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Potential acetylcholinesterase inhibitors to treat Alzheimer's disease

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Abstract. – OBJECTIVE: Alzheimer's disease (AD) is identified by neuropathological symptoms, and there is now no effective treatment for the condition. A lack of the brain neurotransmitter acetylcholine has been related to the etiology of Alzheimer's disease. Acetylcholinesterase is an enzyme that breaks down acetylcholine to an inactive form and causes the death of cholinergic neurons. Conventional treatments were used but had less effectiveness. Therefore, there is a crucial need to identify alternative compounds with potential anti-cholinesterase agents and minimal undesirable effects.

MATERIALS AND METHODS: **Fluoroquinolones and benzimidazole-benzothiazole derivatives offer antimicrobial, anti-inflammatory, anti-oxidant, anti-diabetic, and anti-Alzheimer activities. To enhance the chemical portfolio of cholinesterase inhibitors, a variety of fluoroquinolones and benzimidazole-benzothiazole compounds were evaluated against acetylcholinesterase (AChE) butyrylcholinesterase (BChE) enzymes. For this purpose, molecular docking and adsorption, distribution, metabolism, excretion, and toxicology ADMET models were used for *in-silico* studies for both AChE and BChE enzymes to investigate possible binding mechanisms and drug-likeness of the compounds. The inhibitory effect of docked heterocyclic compounds was also verified *in vitro* against AChE and BChE enzymes. Fluoroquinolones (Z, Z3, Z4, Z6, Z8, Z12, Z15, and Z9) and benzimidazole-benzothiazole compounds (TBIS-16, TBAF-1 to 9) passed through the AChE inhibition assay and their IC_{50} values were calculated.**

RESULTS: **The compound 1-ethyl-6-fluoro-7-(4-(2-(4-nitrophenylamino)-2-oxoethyl)piperazin-1-yl) -4-oxo-1,4 di-hydroquinoline-3-carboxylic acid and 2-((1H-benzo[d]imidazol-2-yl) methyl)-N'-(3-bromobenzyl)-4-hydroxy-2H-thiochromene-3-carbohydrazide 1,1-dioxide (Z-9 and TBAF-6) showed the lowest IC50 values against AChE/BChE (0.37±0.02/2.93±0.03 μM and 0.638±0.001/1.31±0.01 μM, respectively) than the standard drug, donepezil (3.9±0.01/4.9±0.05 μM). During the in-vivo investigation, behavioral trials were performed to analyze the neuroprotective impact of Z-9 and TBAF-6 compounds on AD mouse models. The groups treated with Z-9 and TBAF-6 compounds had better cognitive behavior than the standard drug.**

CONCLUSIONS: **This study found that Z-9 (Fluoroquinolones) and TBAF-6 (benzimidazole-benzothiazole) compounds improve behavioral and biochemical parameters, thus treating neurodegenerative disorders effectively.**

Key Words:
Heterocyclic compounds, Alzheimer's disease, Neuroinflammation, Neuronal loss.

Introduction

Alzheimer's disease is a more severe neurological disorder that steadily affects patients and eventually kills them. Around 70% of elderly people with deteriorating cognitive function have Alzheimer's disease.

There are an estimated 3,60000 new cases each year, which is equal to 980 new cases each day or 40 new cases per hour[1]. Although promising research and development are under progress in early identification and therapy, there is no cure

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for AD. The neurotransmitters acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) are primarily inhibitors and are known to be of use in AD pathology. Both enzymes are present in the brain and are found in neurofibrillary tangles and neurotic plagues. AChE catalyzes the hydrolysis of acetylcholine neurotransmitters to choline and acetic acid, a step that is required to re-activate a cholinergic neuron[2,3].

Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) are two cholinesterase types. The elevation of acetylcholine through inhibition of AChE enzymes was accepted as the most effective strategy for treatment against AD despite its unknown etiology. The inhibitors of AChE and BChE have, therefore, become remarkable options in AD therapy. However, current medicinal products (tacrine, rivastigmine, and donepezil) with AChE-inhibiting activity have some side effects and are only effective against mild AD, and no BChE-inhibiting drugs are available to date. Therefore, to combat AD, new drugs are compulsory to develop. AD reduces AChE enzyme activity levels and increases BChE activities, and the normal BChE and AChE ratio in the brain could change from 0.6 to 11%. It is proposed to enhance the efficacy and expand the indications of the treatment strategy through a dual inhibition strategy for these enzymes. Since ancient times, heterocyclic compounds have been employed to cure many ailments. Precisely, a few examinations have planned and combined different ligands containing, in any event, one heterocyclic framework utilizing the multi-target strategy and investigating new conceivable natural targets. AD is well-known as a multifactorial illness, and its mental origins imply a robust system of novel therapies that can be found through the so-called "multi-target ligands". It is centered on identifying multifunctional compounds to concurrently fulfill two or more goals so that synergistic activities can be achieved and treatment efficiency improved. In addition, a research group[4,5] has proven two N-heterocyclic chemical series for biological activities (triazolothiadiazoles and triazolothiadiazines). Fascinatingly, these compounds effectively inhibited butyrylcholinesterase (BChE) and acetylcholinesterase (AChE)[4,5].

Fluoroquinolones and benzimidazole-benzothiazole derivatives offer antimicrobial, anti-inflammatory, anti-oxidant, anti-diabetic, and anti-Alzheimer activities. Keeping in view the importance of these heterocyclic compounds, the current research work was designed to find potential acetylcholinesterase/butyrylcholinesterase inhibitors to treat Alzheimer's disease. The selection of these compounds was based on their novelty, as no activity was reported against them.

Materials and Methods

The current study has been developed to find out the heterocyclic compound empirical and molecular modeling to locate possible acetylcholinesterase inhibitors to treat Alzheimer's disease.

This study aims to identify potent inhibitors of Alzheimer's acetylcholinesterase/butyrylcholinesterase.

