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Original Research

Azadirachta indica A. Juss Ameliorates Memory Deficits and Reduces Anxiety-Like Behavior by Modulating Cholinergic Neurotransmission in an Animal Model of Depression: *In Silico* and *In Vivo* Studies

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Abstract

Exposure to prolonged and severe stress can lead to negative effects on learning and memory, increased anxiety, reduced motivation, disturbed cholinergic activity, and hippocampal and prefrontal cortical neuronal damage. On the other hand, drugs from natural origin have a beneficial effect on neuronal structure and functions. *Azadirachta indica* (neem), belonging to the Meliaceae family, has been reported to exhibit beneficial effects in wound healing, diabetes management, and antibacterial properties. This study aimed to assess the impact of *Azadirachta indica* on chronic immobilization stress-induced memory impairment in rats. Chronic immobilization stress was induced in rats for 2 hours/day over 10 days. Following this, *Azadirachta indica* was administered at doses of 200, 400, and 600 mg/kg for 14 days. Twenty-four hours after the treatment, behavioral tests, including the novel object recognition test (NORT), T-maze, and elevated plus maze (EPM), were conducted to evaluate memory and anxiety-like behaviors. Acetylcholinesterase (AChE) activity was measured in the frontal cortex, hippocampus, and septum. Additionally, molecular docking studies were performed using Molegro Virtual Docker (MVD-2013, 6.0) to analyze the interaction of 19 active chemical constituents from aqueous neem extracts with various targets, including AChE, brain-derived neurotrophic factor (BDNF), N-methyl-D-aspartate(NMDA) receptors, and anti-cortisol Fab in complex with corticosterone. *Azadirachta indica* treatment significantly enhanced learning and memory in chronically stressed rats, as evidenced by improved performance in NORT and T-maze tests, along with reduced anxiety-like behavior in the EPM test. Treatment also restored AChE activity in the stressed animals. Molecular docking studies indicated that the active constituents of neem extract showed high docking scores to AChE, BDNF, NMDA receptors, and anti-cortisol Fab, correlating with the experimental findings. *Azadirachta indica* exhibited neuroprotective and cholinergic transmission modulation properties, which may underlie its memory-enhancing effects in chronically stressed rats. Treatment enhanced memory in NORT and T-maze test ($p<0.001$). Also, anxiety behavior was reduced in the EPM ($p<0.001$). The correlation between the *in vitro* experimental data and the *in silico* molecular docking results suggests that neem's active compounds could be potential candidates for improving memory and managing stress-induced cognitive impairments.

Keywords: *Azadirachta indica*; Neem; Chronic immobilization stress; Memory impairment; Acetylcholinesterase; Molecular docking; Behavioral tests; Neuroprotection.

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Introduction

Stress is the body's physiological, biological, or psychological response to various stressors [1]. The hypothalamic-pituitary-adrenal (HPA) axis regulates cortisol release in response to stress [2], and prolonged elevation of cortisol due to chronic stress can impair memory [3-6] and cause hippocampal neuronal atrophy [7]. It can reduce dendritic branching, decrease dendrite density, and alter synaptic spine morphology in the hippocampus, impairing cognitive functions [5, 8-10]. Animal studies have shown that chronic stress impairs both short- and long-term memory, as demonstrated by poor performance in tasks like the radial arm water maze [10-12].

Natural nootropics alter the neurotransmitter levels in different parts of the brain [13-14]. They also enhance dopamine release, improve the function of glutamate receptors [15-16], increase choline uptake and cholinergic neurotransmission [17], and alter the activity of different signal transduction pathways [18]. They can act as a positive allosteric modulator for acetylcholine or glutamate receptors [15-17].

Azadirachta indica A. Juss (family: Meliaceae), commonly refered as neem, is a plant regularly used in the Indian traditional medical system [19]. Neem is a large evergreen tree native to Indian subcontinent, but cultivated throughout the tropics and is well-known for its diverse medicinal uses for more than 2000 years. The active phytoconstituents are alkaloids, polyphenols, flavanoids, limonoids, steroids and carotenoids.

The neem ethanolic extract showed neuroprotection against experimental cerebral malaria [20]. Previous studies demonstrated the beneficial effect of neem in cerebral ischemic conditions [21-22]. However, the beneficial effect of *A. indica* aqueous extract on chronic immobilisation stress-induced cognitive deficits is not well understood. Also, we used Molegro Virtual Docker (MVD-2013, version 6.0) to do molecular docking studies of the 19 active chemical constituents in water-based neem extracts ensure the correct experimental results. The present study addresses the potential of *A. indica* extract for the protection of chronic immobilization stress-induced cognitive deficicts in rats, considering the implications for the treatment of chronic stress-associated psychiatric conditions.

Materials and Methods

Experimental animals

Adult male Wistar rats were procured from a registered commercial breeder (Vaarunya Biolabs Pvt. Ltd. Bengaluru, Karnataka, India) (05/BVR/2020). All the animals were housed in a noise-free environment and kept in polypropylene cages, provided with all the requirements as per standard hygienic lab conditions at a temperature of 23 ± 2°C, relative humidity of 55 ± 5% with 12 h light and dark cycle, fed with good quality of standard pellet diet and water *ad libitum*. Institutional Animal Ethics Committee of KLE College of Pharmacy, Bengaluru approved the current study (CPCSEA number: 05/BVR/2020). Maximum efforts were made to reduce the number of animals used and decrease the suffering of experimental animals.

Experimental groups studied

Male Wistar rats (1.5 - 2.0 months old weighing 180-200 g) were used in the current study. Animals were randomly divided into five groups and were kept undisturbed in their cages.The following five groups of 10 animals in each group were used for behavioral and biochemical experiments (Figure 1).

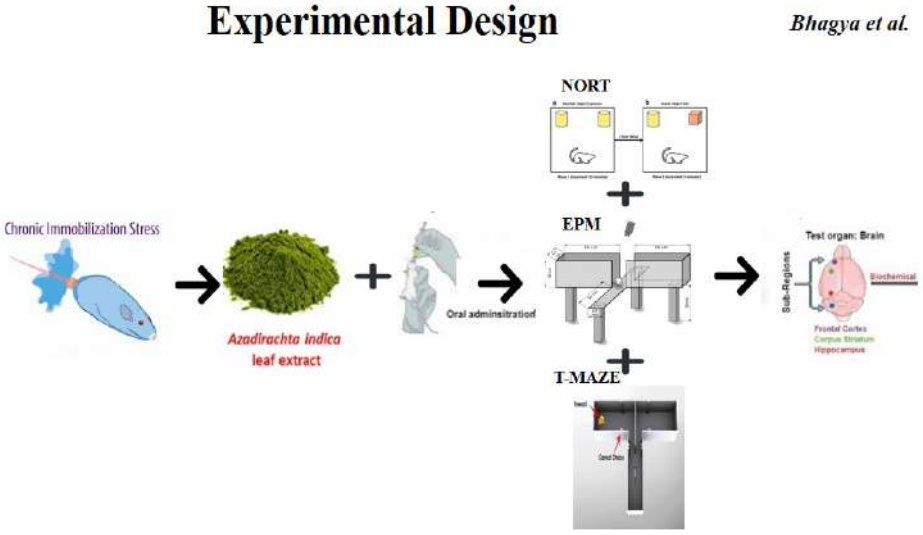


Figure 1. Experimental Design. NORT: Novel Object Recognition Test; EPM: Elevated Plus Maze; T-maze: T-maze alteration task.

Group I: Control group (no stress) + distilled water (0.5 mL)
Group II: Chronic Immobilisation Stress (CIS) group + distilled water (0.5 mL)
Group III: CIS + 200 mg/kg dose of aqueous extract of *A. indica*
Group IV: CIS + 400 mg/kg dose of aqueous extract of *A. indica*
Group V: CIS + 600 mg/kg dose of aqueous extract of *A. indica*

Azadirachta indica plant extract

Aqueous extract of leaves of *A. indica* was received from Green Chem Herbal Extracts and Formulation, Bengaluru, Karnataka, India, as a gift sample.

Experiment design

Induction of stress

Stress was introduced by using immobilization bags as a stressor for 2 h/day for ten days from 10 a.m. to 12 p.m. Rats were placed inside the cone made up of an immobilisation bag and tied from the end using adhesive tape, and a small cut was made on the top for the rats to breathe [5-6].

A. indica treatment

Aqueous extract of leaves of *A. indica* was dissolved in distilled water (100 mg/mL) and given orally for 14 days to all animals except normal and stress groups. Control and CIS groups were administered normal saline solution.

