Govt. of India.

HUMAN AND ANIMAL RIGHTS

All animal procedures were followed according to the US Public Health Service's "Policy on Humane Care and Use of Laboratory Animals," and "Guide for the Care and Use of Laboratory Animals."

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

All the data and supportive information are provided within the article.

FUNDING

None.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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