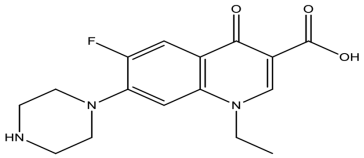
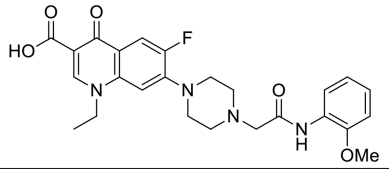
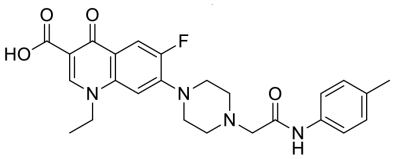
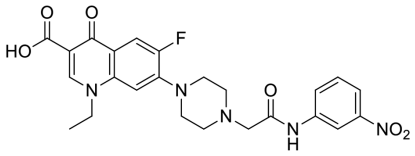
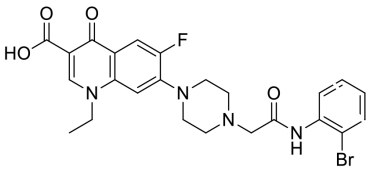
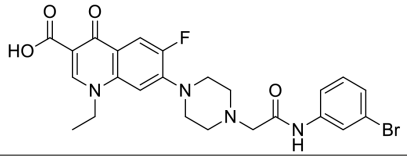
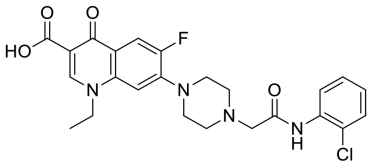
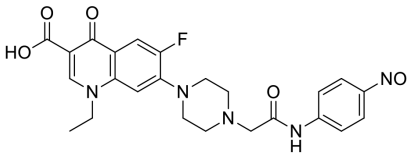
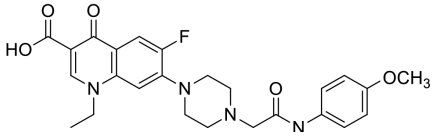
Chemical and Reagents

Chemicals used in this research were purchased from Sigma Aldrich Chemicals Pvt Ltd, Bangalore, India. Dimethyl sulfoxide (DMSO) is a strong solvent that was used to dissolve the heterocyclic compounds. Phosphate-buffered saline (PBS) was used as a buffering agent and to prepare the enzyme and substrate. Acetylcholinesterase (C3389-500UN)/Butyrylcholinesterase (C7512-1.2KU) enzyme, and Acetylcholine iodide (A5751)/S-Butyryl thiocholine iodide (20820-1G) substrate were also purchased from Sigma-Aldrich. DTNB (Ellman's Reagent) was used as a coloring reagent in an enzyme inhibition assay. Donepezil was used as a positive control. Ethanol, Chloroform, Aluminum chloride, Isopropanol, cDNA kit (Thermo Scientific, Chennai, India), triazole (Invitrogen TM), primers (Thermo Fisher Scientific, Waltham, MA, USA), cyber green (SYBR® Green master mix of Bio-Rad, CA, USA) were obtained. The ELISA reader analyzed all chemicals at different wavelengths and the UV spectrophotometer.

Sample Collection

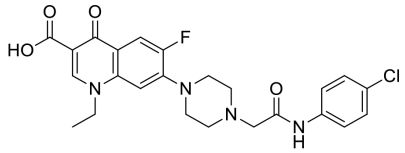
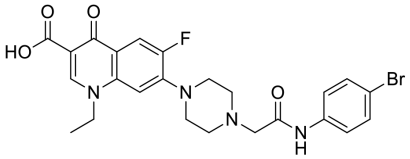
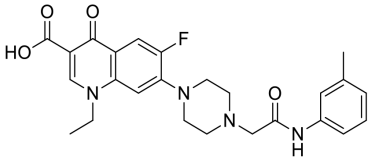
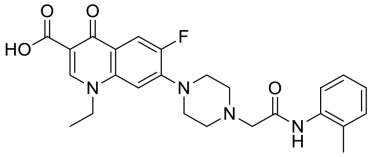
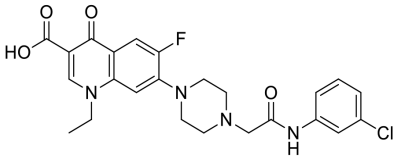
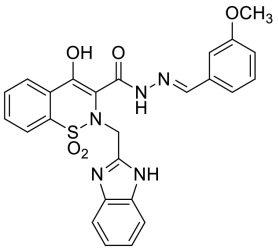
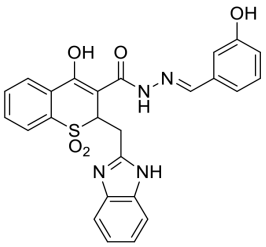
The Department of Medicinal Chemistry laboratory created heterocyclic molecules. The heterocyclic molecules' structures were shown using the CHEMDRAW Ultra 12.0 program (Chennai, Tamil Nadu, India). A total of 300 heterocyclic compounds (fluoroquinolones, benzimidazole-benzothiazole, benzodiazepines, etc.) were analyzed against the acetylcholinesterase/butyrylcholinesterase enzyme. However, this research included only the most powerful series of data (Table I).

Table I. List of heterocyclic compounds.

Sr. No.	Compounds codes	Structures
1	Z	
2	Z3	
3	Z4	
4	Z5	
5 Z6		
6	Z7	
7	Z8	
8	Z9	
9	Z11	

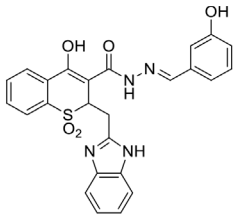
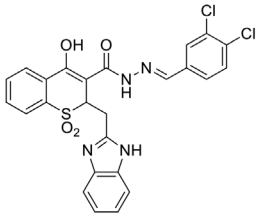
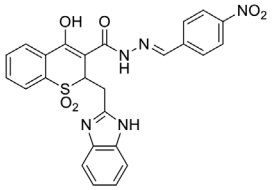
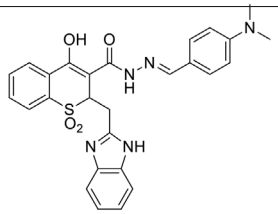
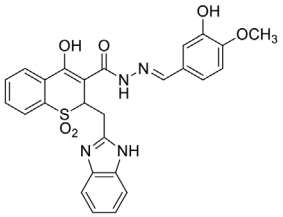
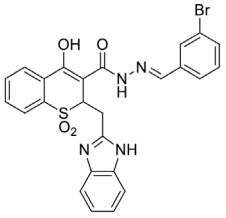
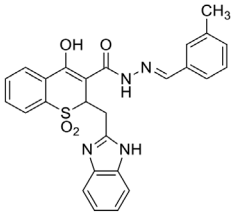
Continued

Table I ***(Continued).*** List of heterocyclic compounds.