Behavioral Studies

Animals were subjected to different behavioral tests, after 24 h after the last treatment of *A. indica.*

Novel Object Recognition Test (NORT)

The NORT is a common behavioral test for investigating various characteristics such as learning and memory in rodents [23]. It is based upon the principle that rodents have a natural preference for novelty, i.e. if a rodent remembers the familiar object will spend more time exploring the novel object [24].
Object selection and experimental setup: Two identical objects (juice mug) and one novel object (juice mug of different colour and design) were slightly different from the identical object and can be discriminated from the identical object used. A square wooden box (60 × 60 × 60 cm) was used as the main arena. The whole activity was recorded with a camera.
Calculation: The calculation is done by subtracting the exploration time on the novel object from the exploration time on the familiar object.
NORT has three-day procedures and consists of:
Day 1- Habituation: In the habituation phase, the rat was taken out from its home cage and placed at the centre of the box used for the experiment, and ten minutes were given to explore the area freely. The apparatus was cleaned with 70% v/v ethanol between the trials.
Day 2- Training: On training day, after 24 h from the habituation phase, two identical objects (juice mugs) were placed at two different corners opposite to each other, and then the rat was taken out from the cage, allowed to explore the similar objects for five minutes. The objects were cleaned with 70% v/v ethanol between the trials.
Day 3- Test: On test day, after 24 h from the training phase, one object was replaced by a similar-looking object (novel object) and placed at the same location used in the training phase. For 5 minutes, the rat was free to investigate both objects. The objects were cleaned with 70% v/v ethanol between the trials.

Elevated Plus Maze (EPM)

The elevated plus maze is a + shaped apparatus used to check the anxiolytic activity in rodents.It is based upon the principle of rodent's aversion to open spaces and height [25]. The rats were taken out from their home cages and placed at the centrer facing towards the open arm and were allowed exploration for 5 min. The activity was recorded using a camera and a stand. At the end of the 5 min, the rats were removed and kept back in their home cages. "The period spent in the open arms (s), time in the closed arms (s), the number of open and total arm entries, the number of vertical rearing, and head dips" were monitored. The apparatus was cleaned using 70% w/v ethanol between the rats [3-6].

T-Maze

The T Maze is a T-shaped behavioral instrument used to assess exploratory behavior in rodents with CNS diseases. This experiment is based on rodents' propensity to visit a new location, i.e., they would rather go to a new maze arm than a known one [26].

The test was divided into two phases.

1. The habituation phase- The right arm of the T-maze was blocked using a steel plate; then the rats were taken out from their home cages and placed at the start of the maze, and explored the area for 5 min. At the end of 5 min, the rats were put back in a home cage; then, the instrument was cleaned with 70 v/v ethanol.
2. The choice phase- In the choice phase, both arms were open. After six hours from the habituation phase, rats were again placed at the start of the maze and explored both arms. At the end of 5 min, the rats were put back in their home cages, and the instrument was cleaned with 70% v/v ethanol.

Calculation: Time spent (in seconds) in each arm was

determined manually.

Estimation of Acetylcholinesterase activity (AChE)

Preparation of test samples

Adult Male rats were sacrificed under euthanasia using ketamine and xyalzine (100 and 10 mg/kg, i.p., respectively), and the brain was removed immediately and rinsed with ice-cold saline solution. The hippocampus, frontal cortex, and septum were dissected quickly on a chilled petri dish containing crushed ice. The tissue was weighed and homogenised in 0.1 M Phosphate buffer (pH 8.0).

Acetylcholinesterase (AChE) activity was estimated from the hippocampus, frontal cortex and septum following Ellman's method. The reaction mixture consisted of 200 µL of PB (0.1 M, pH 8.0), 40 µL aliquot homogenate, and 80µl of 0.01 M 5,5'-Dithiobis (2-nitrobenzoic acid). After adding the substrate 10 µL acetylthiocholine (ATC), a change in the absorbance was noted every 1 min for 10 min at 412 nm using an ELISA reader. All samples were run in duplicate, and enzyme activity is expressed in micromoles/h/mg of protein [27].

Molecular docking studies

Molecular docking was conducted to investigate the actual role of chemical constituents of neem aqueous extracts towards acetylcholinesterase, brain derived neurotropic factor, NMDA receptor and corticosterone using Molegro Virtual Docker (MVD-2013, 6.0) [28-29].This method allows us to evaluate the binding interactions between these constituents and target biomolecules, offering insights into their potential mechanisms of action. The docking simulations aimed to identify optimal binding poses and affinities, enhancing our understanding of the efficacy of these compounds. We have selected these 4 target proteins for the docking study, as these markers play a major role in the stress induced cognitive deficits.

The binding pose of each ligand was assessed by calculating the Root Mean Square Deviation (RMSD) values to determine pose accuracy and stability. Binding scores, rerank scores, and hydrogen bond interactions with key amino acid residues were also analyzed. The resulting RMSD values were used to interpret the reliability of each docking pose and to compare ligand effectiveness across different protein targets.The coordinate file and crystal structure of the following target proteins with PDB IDs: 1EVE/ 1B8M/ 1PBQ/ 8CBZ were obtained from the RCSB-PDB website [30-34].

- Acetylcholinesterase: (PDB ID: 1EVE) three-dimensional structure of the anti-Alzheimer drug, e2020 (aricept), complexed with its target acetylcholinesterase.
- Brain-derived neurotrophic factor, neurotrophin-4: (PDB ID: 1B8M).
- NMDA receptor: (PDB ID: 1PBQ) crystal structure of the NR1 ligand binding core in complex with 5,7-dichloro kynurenic acid (DCKA) at 1.90 angstroms resolution.
- Anti-cortisol fab in complex with corticosterone: (PDB ID: 8CBZ)

Different chemical constituents of aqueous extracts of neem were identified and obtained from the literature review [35-38]. The MolDock Scores and the hydrogen bonding of the test compounds were compared with active ligands and standard drugs like Donepezil and Fluoxetine.

Docking study

1. The selected compounds were built using ChemDraw 12.0.2.
2. The 2D structures were then converted into energy-minimized 3D structures, and saved as MDL MolFile (.mol2).
3. The coordinate file and crystal structure of target proteins (PDB ID: 1EVE/ 1B8M/ 1PBQ/ 8CBZ) were obtained from the RCSB-PDB website.
4. The protein file was prepared by the addition of polar hydrogens, the removal of water molecules, and the removal of other bound ligands.
5. Cavity on the protein was detected using MVD2013 (6.0) software.
6. The binding site of the complexed inhibitor was selected as the active site for docking of the molecules.
7. Using the standard operating procedure, the docking protocol was carried out for synthesized compounds/ligands using MVD2013 (6.0) software.
8. The MolDock scores and the hydrogen bonding of the test compounds were compared with those of the standard ligand and standard drugs (donepezil and fluoxetine).

Phytochemical estimation

Neem extract has been subjected to LC-MS analysis for possible phytoconstituents. The analysis was done by firstly dissolving 1 mg of the plant extract in 1 mL of HPLC grade methanol followed by sonicating for 10 minutes, and finally filtering through 0.22 mm polyvinylidene fluoride (PVDF) membrane syringe filters into a 1 mL LC auto-sampler vial [2]. A sample injection volume of 5 mL was used for chromatographic separation of analytes in reverse phase ultra-high-performance liquid chromatography (RP-UHPLC Waters 2695) through a Inertsil ODS-3 column with dimensions of 4.6 ID×100 mm, 3 µm. The mobile phase A: 0.1% formic acid in water and mobile phase B: 0.1% formic acid in acetonitrile with flow rate: 1 mL/min with following parameters.

TIME (min)	A	B
0.00	90.0	10.0
3.00	2.0	98.0
6.00	2.0	98.0
6.50	90.0	10.0
8.00	90.0	10.0

The mass spectrophotometer (Waters Micromass ZQ) was operated in electrospray ionization mode, with the following parameters: Capillary voltage 3KV, source temperature 140°C, Dissolvation temp 400°C, gas flow 800 L/h. The mass spectrometer was set to scan the mass range from 100 to 1000 m/z and Mass Lynx software used for analysis.

Statistical Analysis

Data were analyzed using GraphPad Prism 5.0 software. Significant differences between groups were determined using one-way ANOVA followed by Tukey's post hoc test, as well as two-way ANOVA with Bonferroni post hoc test for subsequent group comparisons. Data are presented as mean ± SEM.