Sr. No.	Compounds codes	Structures
10	Z12	
11	Z13	
12	Z14	
13	Z15	
14	Z16	
15	Z17	
16	TB1S16	 Molecular Weight: 488.52

Continued

Table I ***(Continued).*** List of heterocyclic compounds.

Sr. No.	Compounds codes	Structures
17	TBAF1	 Molecular Weight: 488.52
18	TBAF2	 Molecular Weight: 541.41
19	TBAF3	 Molecular Weight: 517.51
20	TBAF4	 Molecular Weight: 515.58
21	TBAF5	 Molecular Weight: 518.54
22	TBAF6	 Molecular Weight: 551.41
23	TBAF7	 Molecular Weight: 486.54

In-Silico Screening of Heterocyclic Compounds

Molecular Docking

The molecular docking technique was used to find the best binding between ligand and receptor. A ligand and receptor molecule were prepared for this purpose, as described below.

Preparation of ligand library

ChemDraw Ultra 12.0 software was used to design the structures of all the ligands, which were then saved as MDL files (SDF) to be opened in MOE software (Mumbai, India). The standard donepezil 2D structure was retrieved from NCBI PubChem and saved as an SDF format. Finally, all of the ligands and donepezil structures were 3D protonated, and energy was minimized using default MOE parameters.

Receptor preparation

A PDB database system (available at: http://www.rcsb.org/pdb) with a PDB ID: 1EVE/PDB ID: 5DYW was used to generate the 3D receptor's structure. A Molecular Operating Environment was used to eliminate solvent and ligand residues, energy minimization, 3D protonation, from the retrieved structure.

Docking

The active site on acetylcholinesterase/butyrylcholinesterase was identified using the docking software MOE (PDB ID: 1EVE/ PDB: 5DYW). The active site, which contains Tyr-121 and, Trp-84 has been selected for the acetylcholinesterase enzyme. The MOE software docking algorithm was used to dock the ready-to-dock ligands library with interacting acetylcholinesterase residues. The MOE software validates the exact ligand confirmation to obtain a minimum energy structure. Spatial score and Root Mean Square Deviation (RMSD) values were used to determine the best and highest ligand conformation after docking.

In-silico analysis of drug-likeness and ADMET properties

The best phytochemical docking score was then further selected based on Lipinski's rule of five (Ro5). This was used by the Molinspiration server (available at: http://www.molinspiration.com/cgibin/properties) to determine its physicochemical properties. SwissADME software (available at: http://www.swissadme.ch/) was used to evaluate the drug-like characteristics of applicants. The calculation of ADMET properties, i.e. Absorption, Distribution, Metabolism, Excretion, and Toxicity, is an important indicator of the drug candidate's behavior and toxicity in the human body.

In-vitro Screening of Heterocyclic Compounds

In-vitro acetylcholinesterase assay/ In-vitro butyryl cholinesterase assay

AChE catalyzes the hydrolysis of acetylcholine neurotransmitters to choline and acetic acid, a step that is required to re-activate a cholinergic neuron. Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) are two cholinesterase types. Acetylcholinesterase and butyrylcholinesterase both catalyze acetylcholine (ACh) hydrolysis with similarly high efficiency[6].

Enzyme preparation

The acetylcholinesterase enzyme (C3389-500UN) vial contains 500 units per vial. Therefore, to prepare 233 U/ml stock solution, 1 ml of PBS was added to 1 mg. Furthermore, to prepare 0.015 U/ml working solution, 64.37 µl of stock solution was mixed in 936 µl of PBS and stored at -20°C for further use. Butyrylcholinesterase enzyme (C7512-1.2KU) vial contains 1.2 KU per vial. To prepare 8.7 U/ml (0.087 U/µl) working solution, 1 ml of PBS was added into 1 mg and stored at -20°C for further use.

Substrate preparation

The molecular weight of Acetylcholine iodide (A5751) is 289.18 g/mol. Therefore, 0.1445 mg of the substrate was mixed in 1 ml of PBS to make the 0.5 mM working solution. The molecular weight of S-Butyryl thiocholine iodide (20820-1G) is 317.23 g/mol. Therefore, 0.158 mg of the substrate was mixed in 1 ml of PBS to make the 0.5 mM working solution.

DTNB (Coloring reagent) preparation

The molecular weight of DTNB is 391.85 g/mol. 0.1981 mg of coloring reagent was mixed in 1 ml of PBS to make the 0.5 mM working solution.

Inhibition assay

The developed spectrophotometric approach was used to quantify the *in-vitro* inhibition activity of AChE/BChE. In a nutshell, the reaction mixture consisted of 10 µl of enzyme solution (0.015 unit per well for AchE/0.0087 unit per

well for BChE), 5 µl of test chemical dissolved in DMSO, and 65 µl of phosphate buffer saline. The reaction components were properly mixed and maintained at 37 °C for 10 minutes during the pre-incubation phase. After the pre-incubation phase, the appropriate AchE/BChE solution was then added to a 10 µl solution of acetylthiocholine iodide/S-Butyryl thiocholine iodide (0.5 mM) to start the enzyme-substrate reaction. Additionally, 10 µl of (0.5 mM) DTNB was added to the mixture as a coloring agent. The reaction mixture was incubated for 20 minutes at 37°C, and the absorbance was measured at 405 nm using a microplate reader. Triplicates of the experiments were run with the appropriate controls. A positive control was performed using the reference standard medication donepezil (1 mM/well). The following formula was used to determine the percentage of acetylcholinesterase/butyrylcholinesterase inhibition:

$$\% \text{ of inhibition} = 1 - (At/Ac) \times 100$$

Where 'At' and 'Ac' are the absorbances recorded with and without inhibitors, respectively, after reducing the corresponding background.

In-Vivo Analysis

Experimental animals

Healthy Wistar Rats (100-150 gm) were purchased from the Central Animal House of the College of Pharmacy and placed seven days before the start of the experimentation to acclimatize the animals. The animals were kept in separate cages in groups. All mice were fed for one week under standard environmental conditions (12 h dark/12 h light, temperature 20-25°C, and 30-60% humidity). The Institutional Animal Ethics Committee (IAEC) at the College of Pharmacy gave its approval to conduct the study (IAEC Reference No.: CP/IAEC/PG/3/08/2022).

Induction of Alzheimer's disease in rats

Wistar rats were administered aluminum chloride 300 mg/kg p.o. and D-galactose 150 mg/kg p.o. for 21 days, causing behavioral, biochemical, and molecular defects and chronic aluminum accumulation and disposition in brain tissues[7-9].

Study design

Wistar rats in good health were separated into seven groups (n=7) for this investigation.

Group 1: As a control, they received 1 ml/kg of distilled water.

Group 2: D-galactose 150 mg/kg p.o. and aluminum chloride 300 mg/kg p.o. were administered as a disease control measure.

Group 3: The standard group received donepezil 1.5 mg/kg p.o., D-galactose 150 mg/kg p.o., and aluminum chloride 300 mg/kg p.o.

Group 4: Received treatment with Z-9 1.5 mg/kg p.o., D-galactose 150 mg/kg p.o., and aluminum chloride 300 mg/kg p.o.

Group 5: Received treatment with Z-9 0.75 mg/kg p.o., D-galactose 150 mg/kg p.o., and aluminum chloride 300 mg/kg p.o.

Group 6: Received treatment with D-galactose 150 mg/kg p.o., TBAF-6 1.5 mg/kg p.o., and aluminum chloride 300 mg/kg p.o.

Group 7: Received treatment with D-galactose 150 mg/kg p.o., aluminum chloride 300 mg/kg p.o., and TBAF-6 0.75 mg/kg p.o.

Animals in each group received care every day for 21 days. Behavior and weight were tracked at the start and the end of the 21 days. After receiving the greatest dose for one day while being under mild anesthesia, the animals were killed by cervical dislocation. Brains were removed from their bodies, cleaned with phosphate buffer, and kept at -80°C in triazole for biochemical and histological research.

Behavioural Studies

Morris water maze task

The purpose of the water maze was to examine spatial and continuous memory, task strategy, and learning in AD rodent models. This test was run using Morris' technique. Neuropharmacology frequently employs it to confirm neurocognitive alterations in rodent models. In more modern AD models, it is a sensitive test for spotting spatial disorientation. In this experiment, a round plastic tub with a 1 m diameter and a 1.5 ft depth was employed. Water from the faucet was filled and maintained at a temperature of 26°C. A platform was positioned in the middle of the pool, 1 inch below the water's surface, and it was made visible during training trials. By combining milk powder and water, the forum became opaque, rendering it invisible to the rats during the experiment. North, South, East, and West made up the four quadrants of this pool. Before being put into the pool for 90 seconds, the rats were trained for 5 days (4 trials each day) and taught how to use the platform. Animals were led to a secret platform in the early stages of training, where they remained there for 20 seconds after finding it after four trials in

various directions. The time it took to get out of the water and onto the concealed platform was timed. On the sixth day, the forum was made invisible to the rats, and it took a maximum of 120 seconds for them to escape through the concealed platform from all four directions.

Open field test

In this test, the fear, exploration, and movement of animals were investigated. This test is sensitive to early indicators of memory loss associated with Alzheimer's disease in a mouse model. The regular conduct of rats is to find refuge and prevent the center's openness. Therefore, the early pathogenic change in AD models with increased memory and anxiety, which show the device's declining horizontal and vertical movement, is regarded as a sensitive test. It was composed of the floorboard, 72 x 72 cm in size, and 36 cm walls in a hollow square, white resin chamber. One wall was built of Plexiglas to monitor the movement of rats within the gadget. The floor of this room has 16 identical squares with black lines (18 x 18 cm). A center square with red lines measuring 18 x 18 cm has been designed to separate the central region from other areas in its surroundings. After each animal test, the chamber was cleaned with 70% alcohol. The rat was handled gently with its tail and placed for 10 minutes in the corner of the device, and its four paws' total travel distance and line-crossing patterns were noted. There have been observations of time spent in the middle, stretching posture, cleaning, faces, rearing, and freezing.

Passive Avoidance Test

The contextual short- and long-term memory deterioration was investigated using a shock-motivated task. In this work, animals with AD disease fight against their innate tendency to avoid shocks. The apparatus for this experiment was composed of Plexiglas and included floor grillwork. It measured 27 cm x 27 cm x 27 cm (stainless steel rods of 3 mm thickness, which are 8 mm apart). The grid floor was wired to a battery that supplies 20 V of power. A wooden rostrum (10 cm 7 cm 1.7 cm) was installed at the halfway point. The animals were cautiously handled and set on the platform from their tails. In the first trial, the animal must maintain its position from the rostrum to the floor for 15 to 22 seconds. The second trial started two hours after the first trial ended. He stepped down from a wooden rostrum that had been piled up in the test rats.

Wire Hanging Test

This test was designed to evaluate the animal's neuromuscular power and endurance. The apparatus for this test was 50 cm high, three-inch wide, horizontal stainless steel grids fixed on timber walls. The animals were stabilized while they were put on the grid and carefully held from their tails, allowing them to pick up the grid with their front and hind paws. Up to 30 seconds were required for the animals to stay on the wire. It took between 30 and 60 seconds to hang.

Y-maze test

The Y-maze test is used to identify spatial, short-term, and cognitive defects of the rodents in behavioral neuro-sciences. For this task, the apparatus used was made out of a wooden three-arm with a Y-shaped arm of 120°. These arms had a trigonal median zone and measured 35 cm long, 25 cm high, and 10 cm wide. The animal's 8-minute exploration in the Y-maze was observed. Rats were positioned at the start of each arm of the apparatus, and the numbers of inputs and triads (access into three tiers on successive selections) were recorded to determine the spontaneous change. The following formula calculated the spontaneous alternation:

$$\text{Spontaneous alternation \%} = \frac{\text{Total No. of triads}}{\text{Total No. of arm entries} - 2} \times 100$$

The following equation was used to determine the laterality index used to investigate the animal's side preference[7-9].

$$\text{Laterality index} = \frac{\text{Movements toward left arm} - \text{Movements toward right arm}}{\text{Movements toward left arm} - \text{Movements toward right arm}}$$

Elevated plus-maze task

The elevated plus-maze test is a gold standard procedure in AD rodent models for verifying anxiety-like behaviors and unpredictable behavior patterns. In addition, this task was utilized in an external behavioral model to measure memory and exploration. The unit was designed with two open arms, one with two arms (50 cm x 10 cm), one with a closed arm, and two with a wooden platform with a central platform (10 cm x 10 cm). Animals were gently placed on the bottom of an open arm on the first day (the 20th day of the research). The distance traveled, the number of entries into each arm, the time spent in each arm, and the percent of entries into the open arms were calculated. The same procedure was

carried out 24 hours later (on the 21st day of the study), with the transfer delay being recorded to assess any comprehension, cognitive, and memory problems.