Results

Effect of chronic immobilisation stress and A. indica on anxiety behavior in the elevated plus maze

Statistical analysis revealed that thestressed animals spent more time in closed arms (Figure 2B; F4,25 = 4.39: $p<0.01$) than the open arms (Figure 2A; F4,25 = 28.18: $p<0.001$) compared to the normal control group. CIS rats did not make any notable entries in open arms (Figure 2C; F4,25 = 18.13: $p<0.001$). Compared to the CIS rats, AZ-treated animals (400 mg and 600 mg/kg) spent more time in the open arms and less time in the closed arm when compared to the CIS group. The number of closed-arm entries was more in the stressed group (Figure 2D; F4,25 = 6.401: $p<0.01$). CIS rats preferred closed arms more than open arms indicating anxiety-like behavior. On the other hand, AZ treatment reduced anxiety behavior by increasing the number of open-arm entries. Chronically stressed rats showed less number of rearing (Figure 3A; F4,25 = 11.48: $p<0.001$) and head dips (Figure 3B; F4,25 = 7.125: $p<0.001$) as compared to control rats. Interestingly, AZ treatment restored both rearing and the number of head dips in stressed animals.

A. indica treatment improves recognition memory in stressed animals

The novel object recognition test was used to examine the impact of *A. indica* administration on the recognition memory of stressed rats. It was found that a significant memory improvement was seen in rats treated with *A. indica* in NORT. There was a significant increase in the exploration time of the novel object (Figure 4B; F4,25 = 4.282: $p<0.01$) compared to the familiar object (Figure 4A; F4,25 = 11.96: $p<0.001$) in the normal control group indicating novel object preference. The exploration time with novel objects and familiar objects was similar in the CIS group (Figure 4C; $p<0.001$). The duration of exploration of novel objects was higher in *A. indica*-treated groups and showed a significant increase compared to the

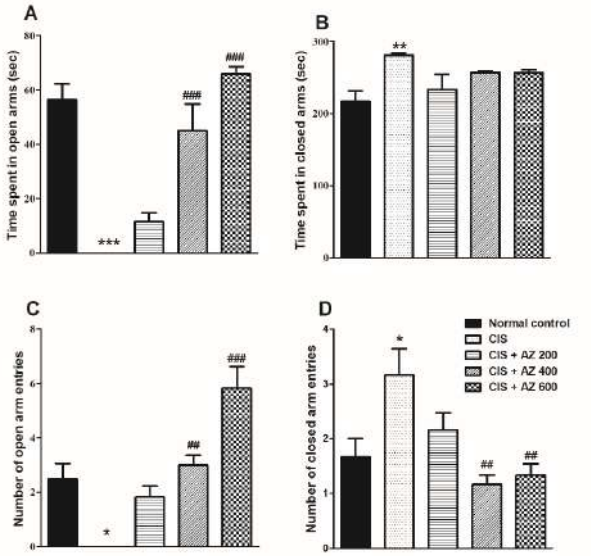


Figure 2. Effect of chronic stress and *Azadirachta indica* treatment on anxiety behavior elevated plus maze. (A) Time spent in open arms (s); (B) Time spent in closed arms (s); (C) Number of open arms entries; (D) Number of closed arms entries. Normal control: Un-stressed rats (n=6) kept at standard home cage condition; CIS: chronic immobilization stress (n=6): animals subjected to CIS for 2h/day for 10 days; CIS + AZ 200 (6), CIS + AZ 400 (6) and CIS + AZ 600 (6): stressed animals treated with *Azadirachta indica* 200 mg, 400 mg and 600 mg/kg/day for 14 days orally. Data were expressed as mean ± SEM. Statistical analysis was performed using One-way ANOVA followed by Tukey's post hoc test. *$p<0.05$, **$p<0.01$, ***$p<0.001$ compared with normal control; $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ compared with CIS.

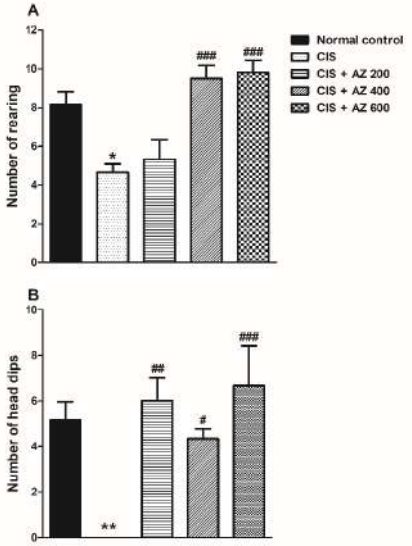


Figure 3. Effect of chronic stress and *Azadirachta indica* treatment on anxiety behavior elevated plus maze. **(A)** Number of rearing in closed arms **(B)** Number of head dips in open arms. Normal control: Un-stressed rats (n=6) kept at standard home cage condition; CIS: chronic immobilization stress (n=6): rats subjected to CIS for 2h/day for 10 days; CIS + AZ 200 (6), CIS + AZ 400 (6) and CIS + AZ 600 (6): stressed animals treated with *Azadirachta indica* 200 mg, 400 mg and 600 mg/kg/day for 14 days orally. Data were expressed as mean ± SEM. Statistical analysis was performed using One-way ANOVA followed by Tukey's post hoc test. *$p<0.05$, **$p<0.01$ compared with normal control; $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ compared with CIS.

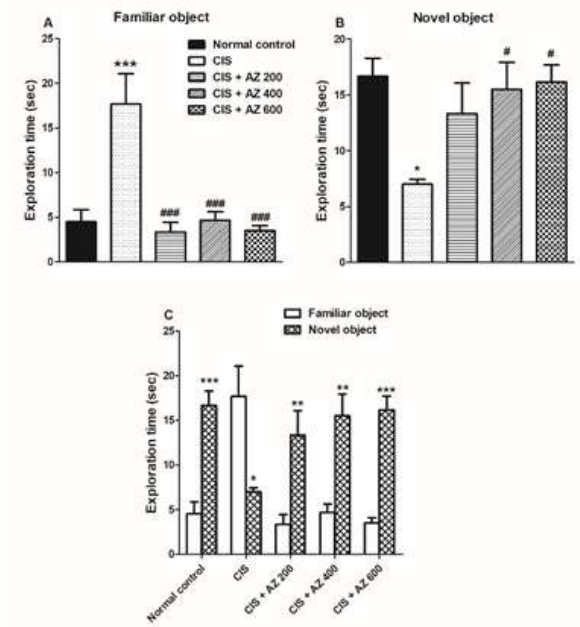


Figure 4. Effect of chronic stress and *Azadirachta indica* treatment on the exploration time for familiar object and novel object. (**A**) Exploration time at the familiar object (sec); (**B**) Exploration time at the novel object (sec). (**C**) Exploration time in both familiar and novel objects. Normal control: Un-stressed rats (n=6) kept at standard home cage condition; CIS: chronic immobilization stress (n=6): Rats subjected to CIS for 2h/day for 10 days; CIS + AZ 200 (6), CIS + AZ 400 (6) and CIS + AZ 600 (6): stressed animals treated with *Azadirachta indica* 200 mg, 400mg and 600 mg/kg/day for 14 days orally. Data were expressed as mean ± SEM. Statistical analysis was performed using One-way ANOVA followed by Tukey's post hoc test. *p<0.05, **p<0.01, ***p<0.001 compared with normal control; $^{\#}p$<0.05, $^{\#\#\#}p$<0.001 compared with CIS.

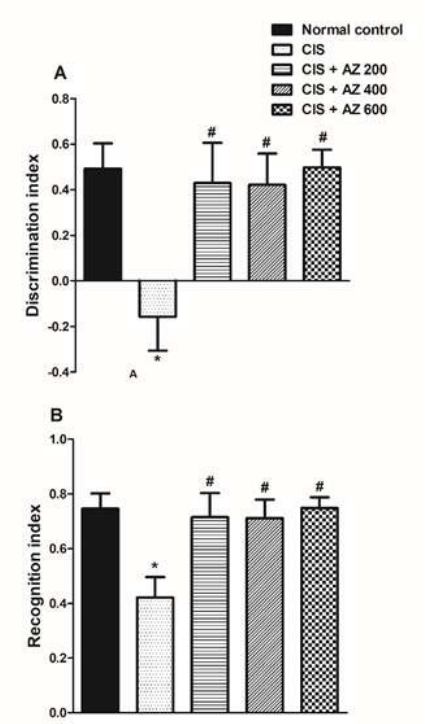


Figure 5. Effect of chronic stress and *Azadirachta indica* treatment on the discrimination index and recognition index in NORT. (**A**) Discrimination index and (**B**) Recognition index. Normal control: Normal control: Un-stressed rats (n=6) kept at standard home cage condition; CIS: chronic immobilization stress (n=6): Rats subjected to CIS for 2 h/day for 10 days; CIS + AZ 200 (6), CIS + AZ 400 (6) and CIS + AZ 600 (6): stressed animals treated with *Azadirachta indica* 200 mg, 400 mg and 600 mg/kg/day for 14 days orally. Data were expressed as mean ± SEM. One-way ANOVA was used in the statistical analysis, followed by Tukey's post hoc test. *p<0.01 compared with normal control; $^{\#}p$<0.05 compared with CIS.