Hole board test

A hole board test is primarily used in mice and rats to evaluate multiple dimensions of unconditioned actions. Therefore, this task is an essential behavioral test in the AD rat model for estimating neophilia, emotion, stress, and anxiety. The floor was split into 16 uniformly-divided holes with a ground height of 1.5 m, a Plexiglas material of 25 cm x 25 cm, and a wall height of 30 cm. During the experiment, a rat was introduced to a pitch and given eight minutes to explore. The rat would insert its head into one of the holes, going deep enough that its ears were at the level of the floor. If the rat lifted its head out of the hole before continuing, this would be considered a fresh head-dipping session.

Statistical Analysis

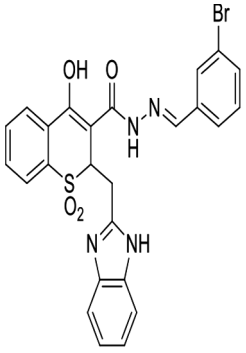
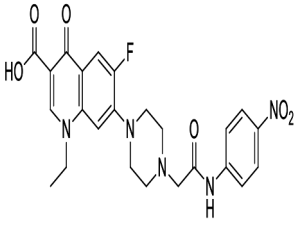
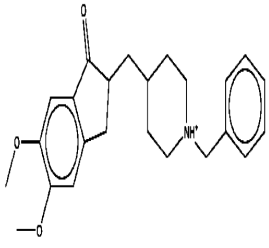
SPSS version 27 (IBM Corp., Armonk, NY, USA) was used to evaluate the data. The experimental study data for intergroup variation were recorded at 5% mean±SEM, one and two-way ANOVA followed by Tukey and Bonferroni post-test correspondingly. Statistically significant $p<0.05$ results were deemed.

Results

In-silico Screening of Heterocyclic Compounds

In silico with a molecular docking approach, the compounds' binding energy and RMSD value have been determined. MOE was used to conduct molecular docking (Table II). MOE is a drug research software platform for integrated visualization, modeling, and simulation by biologists.

Table II. Docking score and RMSD value of selected heterocyclic compounds.

Sr. No.	Compounds ID	Chemical structure	Docking score	RMSD value	Receptors
1	TBAF-6		-11.48	1.27	Thr-B120
2	Z-9		-12.37	1.50	Thr-B120
3	3152 (Donepezil) Standard drug		-11.34	2.08	Gly-B116 Tyr-B332 Thr-B120

Thr-Threonine; Gly-Glycine; Root Mean Square Deviation (RMSD).

In the Molinspiration server, the drug-like characteristics of the suggested inhibitors of acetylcholinesterase/butyrylcholinesterase were anticipated using the ADMET-based drug scan tool. No violation of the five-pill rule and appropriate drug-like features, such as molecular weight (Table III), has been identified by all selected candidates. SwissADME screened all the candidate compounds to evaluate their drug-like qualities (Table IV), which provided a basis for additional drug-like potential validation.

In-vitro Screening of Heterocyclic Compounds

In-vitro acetylcholinesterase and assay results and in-vitro butyryl cholinesterase assay

Butyrylcholinesterase assay is used to check the inhibitory potential of compounds against the butyrylcholinesterase enzyme. The acetylcholinesterase enzyme was used to screen a vast number of heterocyclic compounds. Regarding the screening, 2 Heterocyclic compounds were chosen for further evaluation. These two compounds have potent inhibitory activity against the butyrylcholinesterase enzyme. Tables V and VI show the % inhibition and IC_{50} of Heterocyclic compounds against the butyrylcholinesterase enzyme.

In-Vivo Analysis

Behavioral studies

Behavioral studies were conducted in AD rodent models to investigate contiguous spatial learning, memory, and task strategy. In neuropharmacology, these tests were commonly used to validate neurocognitive alterations in rodent models. The results of these behavioral tasks were recorded (Table VII-IX and Figures 1-5).

Discussion

The most frequent neurodegenerative and predominant dementia form of the elderly is AD. The rise in the synthesis and aggregation of amyloid-beta (Aβ), leading to oxidative stress, neuroinflammation, and neurodegeneration, is one of the primary hallmarks of AD[10-12]. Heterocyclic compounds with acetylcholinesterase/butyrylcholinesterase inhibitory potential are commercially available. For instance, donepezil, galantamine, and rivastigmine are available in local markets to restrict enzyme activity. However, their side effects reduce their usefulness, necessitating the development of a new inhibitor with lower toxicity and fewer adverse effects[13-15]. Fluoroquinolones and benzimidazole-benzothiazole derivatives offer anti-oxidant, anti-inflammatory, anti-diabetic, antimicrobial, and anti-Alzheimer activities. This study was designed to check the *in-silico* anti-Alzheimer activity of synthetic heterocyclic compounds, *in-vitro* screening of these selected compounds as a potential inhibitor of acetylcholinesterase/butyrylcholinesterase (AChE/BChE), and also check their anti-Alzheimer activity and toxicity in a mouse model.

Molecular docking was done to evaluate the inhibitory effect of fluoroquinolones and benzimidazole-benzothiazole substitution compounds against AChE/BChE. Molecular docking results showed that derivatives of fluoroquinolones (Z, Z3, Z4, Z6, Z8, Z12, Z15, and Z9) and benzimidazole-benzothiazole (TBIS-16, TBAF-1 to 9) had a better score and showed maximum interactions with the active site of AChE compared to BChE. On the other hand, Z-9 and TBAF-6 showed significantly better docking results against BChE than donepezil. These heterocyclic compounds are found to be more potent anti-Alzheimer's agents than donepezil against AChE/

Table III. Results of heterocyclic compounds examined for Lipinski rule.

Compound ID	Molecular weight (g/mol)	Number of HBA (nON)	Number of HBD (nOHNH)	Log *p*
TBAF-6	371.01	4	1	1.14
Z-9	422.55	11	2	1.03
3152 (Donepezil) Standard drug	413.87	8	3	5.23

HBA-Hydrogen bond acceptor groups, HBD-Hydrogen bond donor groups.

Table IV. Absorption, metabolism, and toxicity related drug-like parameters of selected compounds.