CIS group. In the test phase, CIS animals showed a lower recognition index (RI) and discrimination index (DI) compared to normal control (Figure 5; p<0.01). Stressed rats treated with *A. indica* showed significantly higher (p<0.001) DI and RI than the CIS group.

Reversal of cognitive deficit in stressed rats by A. indica in T-maze alternation task

Chronic immobilisation stress produced a significant decrease in spontaneous alternation in the T-maze. In contrast, *A. indica*-treated animals showed increased spontaneous alteration, suggesting a reversal of cognitive deficit. The effect of stress and *A. indica* on T-maze exploration time is shown in Figures 6 and 7. All groups preferred a new arm over the familiar arm except the CIS-treated group, which spent more time in the familiar arm. The normal control group showed a significant increase (Figure 6B; F4,25 = 12.18: p<0.001) in exploration time in the new arm compared to the familiar arm (Figure 6A; F4,25 = 17.72: p<0.001). CIS group showed a significant decrease in exploration time in the new arm compared to exploration time in the familiar arm. *A. indica*-treated groups showed a significant increase (Figure 7) in exploration time in the new arm compared to the familiar arm.

Effect of chronic immobilization stress and A. indica treatment on AChE activity

We performed the assay of AChE activity in the hippocampus, frontal cortex, and septum to understand the effect of CIS and *A. indica* treatment on cholinergic transmission. The data were analyzed using a one-way ANOVA and Tukey's posthoc test. It showed a significant difference in the AChE activity between groups in the frontal cortex, hippocampus, and septum. Figure 8 depicts the effect of stress and *A. indica* on AChE levels in the brain. The AChE activity in the frontal cortex was reduced compared to control; the trend did not significantly differ in CIS+ AZ 200. AZ treatment at 400 mg and 600 mg/kg doses significantly restored the AChE activity in stressed animals (Figure 8A; F4,20 = 10.60: p<0.001). AChE activity was significantly decreased in the hippo-

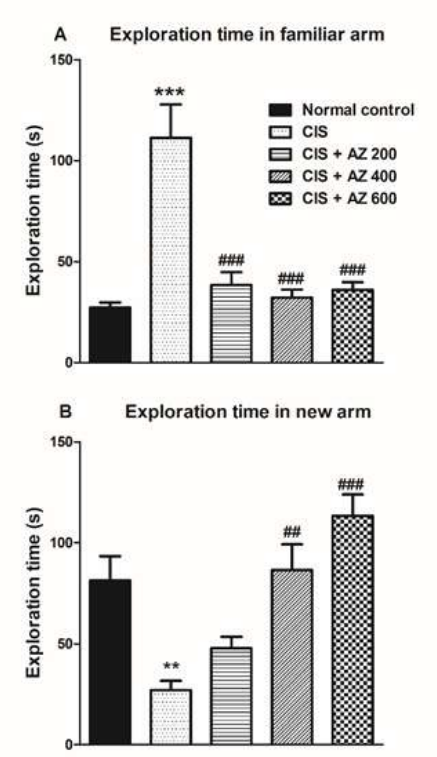


Figure 6. Effect of chronic stress and *Azadirachta indica* treatment on the exploration time in the familiar arm and new arm T-maze task. (A) Exploration time in familiar arm (sec); (B) Exploration time in new arm (sec). Normal control: Un-stressed rats (n=6) kept at standard home cage condition; CIS: chronic immobilization stress (n=6): animals subjected to immobilisation stress for 2h/day for 10 days; CIS + AZ 200 (6), CIS + AZ 400 (6) and CIS + AZ 600 (6): stressed animals treated with *Azadirachta indica* 200 mg, 400 mg and 600 mg/kg/day for 14 days orally. Data were expressed as Mean ± SEM. One way ANOVA followed by Tukey's posthoc used for analysis. $^{**}p<0.01$, $^{***}p<0.001$ as compared with normal control; $^{\#\#}p<0.01$,$^{\#\#\#}p<0.001$ compared with CIS group.

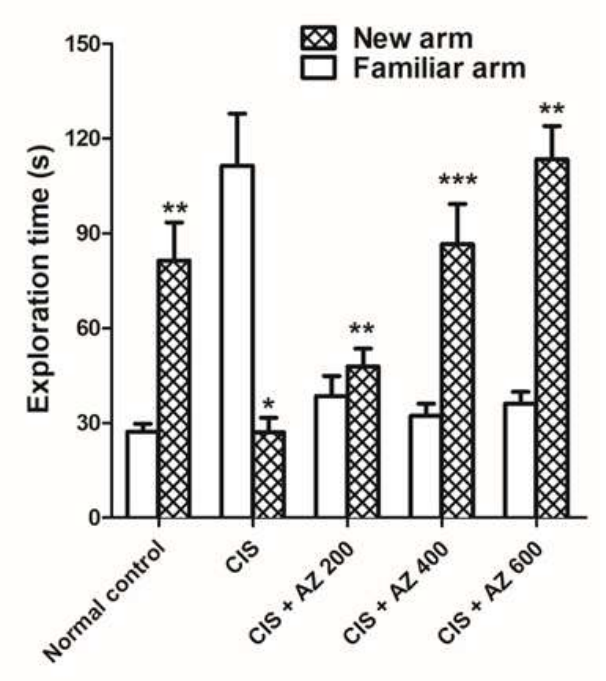


Figure 7. Effect of chronic stress and *Azadirachta indica* treatment on T-maze. Comparison of exploration time in the familiar arm and new arm. Normal control: Un-stressed rats (n=6) kept at standard home cage condition; CIS: chronic immobilization stress (n=6): animals subjected to immobilization stress for 2h/day for 10 days; CIS + AZ 200 (6), CIS + AZ 400 (6) and CIS + AZ 600 (6): stressed animals treated with *Azadirachta indica* 200 mg, 400 mg and 600 mg/kg/day for 14 days orally. Data were expressed as Mean ± SEM. Student 't' test was used to compare the familiar arm vs. the new arm. $^{***}p<0.001$, $^{**}p<0.01$, $^{*}p<0.05$ compared with familiar object.

campus in the CIS group compared to the control group. The AChE activity was restored considerably in all the AZ-treated groups (Figure 8B; F4,20 = 6.79: $p<0.01$). The AChE activity in the septum showed a significant decrease compared with the control group (Figure 8C; F4,20 = 1.298: $p<0.001$).

Molecular docking

The binding mechanism between the chemical components in the neem aqueous extract and the NMDA receptor, brain-derived neurotropic factor, corticosterone, and acetylcholinesterase was assessed using molecular docking. The findings of the molecular docking 3D structure between chemical components and target proteins demonstrated that the ligands may interact on the same binding site with acetylcholinesterase (Figure 9), brain-derived neurotrophic factor (Figure 10), NMDA receptor (Figure 11), and corticosterone (Figure 12) target proteins.

The active ingredients of *A. indica* obtained from the database were used for docking studies. Acetylcholine, Corticosteroids, NMDA receptors, and BDNF are used as target for the above compounds. The associated 3D structure of the protein with PDB Id 1EVE (Acetylcholinesterase), 8CBZ (Corticosterone), 1PBQ (NMDA receptor), and 1B8M (BDNF) were retrieved from the protein data bank.

The MolDock Scores and the amino acid interaction of the test compounds with protein binding site were compared with active ligandsand standard compounds Donepezil and Fluoxetine (Table 1-4).

The docking score of phytochemicals of *A. indica* were found to be better as compared with standard ligand and reference compounds, with Nimbinene (-197.862) showing highest MolDock score as compared reference compounds donepezil (-156.693) and fluoxetine (-118.357) against acetylcholinesterase (1EVE).