Compounds ID's	Blood-brain barrier	Gastro-intestinal absorption	P-glycoprotein substrate	CYP450 1A2 inhibitor	CYP450 2C9 inhibitor	CYP450 2D6 inhibitor	CYP450 2C19 inhibitor	CYP450 3A4 inhibitor
TBAF-6	Yes	High	Yes	No	No	No	No	No
Z-9	No	Low	Yes	No	Yes	No	Yes	Yes
3152 (Donepezil) Standard drug	No	Low	No	Yes	Yes	No	Yes	No

CYP450-Cytochromes P450.

Table V. Inhibition % and IC_{50} of heterocyclic compounds against acetylcholinesterase enzyme.

Sr. No.	Compounds ID	% of Inhibition	IC_{50} (µM)
1	Z-8	25 ± 0.007	7.15 ± 0.015
2	Z-14	87.76 ± 0.16	0.8 ± 0.43
3	Z-3	10 ± 0.55	119.75 ± 0.48
4	Z-11	29.19 ± 0.14	8.05 ± 0.30
5	Z-7	45 ± 0.70	4.87 ± 0.07
6	Z-16	1 ± 0.57	-0.9 ± 0.05
7	Z-15	35 ± 0.61	6.67 ± 0.02
8	Z	75 ± 0.17	2.93 ± 0.29
9	Z-9	97 ± 0.16	0.37 ± 0.02
10	Z-5	75 ± 0.53	9.87 ± 0.08
11	Z-6	17 ± 0.55	0.87 ± 0.01
12	Z-4	29 ± 0.32	11.06 ± 0.015
13	TBAF-1	27.68 ± 0.30	-0.348 ± 0.001
14	TBAF-2	39.76 ± 0.43	18.96 ± 0.56
15	TBAF-3	28.64 ± 0.45	-22.58 ± 0.01
16	TBAF-4	60.15 ± 0.59	-0.34 ± 0.01
17	TBAF-5	17.89 ± 0.41	7.9 ± 0.05
18	TBAF-6	96.35 ± 0.45	0.638 ± 0.001
19	TBAF-7	0 ± 0.7	170.95 ± 0.01
20	TBAF-8	41.21 ± 0.07	-31.58 ± 0.01
21	TBAF-9	55 ± 0.07	15.82 ± 0.03
22	TB1S-9	0 ± 0.61	-0.9 ± 0.09
23	TB1S-6	86.45 ± 0.31	4.87 ± 0.009
24	TB1S-4	27.60 ± 0.007	33.93 ± 0.015
25	TB1S-16	39.41 ± 0.02	23.05 ± 0.03
26	TB1S-14	32.29 ± 0.06	11.06 ± 0.03
27	3152 (Donepezil) Standard drug	68.74 ± 0.07	3.90 ± 0.01

IC_{50}-Half-maximal inhibitory concentration.

Table VI. Inhibition % and IC_{50} of heterocyclic compounds against butyrylcholinesterase enzyme.

Sr. No.	Compounds ID	% of Inhibition	IC_{50} (µM)
1	TBAF-6	66.15 ± 0.10	1.31 ± 0.01
2	Z-9	72.30 ± 0.41	2.93 ± 0.03
3	3152 (Donepezil) Standard drug	81.53 ± 0.37	4.9 ± 0.05

IC_{50}-Half-maximal inhibitory concentration.

Table VII. Effect of heterocyclic compounds on Morris water maze in aluminum chloride-induced Alzheimer's disease model.

Groups	Quadrant 1	Quadrant 2	Quadrant 3	Quadrant 4
Escape latency in seconds				
Control	20.98 ± 0.91	19.56 ± 0.91	19.29 ± 0.91	19.74 ± 0.91
Disease control	42.33 ± 0.45*	41.99 ± 0.31*	42.12 ± 0.31*	41.99 ± 0.31*
Standard Drug	21.22 ± 0.21*	21.25 ± 0.21*	21.07 ± 0.29*	19.56 ± 0.21*
Z-9 (Higher)	32.82 ± 0.37*	32.77 ± 0.52*	32.30 ± 0.51*	32.36 ± 0.37*
Z-9 (Lower)	19.88 ± 0.31**	19.38 ± 0.45**	19.41 ± 0.31**	20.58 ± 0.31**
TBAF-6 (Higher)	35.95 ± 0.15*	36.52 ± 0.21*	35.48 ± 0.15*	35.85 ± 0.15*
TBAF-6 (Lower)	19.80 ± 0.13***	20.1 ± 0.18***	20.22 ± 0.13***	19.32 ± 0.13***

Values are expressed as mean ± SEM, (n=6), ***($p < 0.001$), **($p < 0.01$), *($p < 0.05$) as compared to control.

Table VIII. Effect of heterocyclic compounds on open field test in aluminum chloride-induced Alzheimer's disease model.

Groups	Total No. of lines crossed	Freezing (seconds)	Rearing/ 10 min	Grooming/ 10 min
Control	8.33 ± 0.88	79.33 ± 0.57	0.00 ± 0.00	0.00 ± 0.00
Disease control	27.12 ± 0.58***	0.00 ± 0.00***	4.38 ± 0.41***	3.33 ± 0.39***
Standard Drug	24.44 ± 0.38****	19.33 ± 0.38****	4.41 ± 0.27****	3.74 ± 0.17****
Z-9 (Higher)	24.04 ± 0.27*	24.43 ± 0.27*	4.88 ± 0.19*	5.45 ± 0.11*
Z-9 (Lower)	19.04 ± 0.57***	21.22 ± 0.57***	2.95 ± 0.40***	2.66 ± 0.50***
TBAF-6 (Higher)	25.30 ± 0.21****	23.03 ± 0.02****	5.00 ± 0.21****	4.90 ± 0.22****
TBAF-6 (Lower)	18.56 ± 0.19*	19.26 ± 0.27*	3.10 ± 0.27*	2.75 ± 0.17*

Values are expressed as mean ± SEM, (n=6), ***($p < 0.001$), **($p < 0.01$), *($p < 0.05$) and ns (non-significant) as compared to control.

Table IX. Effect of heterocyclic compounds on Y-maze test in aluminum chloride-induced Alzheimer's disease model.

Groups	Total No. of arm entries	Total No. of triads	% Spontaneous alteration	Laterality index
Control	10.00 ± 0.5	2.00 ± 0.0	37.00 ± 0.5	0.14
Disease control	2.35 ± 0.33***	0.00 ± 0.0***	0.00 ± 0.0***	-0.33
Standard Drug	10.00 ± 0.5^{ns}	2.33 ± 0.3^{ns}	36.00 ± 0.5^{ns}	0.14
Z-9 (Higher)	15.00 ± 1.7^{ns}	3.00 ± 0.0^{ns}	38.18 ± 0.49^{ns}	0.1
Z-9 (Lower)	8.66 ± 0.33***	2.99 ± 0.0^{ns}	35.44 ± 0.4***	0
TBAF-6 (Higher)	16.78 ± 1.8^{ns}	3.33 ± 0.2^{ns}	35.85 ± 1.75^{ns}	0.2
TBAF-6 (Lower)	7.00 ± 0.5***	1.90 ± 0.0**	22.33 ± 0.8***	-0.2

Values are expressed as mean ± SEM, (n=6), ***($p < 0.001$), **($p < 0.01$), and ns (non – non-significant) as compared to control.

BChE. The screening of drug-like properties of screened Z-9 and TBAF-6 showed that these compounds can exhibit high absorption and solubility compared to donepezil. Our findings also predicted that these AChE/BChE inhibitors have physiochemical properties within the reference range of Lipinski's rule and apposite drug-like compounds. The inhibitory effect of docked heterocyclic compounds was also verified *in vitro* against

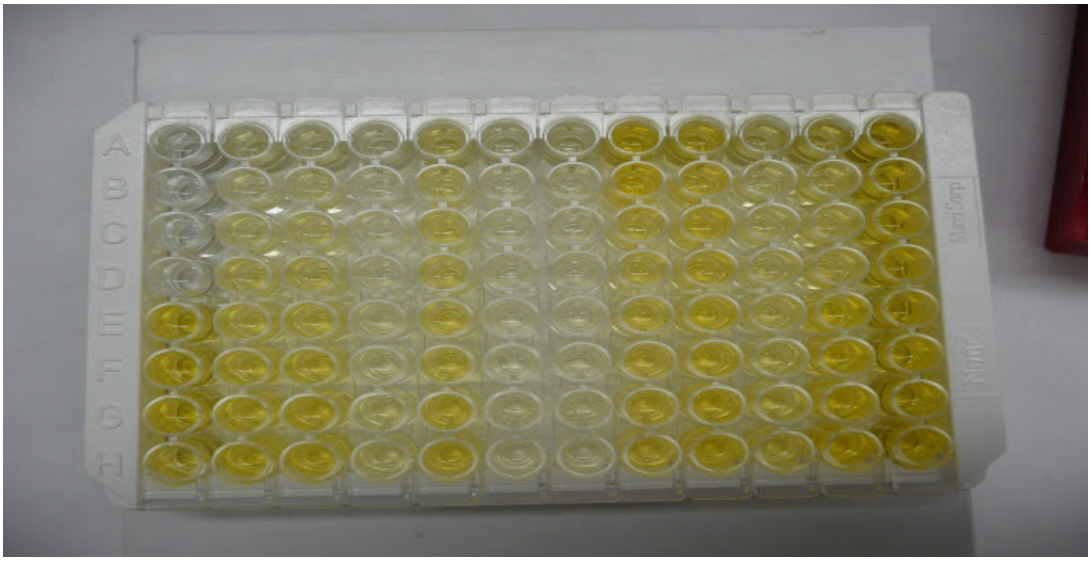


Figure 1. *In-vitro* inhibitory activity of acetylcholinesterase (AChE) butyrylcholinesterase (BChE) enzymes.

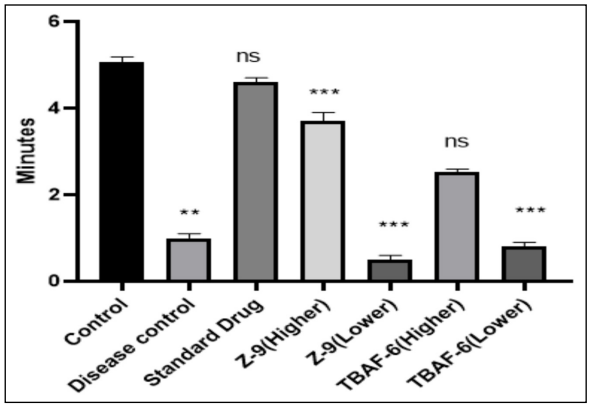


Figure 2. Effect of heterocyclic compounds on passive avoidance test in aluminum chloride-induced Alzheimer's disease model. Values are expressed as mean ± SEM, (n=6), ***(p<0.001), **(p<0.01), *(p<0.05) and ns (non-significant) as compared to control.

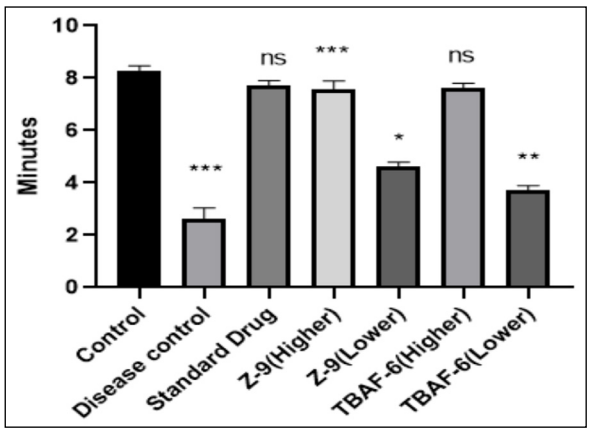


Figure 3. Effect of heterocyclic compounds on hole board test in aluminum chloride-induced Alzheimer's disease model. HD: Head dipping**.** Values are expressed as mean ± SEM, (n=6), ***(p<0.001), **(p<0.01), *(p<0.05) and ns (non – non-significant) as compared to control.

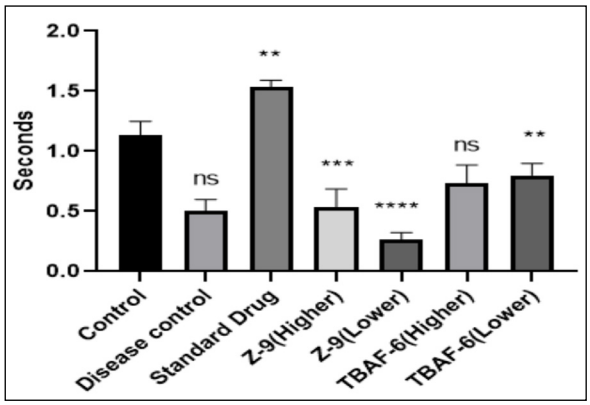


Figure 4. Effect of heterocyclic compounds on a wire hanging test in aluminum chloride-induced Alzheimer's disease model. HT: Hanging time. Values are expressed as mean ± SEM, (n=6), ***(p<0.001), **(p<0.01), *(p<0.05) and ns (non-significant) as compared to control.