The docking score of phytochemicals of *A. indica* were found to be comparable as compared with reference compounds, with rutin (-178.959) showing highest MolDock score as compared to reference compounds

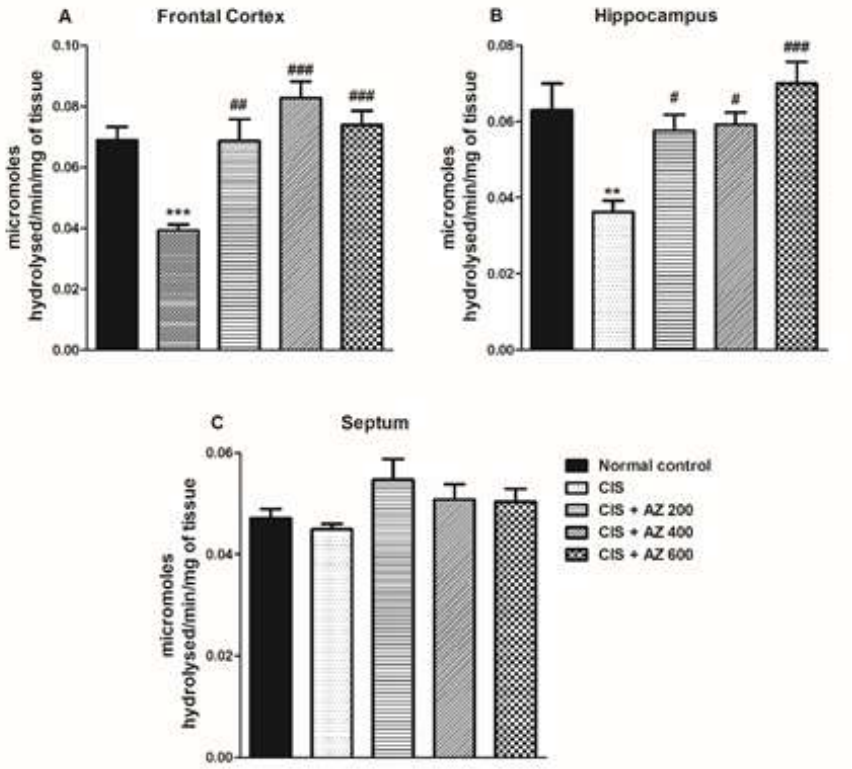


Figure 8. Effect of chronic stress and *Azadirachta indica* treatment on AChE activity in (A) Frontal cortex; (B) Hippocampus and (C) Septum. Normal control: Un-stressed rats (n=5) kept at standard home cage condition; CIS: chronic immobilization stress (n=5): animals subjected to immobilization stress for 2h/day for 10 days; CIS + AZ 200 (5), CIS + AZ 400 (5) and CIS + AZ 600 (5): stressed animals treated with *Azadirachta indica* 200 mg, 400 mg and 600 mg/kg/day for 14 days orally. Data were expressed as Mean ± SEM. One-way ANOVA followed by Tukey's posthoc used for analysis. $^{**}p<0.01$, $^{***}p<0.001$ as compared with normal control; $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ compared with CIS group.

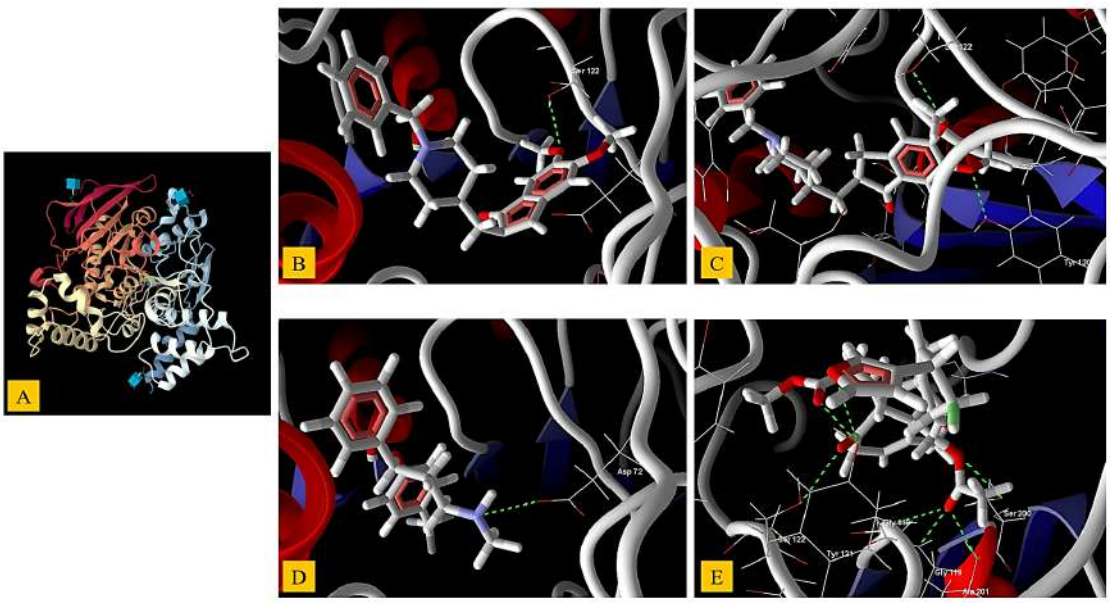


Figure 9. Acetylcholinesterase structure (PDB ID: 1EVE). (A) The three-dimensional structure of the anti-Alzheimer drug, E2020 (aricept), is complex with its target acetylcholinesterase (PDB ID: 1EVE). (B) Ligand 1-BENZYL-4-[(5,6-DIMETHOXY-1-INDANON-2-YL)METHYL] PIPERIDINE docked in best of its conformation (pose) into the binding site of 1EVE. (C) Standard donepezil docked in the best of its conformation (pose) into the binding site of 1EVE. (D) Standard fluoxetine docked in the best of its conformation (pose) into the binding site of 1EVE. (E) Active constituent Nimbinene docked in the best of its conformation (pose) into the binding site of 1EVE.

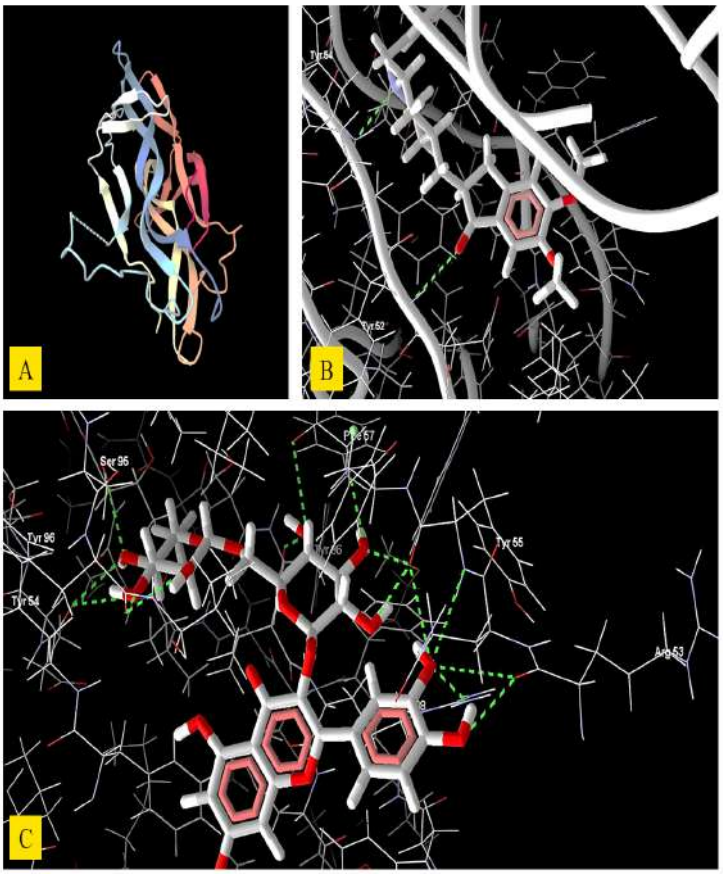


Figure 10. (A) Structure of Brain Derived Neurotrophic Factor, Neurotrophin-4 (PDB ID: 1B8M)(B) Standard donepezil docked in best of its conformation (pose) into the binding site of 1B8M. (C) Active constituent rutin docked in best of its conformation (pose) into the binding site of 1B8M.

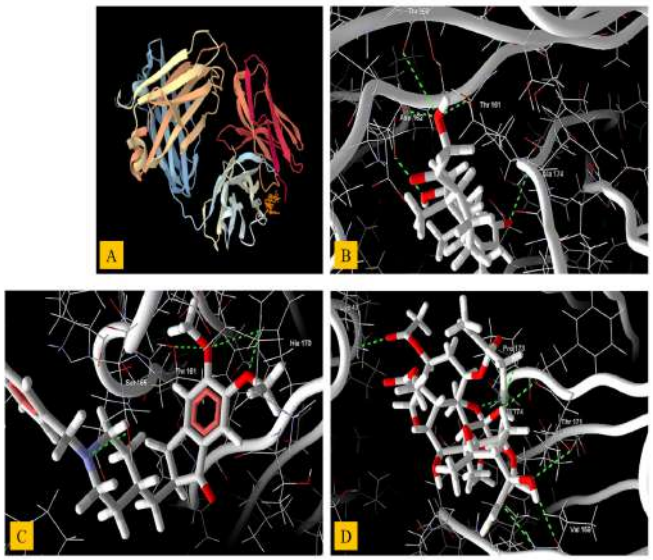


Figure 11. NMRA receptor structure (PDB ID: 1EVE). (A) Crystal structure of the NR1 ligand binding core in complex with 5,7-Dichlorokynurenic acid (DCKA) at 1.90 angstroms resolution (PDB ID: 1PBQ). (B) Ligand 5,7-Dichloro-4-hydroxyquinoline-2-carboxylic acid docked in best of its conformation (pose) into the binding site of 1PBQ. (C) Standard donepezil docked in best of its conformation (pose) into the binding site of 1PBQ. (D) Active constituent Azadirachtin docked in best of its conformation (pose) into the binding site of 1PBQ.

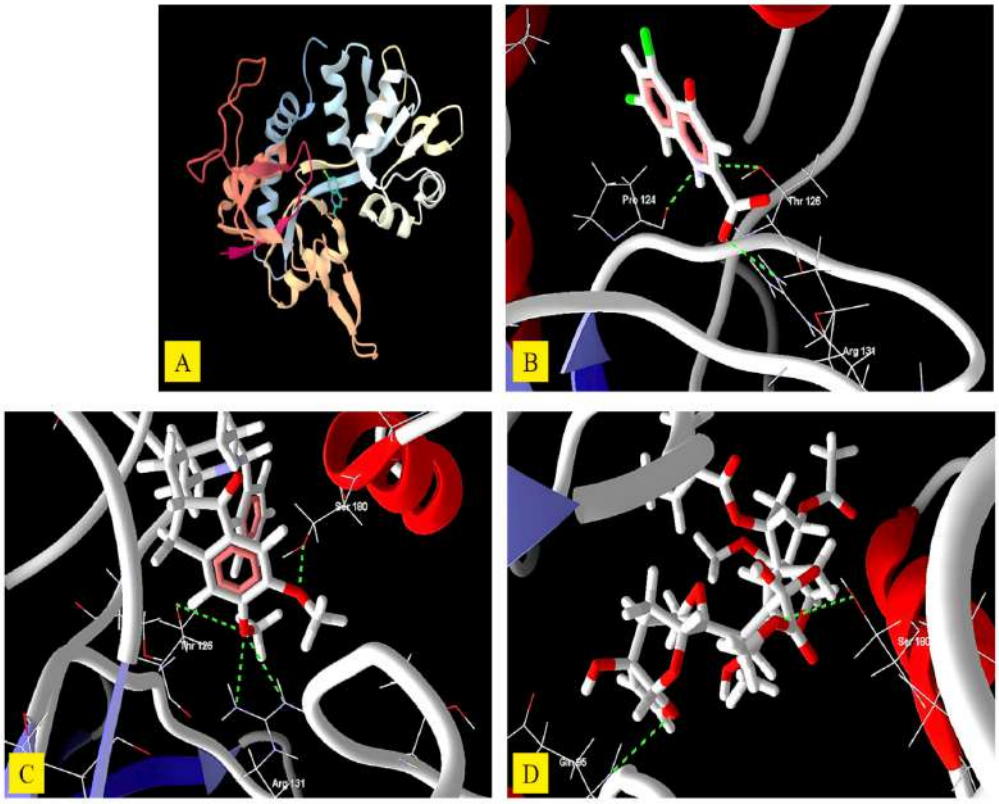


Figure 12. Corticosterone docking (PDB ID: 8CBZ). (A) Crystal structure of Anti-cortisol Fab in complex with corticosterone (PDB ID: 8CBZ). (B) Ligand corticosterone docked in best of its conformation (pose) into the binding site of 8CBZ. (C) Standard donepezil docked in best of its conformation (pose) into the binding site of 8CBZ. (D) Active constituent azadirachtin docked in best of its conformation (pose) into the binding site of 8CBZ.

erence compounds donepezil (-104.32) while fluoxetine did not form any H-bond at target against corticosterone (8CBZ).

The Mol Dock score and amino acid interaction between different neem's chemical constituents and target proteins were shown in tables 1, 2, 3, 4 and 5.

Phytochemical analysis of A. indica extract by LC-MS

LC-MS analysis of aqueous extract of neem reveals that the following phytoconstituents, including terpenois, flavonoids.

Discussion

This study aimed to check whether *A. indica* positively influences learning and memory in chronically stressed rats. The current study showed that ten days of chronic immobilization stress impaired learning and memory in NORT and T-maze tasks. In the NORT, chronic stress resulted in a reduction in recognition memory. Rats treated with a low dose of *A. indica* (200 mg/kg) did not restore impaired recognition memory in stressed animals. *A. indica* at 400 and 600 mg/kg doses completely restored recognition memory as animals spent more time with novel objects compared to the CIS group. In the T-Maze, spatial working memory was compromised in chronically stressed animals compared with the control group. CIS group tends to spend more time in the familiar arm than the new arm. CIS + AZ groups showed enhanced exploration in the new arm compared to the familiar arm. *A. indica* demonstrated a dose-dependent effect; higher dose (600 mg/kg) animals spent more time in the new arm compared to the low dose group (200 mg/kg).

In addition, chronic stress resulted in heightened anxiety-like behavior in the EPM. CIS animals made more entries in closed arms than the number of entries in open arms. Also, the time spent in the closed arms was significantly more than the time spent in the open arms. *A. indica* treatment resulted in anxiolytic activity, and the number of open-arm entries was restored to normal. The number of rearing and head dips were normalised in the *A. indica*-treated animals. Chronic immobilization stress reduced AChE activity in the frontal cortex and hippocampus without affecting septal AChE activity. Interestingly, *A. indica* administration at three different dosages, 200, 400, and 600 mg/kg, restored hippocampus and frontal cortical AChE activity.

Previous research demonstrated the connection between

Table 1. MolDock Scores of active chemical constituents of Aq. Neem extract on three-dimensional structure of the anti-Alzheimer drug, E2020 (aricept), complexed with its target acetylcholinesterase (PDB ID: 1EVE)

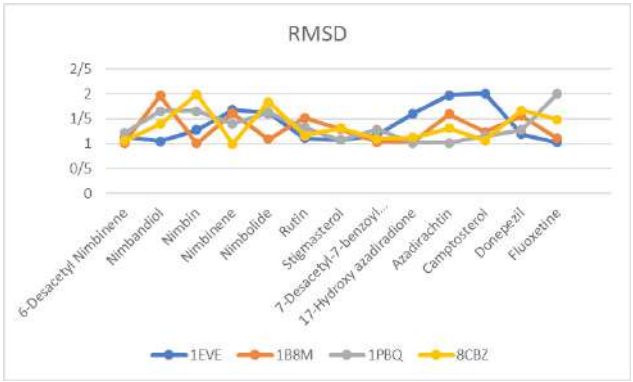
Name of Compound	MolDock Score	Rerank Score	Binding affinity	RMSD value	HBond	Amino acid
6-Desacetyl Nimbinene	-177.279	-119.095	-23.6958	1.13119	-7.12961	Tyr 334, Phe 331, Tyr 121,
Nimbandiol	-165.697	-42.4369	-22.4498	1.04524	-11.6232	Ser 200, Ser 122, Tyr 121, Gly 119, Asn 85, ,Trp 84, Asp 72
Nimbin	-194.964	-119.712	-27.9707	1.27154	-7.94917	Gly 123, Ser 122, Tyr 121, Tyr 70
Nimbinene	-197.862	-132.041	-28.2831	1.67425	-12.7578	Ala 201, Ser 200, Ser 122, Tyr 121, Gly 119, Gly 118,
Nimbolide	-195.647	-137.483	-25.3411	1.61579	-5.69826	Ser 122, Tyr 121
Rutin	-176.904	-78.5725	-29.7921	1.10491	-30.4815	His 440, Phe 330, Arg 289, Phe 288, Ser 200, Glu 199, Tyr 130, Tyr 121, Gly 119, Asn 85, Ser 81, Asp 72
Stigmasterol	-163.726	-122.957	-11.1962	1.07147	-1.44216	Phe 331
7-Desacetyl-7-benzoyl gedunin	-195.722	-139.232	-27.2574	1.17052	0.184052	Phe 288, Tyr 121
17-Hydroxy azadiradione	-165.881	-123.197	-19.3429	1.60399	-5	Ser 122, Tyr 121
Azadirachtin	-196.734	35.682	-38.0636	1.97447	-16.9751	Phe 288, Gly 123, Tyr 121, Trp 84, Asp 72, Gln 69
Camptosterol	-166.474	-122.266	-9.27584	2.00612	0	No H bonding with A.A
Donepezil	-156.693	-120.599	-29.1752	1.19447	-3.76502	Tyr 130, Ser 122
Fluoxetine	-118.357	-94.1607	-37.7938	1.02187	-0.640119	Asp 72

long-term stress and alterations in neurochemistry and behavior. Stress impairs learning and memory by altering the morphology of hippocampus pyramidal neurons. In the hippocampus, prolonged stress increases glucocorticoid production, reduces AChE activity, slows neurogenesis, decreases BDNF levels, and induces dendritic atrophy [4-7,39-43]. Chronic immobilization stress causes hippocampus shrinkage and prefrontal cortical neuron hypofunction [6,44-45]. In the prefrontal cortex and hippocampal regions, ACh plays a role in memory formation. ACh is hydrolyzed by acetylcholinesterase (AChE), and the presence of AChE is a reliable indicator of altered cholinergic neurotransmission in different brain areas under long-term stress. Persistent and repeated stress lowers the activity of AChE in the hippocampus [43].