AChE and BChE enzymes. Fluoroquinolones (Z, Z3, Z4, Z6, Z8, Z12, Z15, and Z9) and benzimidazole-benzothiazole compounds (TBIS-16, TBAF-1 to 9) passed through the AChE inhibition assay and their IC_{50} values were calculated. The compounds Z-9 and TBAF-6 showed the lowest IC_{50} values against AChE/BChE (0.37±0.02/2.93±0.03 µM and 0.638±0.001/1.31±0.01 µM, respectively) compared to donepezil (3.9±0.01/4.9±0.05 µM)[16]. Further, the results revealed that heterocyclic compounds have greater potential to inhibit AChE and BChE activity than commercially available medicines. Neurodegenerative disorders, especially AD, have gained momentum in modern healthcare systems. Our planet's crust is largely made of aluminum, which has been linked to the etiopathogenesis of neurodegenerative diseases marked by neuropsychiatric symptoms, behavioral changes, and cognitive deficits like impaired working memory and semantic memory as well as a lack of interest in learning new information[17]. Aluminum chloride impairs glucose uptake, damages lipids and proteins through peroxidation, alters phosphoinositide metabolism, alters protein phosphorylation, and produces more free radicals or reactive species[18,19].

Behavioral trials were performed to analyze the neuroprotective impact of fluoroquinolone (Z-9) and benzimidazole-benzothiazole (TBAF-6) compounds on AD mouse models. The results of the Morris water maze test were comparable to those of Petrasek et al[20]'s earlier investigations, which demonstrated increased escape latency in the illness control group and improved latency in treatment groups. In the Y-maze task, the use of chronic aluminum chloride affected sustaining cognition, as well as the shorter-term and inherent ability of mice to alternate arms or neophilia. The groups treated with fluoroquinolone and benzimidazole-benzothiazole compounds had better cognitive behavior compared to standard drugs. The findings of the Y-maze test demonstrated that chronic administration of aluminum chloride reduced rodents' sustained cognition, short-term memory, and innate ability to alternate arms or neophilia. Our findings show that the consumption of fluoroquinolone (Z-9) and benzimidazole-benzothiazole (TBAF-6) compounds increased muscular strength in rats due to their multifunctional pharmacological actions.

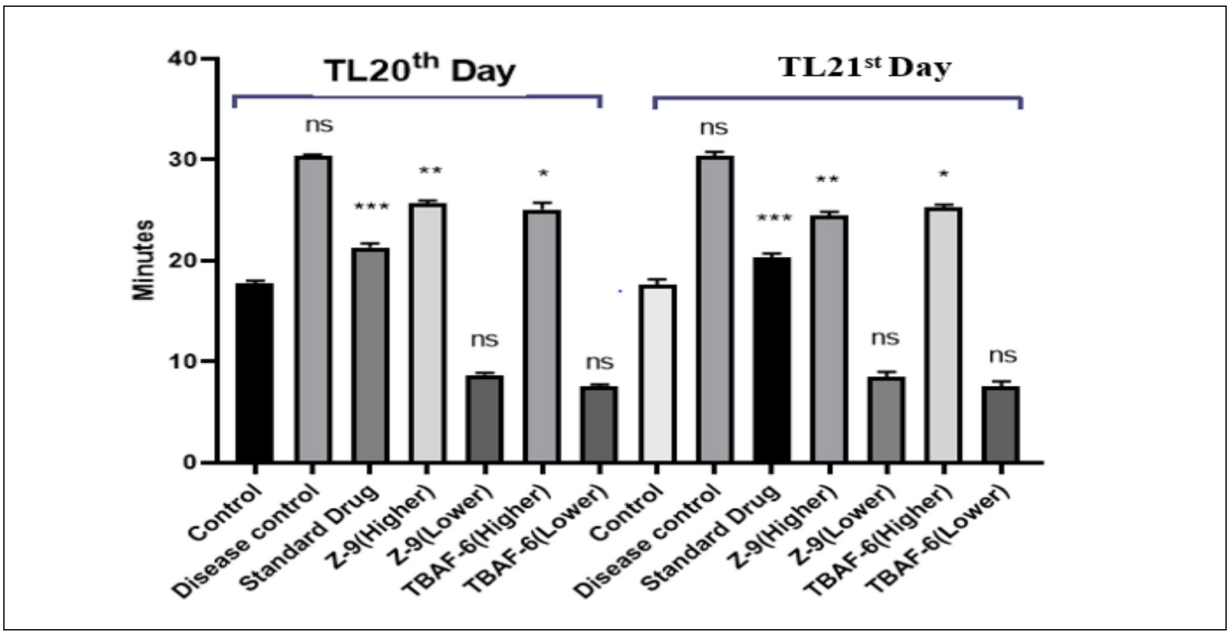


Figure 5. Effect of heterocyclic compounds on elevated plus-maze in aluminum chloride-induced Alzheimer's disease model. TL 20th day: transfer latency at 20th day, TL 21st day: transfer latency at 21st day. Values are expressed as mean ± SEM, (n=6), two ways ANOVA was applied, ***(p<0.001), **(p<0.01), *(p<0.05) and ns (non-significant) as compared to control.

Conclusions

It is concluded that Z-9 (Fluoroquinolones) and TBAF-6 (benzimidazole-benzothiazole) compounds had better cognitive behavior than standard drugs. Further research into these compounds would be helpful in identifying a potential therapeutic approach to manage neurodegenerative disorders.

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Ethics Approval
The Institutional Animal Ethics Committee (IAEC) at the College of Pharmacy (CP/IAEC/PG/3/08/2022) approved all experimental procedures.

Conflict of Interest
The author declares that there is no potential conflict of interest with this paper.

Authors' Contribution
S. Ezhilarasan conceived and designed research, conducted experiments. Saud Alqahtani, Taha Alqahtani, Krishnaraju analyzed data and wrote the manuscript. Kalpana Krishnaraju Malarkodi Velraj, Vinoth Prabhu Veeramani, Saleh F Alqifari, Ezhilarasan, and S. Durgaramani reviewed the manuscript. All authors read and approved the manuscript.

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Availability of Data and Materials
Data are available on request to the corresponding author.

Informed Consent
Not applicable as the research does not involve any human study.

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