The present investigation employed a chronic immobilization stress paradigm to assess *A. indica*'s memory-enhancing properties. The behavioral assessments were employed by the novel object recognition test, T-maze task, and EPM test. The findings demonstrate that two weeks of *A. indica* therapy significantly improves memory under stressful circumstances. Stressed animals showed better curious behavior after being treated with an aqueous extract of *A. indica* leaves. Both the time spent exploring

Table 2. MolDock Scores of active chemical constituents of neem aqueous extract on brain derived neurotrophic factor, neurotrophin-4 (PDB ID: 1B8M)

Name of Compound	MolDock Score	Rerank Score	Binding affinity	RMSD value	H Bond	Amino acid
6-Desacetyl Nimbinene	-132.19	296.349	-24.6013	1.01289	-6.97628	Arg 98, Tyr 55
Nimbandiol	-115.186	269.264	-20.4847	1.96552	-8.04226	Arg 98, Tyr 55
Nimbin	-156.943	222.235	-28.0186	1.00947	-9.15112	Arg 98, Arg 88, Tyr 55
Nimbinene	-155.672	154.963	-26.4589	1.61081	-7.77474	Arg 98, Arg 88
Nimbolide	-113.122	253.432	-24.3946	1.0857	-1.94504	Leu 90, Arg 88
Rutin	-178.959	84.8131	-29.3969	1.52112	-26.4161	Tyr 96, Ser 95, Arg 88, Tyr 86, Phe 57, Tyr 55, Tyr 54, Arg 53
Stigmasterol	-126.669	136.905	-11.5858	1.28811	-2.44415	Arg 88, Arg 53
7-Desacetyl-7-benzoyl gedunin	-152.1	128.459	-29.6905	1.03121	-4.71214	Phe 57, Tyr 55, Tyr 52
17-Hydroxy azadiradione	-106.891	99.2879	-18.9848	1.03069	-0.832926	Trp 100, Ser 45
Azadirachtin	-65.2813	473.667	-30.1763	1.59212	-3.7084	Trp 110, Trp 100, Ala 47
Camptosterol	-126.559	117.12	-13.9851	1.23668	-6.90139	Arg 88, Tyr 55, Arg 53
Donepezil	-145.759	30.0271	-30.0005	1.56116	-4.18217	Tyr 54, Tyr 52
Fluoxetine	-137.39	-113.661	-38.3236	1.10914	0	No H bonding with A.A



Graph. RMSD values for compounds on four target proteins

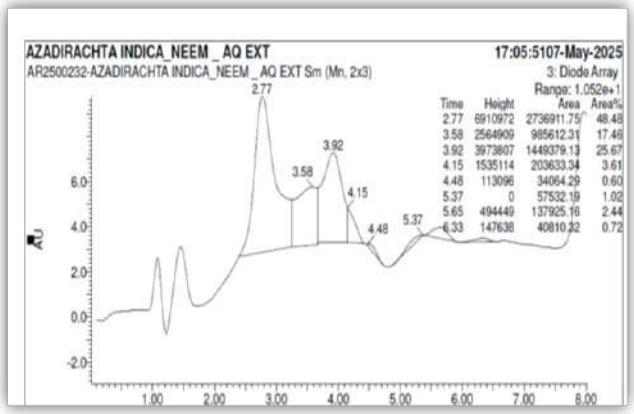


Figure 13. LC-MS of *Azadirachta indica* extract

Table 3. MolDock Scores of active chemical constituents of neem aqueous extract on crystal structure of the nr1 ligand binding core in complex with 5,7-dichlorokynurenic acid (dcka) at 1.90 angstroms resolution NMDA receptor (PDB ID: 1PBQ)

Name of Compound	MolDock Score	Rerank Score	Binding affinity	RMSD value	H Bond	Amino acid
6-Desacetyl Nimbinene	-152.755	-21.2475	-23.4784	1.20949	-4.46367	Arg 131, Thr 126
Nimbandiol	-128.259	-86.854	-21.3229	1.65621	-9.8691	Ser 180, Phe 246, Gln 144
Nimbin	-148.165	-88.4098	-29.4719	1.6557	-11.0254	Ser 180, Glu 130, Asn 129
Nimbinene	-145.165	-69.2158	-23.2712	1.40462	-2.49697	Ser 180
Nimbolide	-130.244	-83.5256	-24.9887	1.62963	-5.23647	Ser 180, Arg 131, Gln 95
Rutin	-129.733	-103.199	-27.4781	1.32808	-24.4747	Asp 224, Glu 204, Val 181, Lys 177, Gln 144, Glu 96, Thr 94, Gly 93, Phe 92, Gln 13
Stigmasterol	-142.028	-109.225	-13.1806	1.0748	-5.69833	Asn 107, Thr 94
7-Desacetyl-7-benzoyl gedunin	-136.273	-46.0977	-29.8441	1.28306	-5.55859	Ser 180, Arg 131, Asn 107, Thr 94
17-Hydroxy azadiradione	-140.468	-59.2979	-20.9465	1.01357	-8.81867	Ser 180, Arg 131, Thr 126
Azadirachtin	-156.244	-67.8212	-26.674	1.01217	-3.60831	Ser 180, Gln 95
Camptosterol	-130.445	-86.0441	-9.91052	1.15342	-1.3971	Thr 94
Donepezil	-126.51	-65.2965	-30.3196	1.27851	-5.52251	Ser 180, Arg 131, Thr 126
Fluoxetine	-107.778	-86.9328	-37.483	2.00803	0	No H bonding with A.A

the new arm and the motivation to do so were increased in the T-maze. The EPM revealed anti-anxiety action in the *A. indica*-treated group. In stressed rats, *A. indica* treatment modulated cholinergic neurotransmission by restoring AChE activity in the hippocampus and prefrontal cortex.

Prior research has demonstrated the neuroprotective effects of *A. indica* under different conditions. *A. indica* inhibits oxidative-nitrosative stress, the production of pro-inflammatory cytokines, and apoptosis to exert its neuroprotective effects against neuropathic pain caused by partial sciatic nerve ligation [49]. *A. indica* greatly alleviates the functional impairments brought on by hypoperfusion, lowers anxiety, enhances memory and learning, and lessens gliosis [21]. By lowering neuroinflammation and acting as an antioxidant, *A. indica* enhanced functional recovery in the rat Parkinson's disease model caused by 6-hydroxydopamine [50]. In an experimental Alzheimer's disease model, *A. indica* pretreatment enhanced reference memory, working memory, and spatial learning and showed anxiolytic action [48]. In the chronically stressed rats, there was reduced acetylcholine esterase activity. This supports the fact that chronic stress damages the cholinergic neurons. *A. indica* treatment restored AChE activity in the hippocampus and prefrontal cortex.

The brain's cholinergic neurons are destroyed by prolonged, severe stress, and the primary treatment strategy for chronic stress is to increase cholinergic neurotransmission. The standard medications (fluoxetine and donepezil) that were studied in AChE (PDB ID: 1EVE) showed docking scores that were lower than the chemical ingredient of neem, camptosterol (Table 1, Figure 9). The findings showed that neem extract showed an approximately comparable potency to conventional medications. These results follow those produced by the Molegro Virtual Docker application.

Modified neuroplasticity pathways have been associated with chronic stress disorders. Corticosteroids, NMDA receptors, BDNF, and impaired memory are linked to stress induced cognitive deficits. Previous studies showed that the BDNF levels were reduced in the hippocampus of animals subjected to chronic immobilization stress [5-6]. Prolonged stress activates HPA axis and enhances the secretion of corticosterone in experimental animals [47]. Also, previously it was shown that chronic stress reduces

acetylcholinesterase activity in the hippocampus indicating importance of cholinergic neurotransmission in the stress-induced cognitive deficits [43]. Glutamate NMDA receptors associated excitotoxicity play a major in hippocampal dendritic atrophy [46].

The neem extract displayed a higher docking score for the three stress-associated targets. The neem aqueous extract exhibits greater efficacy against chronic stress targets as compared to standard drugs. These observations were confirmed by the Molegro Virtual Docker program results (Tables 2, 3, 4 and Figures 10, 11,12). Molecular docking studies of the title compounds were carried out using Molegro Virtual Docker (MVD-2013, 6.0) software and results are given in Table 1, 2, 3, 4 and 5. The chemical constituents exhibited well-conserved hydrogen bonds with one or more amino acid residues in the active pocket of targets acetylcholinesterase, corticosterone, NMDA receptors, BDNF. The MolDock score of phytoconstituents ranged from -163.726 to -197.862 on Acetylcholinesterase, -65.2813 to -178.959on BDNF, -128.259to -156.244 on NMDA receptor and-115.073 to -162.129 on corticosterone (Table 5).

Table 4. MolDock Scores of active chemical constituents of neem aqueous extract on crystal structure of anti-cortisol Fab in complex with corticosterone (PDB ID: 8CBZ)

Name of Compound	MolDock Score	Rerank Score	Binding affinity	RMSD value	H Bond	Amino acid
6-Desacetyl Nimbinene	-131.855	-44.6084	-25.7827	1.05916	-9.27907	Thr 171, His 170, Gln 163, Asp 162, Thr 161
Nimbandiol	-127.333	-78.0426	-22.1132	1.3891	-11.8732	Thr 171, Lys 166, Ser 165, Asp 162
Nimbin	-154.154	-90.0342	-24.796	1.98786	-0.643575	Thr 171, His 170
Nimbinene	-141.083	-76.0529	-25.3992	1.00096	-7.27126	Thr 171, His 170
Nimbolide	-145.561	-93.8127	-26.6626	1.8384	-9.06434	Thr 171, Ser 165
Rutin	-140.505	-96.5951	-26.2362	1.16393	-21.041	His 170, Val 169, Ser 165, Asp 164, Asp 162
Stigmasterol	-120.387	-92.952	-13.2307	1.30578	-6.05683	Thr 84, Gln 37
7-Desacetyl-7-benzoyl gedunin	-132.32	-9.61485	-32.1954	1.09892	-10.3725	Thr 171, Ser 171, His 170, Thr 161
17-Hydroxy azadiradione	-131.834	-58.1924	-20.2586	1.12535	-4.12481	Ser 165, Gln 163, Glu 102, Lys 100
Azadirachtin	-162.129	-57.8274	-30.9054	1.31162	-11.4839	Ala 174, Pro 173, Thr 171, Val 158, Lys 43
Camptosterol	-115.073	-80.363	-10.2213	1.06619	-2.46007	Val 158
Donepezil	-104.32	-74.5225	-30.0444	1.67002	-5.55117	His 170, Ser 165, Thr 161
Fluoxetine	-113.13	-85.0785	-37.6821	1.48438	0	No H bonding with A.A

Table 5. LC-MS data of *Azadirachta indica* extract

Retention Time (min)	m/z Values	Tentative Compounds	Class of phytochemicals
2.77	165.01, 179.20, 289.16, 295.04, 449.35, 464.53, 505.52, 557.53,	Nimbin, Limonoid glycosides	Limonoids
3.58	229.17, 259.40, 319.33, 337.48, 425.53, 439.45, 520.70	Quercetin derivatives, flavonoids	Flavonoids
3.92	155.05, 205.08, 265.34, 293.32, 339.33, 353.58, 415.49, 423.41	Kaempferol derivatives,	Flavonoids
4.15	219.33, 259.33, 291.34, 421.50	Azadirachtin fragments, Neem limonoids	Limonoids
4.48	249.10, 293.52, 325.33, 356.35, 439.52, 467.77, 555.75, 567.56	Gedunin or derivatives	Triterpenoids
5.37	521.62, 609.80, 610.79, 688.41	Complex limonoids	Polyphenols
5.65	619.97, 633.70, 715.54, 734.15	High-MW glycosides	Glycosides
6.33	700.03, 747.49, 752.04	Polyphenolic fragments or glycosides	Polyphenols/ Glycosides

All phytoconstituents like 6-desacetyl nimbinene, nimbandiol, nimbin, nimbinene, nimbolide, rutin, stigmasterol, 7-desacetyl-7-benzoyl gedunin, 17-hydroxy azadiradione, azadirachtin showed the highest MolDock score as compared to standard drugs donepezil and fluoxetine on targets Acetylcholinesterase, NMDA receptor and corticosterone. Phytoconstituent camptosterol showed no H-bonding against acetylcholinesterase, and lower MolDock scores as compared to standard drugs against BDNF target. However, displayed a higher MolDock score as compared to standard drugs donepezil and fluoxetine against NMDA receptor and corticosterone.

The RMSD values obtained from docking studies across four target proteins (1EVE, 1B8M, 1PBQ, and 8CBZ) indicate the quality and stability of ligand binding poses. Compounds such as 6-desacetyl nimbinene, rutin, stigmasterol, and 7-desacetyl-7-benzoyl gedunin demonstrated consistently good and reliable binding, with RMSD values ranging between 1.01 to 1.31 Å, indicating close alignment with their predicted poses and stable docking conformations. These ligands are likely to form accurate and meaningful interactions within the binding pocket.

Most other ligands, including nimbandiol, nimbin, nimbolide, and donepezil, displayed RMSD values in the acceptable range of 1.0 to 2.0 Å, suggesting acceptable and reliable docking results as shown in the graph.

The binding affinity analysis across four protein targets shows that azadirachtin, rutin, and 7-desacetyl-7-benzoyl gedunin exhibit strong interactions, often surpassing the standard drug donepezil. These compounds formed multiple hydrogen bonds with key residues, contributing to stable binding. Fluoxetine also displayed high binding affinity, particularly with 1PBQ and 8CBZ. In contrast, stigmasterol and camptosterol showed weaker affinities and fewer interactions, indicating limited potential. Overall, the phytochemicals demonstrated competitive binding efficiencies, suggesting their suitability as potential inhibitors. Azadirachtin and rutin, with their consistent performance, highlight the promise of natural compounds in targeting these proteins effectively.

However, only a few of them showed the highest MolDock score than the standard drugs against BDNF target and in contrast, other constituents were comparable when compared with standard drugsat the cavity.These results show that the chemical constituents of neem aqueous extract possess higher affinity than standard ligands and reference drugs towards the active site of the target proteins acetylcholinesterase, corticosteroids, NMDA receptors, and were comparable to target BDNF. Thus, the phytoconstituents of aq. Extracts of neem could bind with some of the residues of the active site and can be further developed into potential pharmacological agentsfor chronic stress disorders and impaired memory.

The Ayurvedic Rasayana *A. indica* works in the chronic stress model to reverse the neurobehavioral alterations, lessen cognitive impairments, and increase cholinergic activity. Since this research is preliminary, it is unable to pinpoint the precise mechanism behind its protective function in the chronic restraint stress paradigm. We conclude that 14 days of treatment with *A. indica* attenuates

the impact of CIS on memory and cholinergic activity. Despite the promising results observed in this study, there are several limitations that should be considered. One notable limitation is the lack of a complete phytochemical analysis of the neem aqueous extract. Although we have identified key phytoconstituents, a detailed profile of all the active compounds would provide a better understanding of the specific molecules responsible for the observed effects. Additionally, the study primarily focused on *in silico* docking studies, and further *in vivo* and clinical investigations are needed to validate these findings. The absence of dose-response data and the effects of long-term use are also aspects that require future attention to establish the clinical relevance of neem-derived compounds for memory impairment.

Conclusion

Based on the obtained results, it can be concluded that the phytoconstituents present in the aqueous extract of neem exhibited the highest MolDock scores compared to standard drugs and ligands across various molecular targets. An RMSD value of less than 2Å in the re-docking analysis indicates accurate prediction of ligand binding conformations by the docking algorithm and scoring functions. Compounds exhibited lower RMSD values and higher binding affinities, suggesting that they form more stable interactions with the protein targets, which indicates a stronger inhibitory potential. These findings suggest that neem-derived compounds hold promise as potential therapeutic agents for managing memory impairment associated with chronic stress disorders. However, further *in vivo* and clinical studies are necessary to validate these results and assess their safety, efficacy, and mechanism of action.

Conflict of Interests

None.

Acknowledgements

None.

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